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DNA-Binding Properties and Photocleavage Activity of Cationic Water-Soluble Chlorophyll Derivatives

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Abstract: Three cationic water-soluble chlorin e_6 derivatives, that is, 6a-,γb-,7c-tris(2-trimethylammonioethyl)chlorin e_6 (**1**), 6a-,γb-,7c-tris(3-methylpyridiniummethyl)chlorin e_6 (**2**), and 6a-,γb-,7c-tris(2-trimethylammonioethyl)-2-(3-trimethylammonioprop-1-enyl)chlorin e_6 (**3**), have been designed and synthesized to allow the study of their DNA-binding and -photocleavage activities. The DNA-unwinding assay, measurements of melting temperatures of double-stranded DNA, and the induced CD and visible absorption spectra have revealed that **1** and **3** are intercalated into the base pairs of the double-helical DNA, while **2** is bound to outside the minor groove of the double-helical DNA. The cationic water-soluble chlorin e_6 derivatives effectively cleave the double-helical DNA under photoirradiation and the DNA-photocleavage activity increases in the order **3**>**1**>**2**. The DNA-binding and -photocleavage characteristics of the three cationic water-soluble chlorin e_6 derivatives are influenced by aspects of their molecular structure, such as the kind, number, and position of the cationic substituents.

Keywords: bioorganic chemistry · chlorins · DNA recognition · photobiology · porphyrinoids

Introduction

In the past two decades there has been considerable interest in small-molecule DNA binders because of their therapeutic use for cancer and genetic diseases. In particular, cationic porphyrins have been intensively studied because they are potentially useful as photonucleases,[1] structural probes of DNA,[2] inhibitors of human telomerase,[3] and photosensitizers for photodynamic therapy (PDT).[4–6] On the other hand, a few chlorophyll derivatives have also received a great deal of attention as effective photonucleases and/or useful photosensitizers because of their strong absorption in the visible region, absorption that originates from the π–π* transition in the chlorin ring.[5,6] In view of their characteristic strong absorption in the so-called Soret and Q-band regions and their selective accumulation on cancer cells,[7] chlorophyll derivatives are very attractive as photosensitizers in the next generation in place of some of the porphyrin derivatives currently used in PDT.[6] Therefore, new chlorin e_6 derivatives have been extensively investigated in recent years with the aim of improving their tumor selectivity and therapeutic effectiveness.[8] The DNA binding of a few chlorophyll derivatives, for example, pheophorbide a,[9] cationic derivatives of pyropheophorbide a,[10,11] and cationic chlorin e_6 trimethyl ester,[12] has also been focused on with a view towards the development of DNA-targeting photosensitizers. In previous reports it has been revealed that the chlorophyll derivatives bind to DNA and give rise to the efficient cleavage of the DNA by a type I or type II mechanism under irradiation by visible light.[9,11] However, almost all of the chlorophyll derivatives studied so far are only slightly soluble in water and they frequently aggregate in the buffer solution because most of them are monocationic and/or possess more hydrophobic moieties as the peripheral groups on the chlorin ring. Unfortunately, the poor solubility or self-aggregation of the chlorophyll derivatives in the buffer solution not only hinders their intercalation into the base pairs of the double-helical DNA but also deactivates the photoexcited dye molecules.[13] In addition, this problem makes it difficult to elucidate the influence of the molecular structure on the interaction between the chlorophyll derivatives and DNA as well as to develop a new effective photonuclease as a useful photosensitizer. Therefore, only a few studies have dealt with the question of how the DNA interaction or DNA photocleavage of cationic water-soluble chlorophyll derivatives is influenced by the kind, charge number, and position of the

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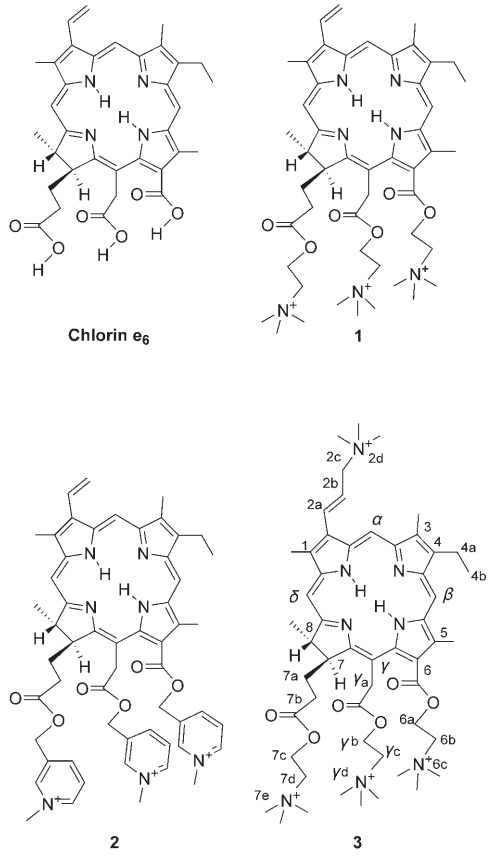
peripheral substituents. The lack of fundamental information that is indispensable for the development of useful photosensitizers in PDT has prompted us to systematically study the properties of DNA binding and the activities of DNA photocleavage of cationic water-soluble chlorophyll derivatives.

In an attempt to avoid the aggregation of the dye molecules and to enhance their affinity to DNA, we have designed and synthesized tricationic esters of chlorin e_6 (Scheme 1, **1** and **2**).[14] These tricationic esters are readily



Scheme 1. Structures of chlorin e_6 and cationic water-soluble chlorin e_6 derivatives **1**–**3**.

soluble in water as monomers because cationic substituents such as alkyltrimethylammonium or methylpyridinium-3-yl groups are introduced on the parent chlorin e_6. In the present work, we have designed and synthesized another new tetracationic ester of chlorin e_6, namely, 6a-,γb-,7c-tris(2-trimethylammonioethyl)-2-(3-trimethylammonioprop-1-enyl)-chlorin e_6 (Scheme 1, **3**) in order to study the DNA-binding and -photocleavage activities of cationic water-soluble chlorophyll derivatives with peripheral substituents that are different in kind, charge number, and position. In the design of the cationic water-soluble chlorin e_6 derivatives, we have taken into consideration three major binding modes, that is, intercalation and two types of outside binding. These cationic water-soluble chlorin e_6 derivatives (Scheme 1) are expected to be potential intercalators because their chlorin rings are rich in π electrons and they are free to behave as single molecules in buffer solution, rather than aggregates. A favorable aromatic stacking interaction of the chlorin ring with the nucleic acid bases is crucial in macrocyclic intercalators such as chlorophyll derivatives. In the cases of outside binding, one type is outside groove binding, involving placement of dye molecules in the minor groove, and the other type is outside binding with self-stacking, in which the dye molecules are stacked along the DNA helix. The interaction of the cationic water-soluble chlorin e_6 derivatives with DNA has been investigated by DNA-unwinding assays, melting temperature measurements on double-stranded DNA, and induced circular dichroism (CD) and UV/Vis absorption spectroscopy. The activity and mechanism of the DNA photocleavage in the presence of the cationic water-soluble chlorin e_6 derivatives have been studied in terms of the cleavage of plasmid DNA induced under irradiation with visible light and monitored by agarose gel electrophoresis.

Results and Discussion

Binding mode evidenced by DNA-unwinding assay: The unwinding assay with topoisomerase I is an essential means to assess the ability of small-molecule DNA binders to intercalate into double-helical DNA. In the present study, plasmid DNA was relaxed by incubation with topoisomerase I prior to addition of the dye and this was followed by further incubation with the dye. After topoisomerase I was deactivated and the dye was removed, the DNA was analyzed by agarose gel electrophoresis.[15] The experimental results obtained for **1**–**3** in the DNA-unwinding assay are shown in Figure 1. As can be seen from Figure 1, the plasmid DNA (pBR322 DNA, Form I) was fully relaxed by topoisomerase I (Figure 1, lane 2), and the relaxed Form II DNA was fully unwound through interaction with **1** and **3**. However, the Form II DNA was not fully unwound by **2**. The above results of the DNA-unwinding assay revealed that **1** and **3** intercalate into the base pairs of the double-helical DNA, but **2** binds to the outside of the double-helical DNA. The helix-unwinding angle can be estimated from the electrophoretic mobility because it is reflected in the number of superhelical turns in plasmid DNA.[16] The unwinding angle (ϕ) of **1**–**3** was estimated by the following method. According to Figure 1, the center of the topoisomer distribution, which corresponds to the writhing number (τ), in Form II DNA fully relaxed by topoisomerase I (Figure 1, lane 2) is $+1$. The sample (Figure 1 A , lane 7) contains 2.9 μM **1** and 5.0×10^{-3} μM (in DNA molecules) pBR322 DNA and reactive topoisomerase I. Since the equilibrium constant (K_{app}) is sufficiently large (1.5×10^{4} M^{-1}, see Table 1), the concentration of DNA-bound **1** in this buffer solution can be equal to the total concentration of **1** (2.9 μM). This means that the m value, that is, the number of the dye molecules bound to one pBR322 DNA molecule, is 573. In addition, the $\Delta\tau$

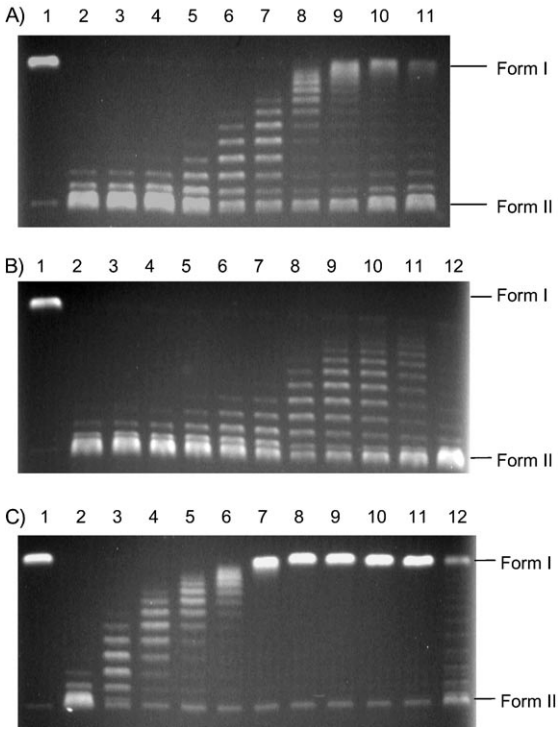


Figure 1. Agarose gel electrophoresis of pBR322 DNA relaxed by topoisomerase I in the presence of A) **1**, B) **2**, and C) **3**. Lane 1: pBR322 DNA (Form I); Lanes 2–12 (lanes 2–11 in A): pBR322 DNA (Form I, 0.5 μg in 35 μL) treated with topoisomerase I (6.6 units) in the presence of 0, 0.76, 1.5, 2.2, 2.5, 2.9, 3.8, 6.0, 8.0, and 10 μM **1**; 0, 0.76, 1.5, 2.2, 2.5, 2.9, 3.8, 6.0, 8.0, 10, and 15 μM **2**; and 0, 0.44, 0.66, 0.88, 1.1, 1.5, 1.9, 2.5, 3.8, 6.0, and 10 μM **3**, respectively.

Table 1. Spectroscopic parameters of cationic water-soluble chlorin e_6 derivatives and their apparent equilibrium constants with CT-DNA.

Dye	Induced CD	UV/Vis					
	$\Delta\varepsilon$ [$cm^{-1}M^{-1}$] (λ_0 [nm])[a]	λ_0 [nm]		ε_{max} [$cm^{-1}M^{-1}\times10^4$]		ΔH [%][b]	$K_{app}\times10^3$ [M^{-1}]
		free	DNA-bound[a]	free	DNA-bound[a]		
1	−20.2 (399.5), +9.30 (413.5)	400.5	407.5	14.0	7.67	45.2	15
2	+3.84 (412.5)	404.5	408.0	9.55	6.03	34.0	7.5
3	−18.6 (401.0), +5.74 (417.0)	402.0	409.0	11.7	7.01	40.1	27

[a] In the presence of CT-DNA ($R=0.01$). [b] Hypochromicity in the Soret region ($R=0.01$).

value is determined to be 5, since the τ value (Figure 1 A, lane 7) is −4. Thus, the ϕ value of **1** is calculated to be 3.1° according to [Eq. (1)] (see the Experimental Section). When similar calculations were repeated for lanes 6 and 8 in Figure 1, the ϕ value of **1** was determined to be $3.3\pm1.0°$. By contrast, the ϕ value of **3** is calculated to be $16\pm2°$ from Figure 1 C, lanes 3–5. Interestingly, the ϕ value of **3** is comparable to that of 19° for 5,10,15,20-tetrakis(*N*-methylpyridinium-4-yl)porphyrin (TMPyP),[17] which is known to be a relatively bulky DNA intercalator, although the chlorin ring is more structurally hindered at the 7–8 positions than the porphyrin ring. This structural hindrance may hardly contribute to the DNA-unwinding angle. On the other hand, this result shows that **3** has a higher ability for DNA unwinding than **1**. A relatively large distortion from the ideal B-form DNA is required for intercalation of **3** into the base pairs as compared to that required for intercalation of **1**. The difference in ϕ values for **1** and **3** is due to the difference at the 2b position. Consequently, it can be stated that the binding mode of the cationic water-soluble chlorin e_6 derivatives to DNA is strongly influenced by the stereochemistry of the peripheral substituents of the chlorin ring and also the carbon-chain length between the ester moiety and the trimethylammonium or methylpyridinium ion.

Physicochemical evidence for DNA binding: The thermal denaturation of double-helical polynucleotides from double-stranded to single-stranded DNA is manifested as hyperchromism in the UV absorption of the DNA base pairs at 260 nm. The melting temperature (T_m) of DNA is sensitive to its double-helix stability and the binding of dyes to DNA alters the T_m value, with dependence on the strength of the interactions.[18] Upon binding of small dye molecules to calf thymus DNA (CT-DNA), the T_m value of the B-form DNA should become higher, as compared to that of unbound or free CT-DNA. In addition, as the dyes bind more strongly to the DNA, the increase in the T_m value will become larger.[19] Therefore, the T_m value can be used as an indicator of the binding properties and binding strengths of dyes with DNA. Under the present experimental conditions, the melting curve (absorbance versus temperature) has a transition, and the T_m value of free double-stranded CT-DNA is 66.1 °C; this value corresponds to the half dissociation of the Watson–Crick base-paired duplex. Upon addition of the cationic water-soluble chlorin e_6 derivatives **1–3** to the DNA solution, the profile of the melting curve did not change drastically although the T_m value was higher than that of free CT-DNA (Figure S1 in the Supporting Information). The increase in melting temperature (ΔT_m), that is, the difference in the melting temperatures in the absence and presence of chlorin, is plotted against the molar ratio (R) of [chlorin] to [DNA in base pairs]. Increased addition of the cationic water-soluble chlorin e_6 derivatives **1–3** to the buffer solution of CT-DNA raises the T_m value to some extent, thereby indicating that the double-stranded CT-DNA is stabilized by the binding of **1–3** (Figure 2). A comparison of the increase in the melting temperature (ΔT_m) revealed that, at the same R value, the ΔT_m value for **1** is the largest and that for **2** is the smallest of the three cationic chlorin e_6 derivatives. Interestingly, tricationic **1** exhibited a larger ΔT_m value than tetracationic **3**, although in general **3**, with four positive charges, would stabilize the duplex structure of DNA more than **1**, with three positive charges.[20] This result probably occurs because **1** intercalates into certain site(s) of the base pairs of the double-helical DNA where it is favorable for the trimethylammonium substitu-

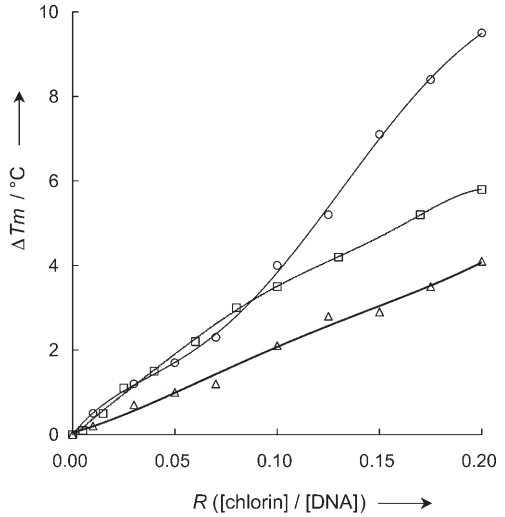


Figure 2. Plots of the increase in melting temperature (ΔT_m) versus R-([chlorin]/[CT-DNA in base pairs]) for cationic water-soluble chlorin e_6 derivatives **1** (○), **2** (△), and **3** (□).

ents of **1** to electrostatically interact with the phosphate anion sites of CT-DNA. In view only of the magnitudes of the ΔT_m values for the cationic water-soluble chlorin e_6 derivatives, compounds **1** and **3** are taken to be intercalators, while **2** is considered to be an outside groove binder. This interpretation is consistent with the conclusion drawn from the experimental results obtained from the unwinding assay and the other methods.

Spectroscopic evidence for DNA binding: Spectral changes in circular dichroism (CD) were observed upon increased addition of CT-DNA to a buffer solution of the cationic water-soluble chlorin e_6 derivatives. The induced CD spectra shown in Figure 3 were calculated from the original CD spectra (Figure S2 in the Supporting Information). The induced CD spectral data obtained are summarized in Table 1. In the induced CD spectra of both **1** and **3**, an intense negative signal with a weak positive signal appeared at relatively small values of R, for example, 0.01. By contrast, only a weak positive signal was observed in the induced CD spectrum of **2** under the same experimental conditions. Thus, the induced CD spectra, as well as the DNA-unwinding assay and the other physicochemical measurements, suggest that **1** and **3** are classified as intercalators, while **2** is as an outside groove binder.

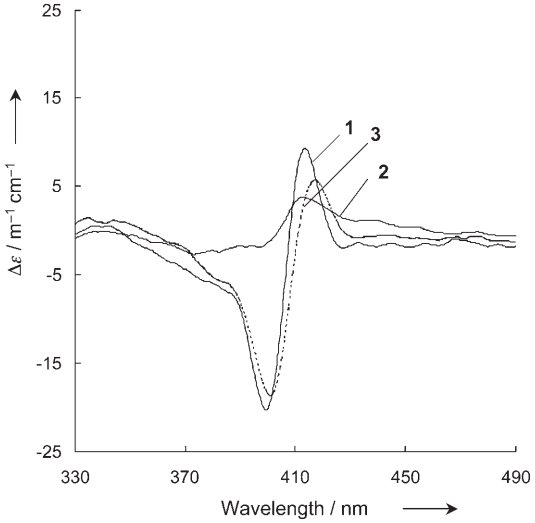


Figure 3. Induced CD spectra of DNA-bound **1**–**3** in TE buffer (10 mM tris(hydroxymethyl)aminomethane-HCl (Tris-HCl) and 1 mM ethylenediaminetetraacetate (EDTA), pH 7.6, at 25 °C) at $R = 0.01$.

A buffer solution of the cationic water-soluble chlorin e_6 derivatives was spectrophotometrically titrated with a CT-DNA buffer solution. The absorption-spectrum changes over the course of titration are reproduced for **1** (Figure 4 and Figure S3 in the Supporting Information). At the beginning of the spectrophotometric titration, substantial hypochromism with an isobestic point was found in the Soret and Q bands at $R \geq 0.67$, and then significant hyperchromism with a new isobestic point and a large red shift was observed at $R \leq 0.67$. With respect to **2** and **3**, a similar spectral feature to that for **1** was also confirmed, as shown for **2** and **3** in Figures S4 and S5 in the Supporting Information. For brevity, only their spectroscopic parameters and characteristics are listed in Table 1. Judging from the results of spectrophotometric titration, the interaction of **1**–**3** with CT-DNA proceeds in two steps (Figure 4 and Figures S3–S5 in the Supporting Information). In the first step, the absorption spectra of **1**–**3** exhibit hypochromicity without shift of the Soret band as the R values decrease for **1** or **2** in the range $R \geq 0.67$ and for **3** in the range $R \geq 0.50$. The absorption-spectrum changes are very fast and reach equilibrium within a few minutes after mixing **1**–**3** with CT-DNA in the buffer solution. In addition, the absorption spectra were influenced by the ion strength of the buffer solution and thus the addition of sodium chloride hindered the change of the spectra

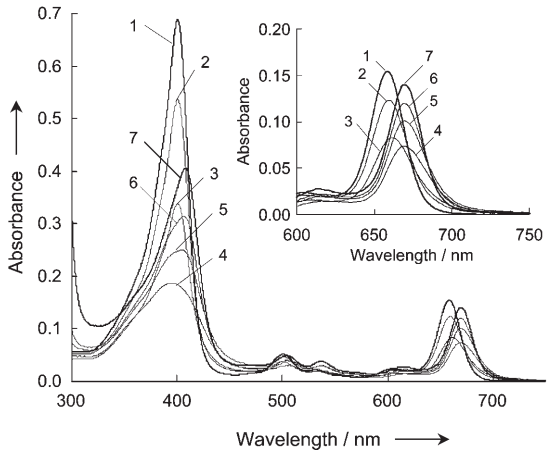


Figure 4. Changes in the visible absorption spectrum for **1** with increased addition of CT-DNA at 25 °C. The numbers attached to the absorption spectra correspond to decreasing R values (1 = ∞, 2 = 7.11, 3 = 4.97, 4 = 1.00, 5 = 0.67, 6 = 0.069, and 7 = 0.010). The corresponding data for **2** and **3** are provided as Figures S4 and S5 in the Supporting Information.

to some extent (data not shown). These features of the absorption-spectrum changes suggest that the first step of the process is electrostatic interaction of the cation site of **1**–**3** with the phosphate anion site of CT-DNA. In the second step, the negative charges on the phosphate backbone of CT-DNA become stoichiometrically in excess at $R \leq 0.67$ for **1** or **2** and $R \leq 0.50$ for **3**, so the tangling of CT-DNA electrostatically neutralized by cationic **1**–**3** will be relaxed to retain the solubility of CT-DNA in the buffer solution. As a result, the absorption spectra of **1**–**3** exhibit hyperchromicity with a new isobestic point and a red shift of the Soret band as the *R* values decrease in the second step. The absorption-spectrum changes of the second step are relatively slow compared to those of the first step, and the hyperchromicity and red shift in the Soret band are not so significant any more and are almost independent of the decrease in the *R* values when $R \leqq 0.01$. From the features of the spectra, it is suggested that **1**–**3** are relocated to another site of the double-helical DNA. On the other hand, **1** and **3** exhibited a substantial hypochromicity of 45 and 40%, respectively, at an *R* value of 0.01, which is reasonable but a little bit larger than that reported for intercalators such as TMPyP.[21] The red shift in the Soret band of **1** and **3** was 7 nm at $R = 0.01$, while that in the Q band was 11.0 nm for **1** and 9.5 nm for **3**. By contrast, the hypochromicity in the Soret band of **2** was 34% under the same experimental conditions and the red shifts in the Soret and Q bands were 3.5 nm and 7.0 nm, respectively. The large hypochromicity and red shift in the Soret band for **1** or **3** are certainly due to the π–π interactions between the chlorin ring and the nucleic acids bases. On the other hand, the small hypochromicity and bathochromicity for **2** are associated with minor conformation changes through the electrostatic interaction with the CT-DNA. The spectral features observed indicate that, in the region of relatively small *R* values, **2** is bound outside the CT-DNA while **1** and **3** are intercalated into the base pairs of the double-helical CT-DNA. This interpretation is in good agreement with that based on the results of the unwinding assay and the other physicochemical and spectroscopic measurements. Consequently, it can be said that the first step of the process is electrostatic interaction of the cation site of **1**–**3** with the phosphate anion site of CT-DNA (at $R \geq 0.67$ for **1** or **2** and $R \geq 0.50$ for **3**) and the second step is intercalation of **1** and **3** into the base pairs of CT-DNA or outside binding of **2** at the groove of CT-DNA (at $R \leq 0.67$ for **1** or **2** and $R \leq 0.50$ for **3**). It is rare that the electrostatic binding process of the cationic water-soluble chlorin e_6 derivatives with DNA can be explicitly differentiated from the intercalative one spectrophotometrically, although the interactions of some cationic porphyrins with DNA proceed in two steps.[22] This phenomenon must be due to the fact that the positive charges of the cationic water-soluble chlorin e_6 derivatives are asymmetrically localized on the peripheral groups attached to the chlorin ring, while those of most cationic porphyrins are symmetrically located at the *meso* position. Indeed, the kind and the position of the cationic substituents affect the electron density of the porphyrin ring or the charge distribution in the molecule.[23] Thus, the kind, number, and position of the cationic peripheral groups influence the binding mode of the cationic water-soluble chlorin e_6 derivatives with CT-DNA.

The apparent equilibrium constants (K_{app}) in the second step were calculated in the range of $R = 0.1$–0.01 by [Eq. (2)] and are listed in Table 1 (see the Experimantal Section). The increasing order for the equilibrium constants is **3** > **1** > **2**. The equilibrium constant of **3** is larger than that of **1** because **3** has more charges in its molecular structure than **1**. On the other hand, the equilibrium constant of **1** is larger than that of **2** because **1** is an intercalator while **2** is an outside binder of DNA. The π–π interaction between the chlorin ring and the base pairs of CT-DNA must contribute to the DNA binding in intercalative **1**, as compared to that in **2** in which no intercalative interaction is expected because of the structural hindrance. Compound **2**, unlike **1** or **3**, outside binds to CT-DNA because the warped chlorin ring hinders the intercalation into the base pairs. The chlorin ring of **2** is warped to minimize the molecular force field, as compared to **1** which has a planar chlorin ring. In fact, the three pyridinium groups attached to the chlorin ring are bulky, as demonstrated in the Corey–Pauling–Koltun modeling with the MOPAC software (Figure S6 in the Supporting Information). As a consequence, it has also been confirmed that the kind, number, and position of cationic peripheral groups affect the binding mode of the cationic water-soluble chlorin e_6 derivatives with CT-DNA.

DNA cleavage under irradiation with visible light: The DNA-photocleavage activity of the cationic water-soluble chlorin e_6 derivatives was assessed by the photocleavage of plasmid DNA (pBR322 DNA) from supercoiled Form I to open-circle Form II or linear Form III and was monitored by gel electrophoresis and densitometry.[24,25] The gel electrophoresis patterns were obtained for the photocleavage of supercoiled pBR322 DNA under irradiation with visible light at various concentrations of dyes (Figure 5). The so-called single-strand breaks increased with the addition of the cationic water-soluble chlorin e_6 derivatives, and the DNA cleavage in the presence of the cationic water-soluble chlorin e_6 derivatives is induced only by light irradiation. In a comparison of DNA photocleavage at the same *R* value (for example, $R = 0.229$), tetracationic **3** gives rise not only to cleavage to Form II but also to double-strand breaks to yield Form III. In addition, only the single-strand breaks without production of Form III are caused by photoirradiation in the presence of even 88 μM tricationic chlorin e_6 (**1** or **2**), while photoirradiation in the presence of only 8.8 μM tetracationic **3** gives rise to double-strand breaks to produce Form III DNA. The DNA-photocleavage activity of tetracationic **3** is much higher than that of tricationic **1** or **2**. Interestingly, the outside binder **2** has the lowest DNA-photocleavage activity of the three cationic water-soluble chlorin e_6 derivatives. Therefore, the increasing order of effectiveness as photosensitizers, that is, of the DNA-photocleavage activity, is **3** > **1** > **2**. This increasing order of DNA-photo-

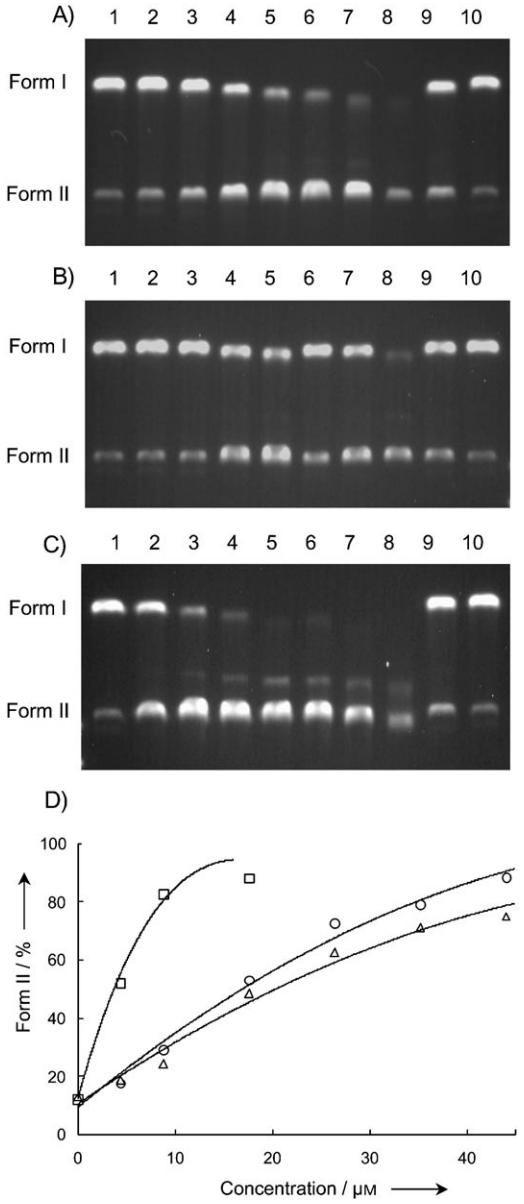


Figure 5. Agarose gel electrophoresis patterns of the photocleavage of pBR322 DNA (1.0 μg in 8.0 μL) after 5 h irradiation (at 404.5 and 436.0 nm) in TE buffer (pH 7.6 at 25 °C) in the presence of A) **1**, B) **2**, and C) **3**. Lanes 1 and 10: controls; lanes 2–8: in the presence of 4.40, 8.80, 17.6, 25.4, 35.2, 44.0, and 88.0 μM dye, respectively; lane 9: in the presence of 44.0 μM dye in the dark. D) Plots of the percentage of Form II DNA against the concentrations of **1** (○), **2** (△), and **3** (□).

cleavage activity is in parallel with the magnitude of the equilibrium constant. As a result, the larger the equilibrium constant and the charge number of the cationic water-soluble chlorin e_6 derivative, the higher the DNA-photocleavage activity. In addition, a comparison of the photocleavage activity revealed that an intercalator is more effective as a DNA photocleaver than a groove binder. This is probably because the former has a larger equilibrium constant than the latter. In view of usefulness and effectiveness as a photosensitizer, it is important to compare the DNA-photocleavage activities of the cationic water-soluble chlorin e_6 derivatives with that of a representative cationic porphyrin such as TMPyP.[26] The DNA-photocleavage assay for TMPyP was carried out under the same experimental conditions as those for the cationic water-soluble chlorin e_6 derivatives. The photocleavage yields of Form II obtained for TMPyP were 51, 60, and 71 % upon treatment with 8.80, 17.6, and 44.0 μM TMPyP, respectively, and irradiation for 5 h (data not shown). It has been reported for cationic porphyrins that the DNA-photocleavage activity is correlated to the number of positive charges.[25,27] Therefore, the photocleavage yield of Form II should be compared between tetracationic **3** and TMPyP. It is worth noting that the DNA-photocleavage activity of **3** is higher than that of TMPyP. Thus, it has been demonstrated that the cationic water-soluble chlorin e_6 derivatives must be useful as photonucleases.

Mechanism of DNA photocleavage: DNA photocleavage sensitized by dyes has been widely studied and is mainly understood by type I or type II mechanisms including the electron-transfer process and/or generation of a hydroxyl radical ($OH^{\bullet}$) and singlet oxygen ($^1O_2$).[1,28] In this research, the mechanism of the DNA photocleavage induced by the cationic water-soluble chlorin e_6 derivatives has been examined according to the previously reported protocol.[11,29] The percentages of Form II DNA, as determined by densitometry, are shown in (Figure 6). The percentages of Form II DNA after photoirradiation in the presence of **1**–**3** were decreased by the addition of mannitol, which is known to be a scavenger of $OH^{\bullet}$.[1] This indicates that a hydroxyl radical is involved in the DNA photocleavage. However, the hydroxyl radical should not play a major role in the mechanism of the DNA photocleavage, because the effect of mannitol as a scavenger of $OH^{\bullet}$ is relatively small. In addition, singlet oxygen is hardly involved in the DNA photocleavage since the addition of *N*-acetylhistidine, which is known as a scavenger of $^1O_2$, did not significantly affect the DNA photocleavage. On the other hand, the DNA photocleavage was increased upon going from a buffer solution of **1**–**3** in H_2O to one in D_2O. A substantial increase in the DNA-photocleavage efficiency in D_2O is usually due to the fact that the lifetime of $^1O_2$ generated in D_2O is longer than that in H_2O. However, the enhanced photocleavage yield observed for the cationic water-soluble chlorin e_6 derivatives may be associated with an increase in the excited states of the dyes.[1] When the sample solutions were thoroughly degassed with nitrogen gas and maintained under N_2 during the experiment, the DNA photocleavage was slightly decreased in **1** and **3** as compared with that in air, while the percentage of Form II DNA was rather increased in **2**. These results suggest that there is an anaerobic pathway to produce Form II DNA. For instance, the DNA photocleavage by **1**–**3** may be due to the participation of guanine radical cations produced by energy transfer from the exited chlorin. Thus, the DNA photocleavage by **1**–**3** can be explained by the following two mechanisms.[28] 1) A hydroxyl radical abstracts a hydrogen atom from the DNA 2-deoxyribose phosphate backbone, thereby resulting in DNA cleavage at every nucleotide. In addition, $OH^{\bullet}$ also

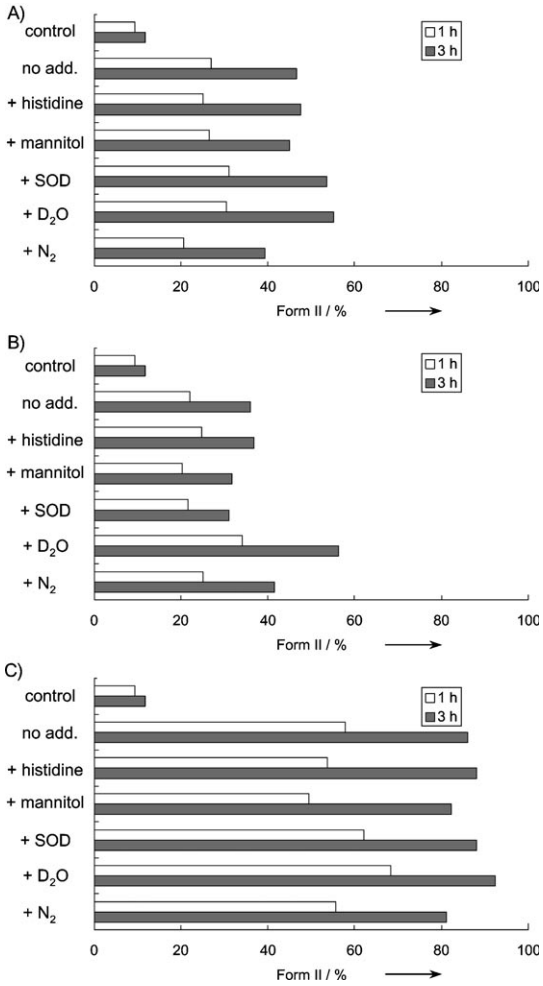


Figure 6. Influence of additives on DNA cleavage induced by light irradiation (at 404.5 and 436.0 nm) in TE buffer in the presence of 25.4 μM **1** (A), **2** (B), and **3** (C). The concentrations of the additives were 100 mM *N*-acetylhistidine and mannitol, 75 U mL^{-1} superoxide dismutase (SOD), and 87.5% D_2O, respectively. The samples for the experiment in N_2 were prepared with oxygen-free buffer solution and the microtubes were sealed with a polypropylene film in a chamber purged with N_2. Samples prepared with the air-saturated buffer were sealed in the air in the same manner.

causes addition to the DNA bases to yield a variety of oxidation products. 2) Under these rather mild conditions of oxidation and without treatment by piperidine or alkali, guanine was specially oxidized by electron transfer through the excited chlorin to produce 8-oxo-7,8-dihydroguanine.[29] The experimental protocol used here is useful in the preliminary evaluation of the mechanism of DNA photocleavage, although it lacks the accuracy to elucidate the mechanism of DNA photocleavage in some respects.[1] Therefore, these conclusions will have to be checked by a more precise protocol with the use of end-labeled oligodeoxynucleotides in the future.[11,30]

Conclusion

Three types of the cationic water-soluble chlorin e_6 derivatives (**1**–**3**) have been designed and synthesized. Their interaction with DNA has been characterized by a DNA-unwinding assay, DNA melting temperature experiments, and measurement of the induced CD and UV/Vis spectra. Tricationic **1** and tetracationic **3**, with the chlorin ring rich in π electrons, are both intercalated into the base pairs of the double-helical DNA, while tricationic **2**, with the bulky peripheral pyridinium groups, is outside bound to the minor groove of the double-helical DNA. In addition, tetracationic **3**, with a trimethylammmonium group at the 2b position, intercalates deeply into the DNA base pairs to strongly interact with the nucleobases and has higher ability to unwind DNA than tricationic **1**. On the other hand, all the cationic water-soluble chlorin e_6 derivatives give rise to DNA photocleavage and serve as photonucleases under irradiation with visible light. The increasing order of efficiency for DNA photocleavage is **3**>**1**>**2**. DNA photocleavage by the cationic water-soluble chlorin e_6 derivatives could be accounted for, at least partly, by a type I mechanism. The cationic water-soluble chlorin e_6 derivatives with a large number of charges and intercalative nature have a high affinity for DNA binding and photocleavage. Thus, it is necessary for the design of water-soluble chlorophyll derivatives to introduce as many small cationic substituents as possible, so that the original chlorin ring becomes able to easily intercalate into the double-helical DNA.

Experimental Section

Chemicals and instruments: Oxalyl chloride and dithiothreitol (DTT) were supplied by Nacalai Tesque, Inc. Ethidium bromide and 3-pyridinemethanol were purchased from Merck, Ltd. Eschenmoser's salt (*N*,*N*-dimethylmethyleneammonium iodide) and 2-dimethylaminoethanol were obtained from Aldrich. Iodomethane was supplied by Tokyo Kasei Kogyo Co., Ltd. Tris(hydroxymethyl)aminomethane (Tris) and TE-saturated phenol were purchased from Kanto Chemical Co., Ltd., and Fluka, respectively. Orange G, sodium dodecyl sulfate (SDS), and *N*-acetylhistidine monohydrate were obtained from Wako Pure Chemical Industries, Ltd. D-Mannitol was supplied by Katayama Chemical Industries Co., Ltd. Calf thymus DNA (CT-DNA; catalogue number 091 K7030) and superoxide dismutase (SOD) were purchased from Sigma. Deuterium oxide (D_2O) and wheat-germ topoisomerase I (catalogue number 19646901) were obtained from Acros and Promega, respectively. Agarose for electrophoresis (Agarose S) was supplied by Nippon Gene Co., Ltd. Plasmid DNA (pBR322 DNA in Form I) was obtained from Nippon Gene Co., Ltd, or BioLabs Inc. 5,10,15,20-Tetra(4-pyridyl)porphyrin was obtained from Aldrich and treated with iodomethane to give 5,10,15,20-tetrakis(*N*-methylpyridinium-4-yl)porphyrin (TMPyP). Buffer solutions were prepared by using ultrapure water treated by an ultrapure Millipore distillation apparatus (Milli-Q Labo, Nippon Millipore, Ltd.). Other chemicals were used as received without further purification and all solvents were of analytical reagent grade unless specified.

The UV/Vis absorption spectra were recorded in solution on a JASCO V-570 spectrophotometer equipped with a JASCO ETC-505T temperature controller by using a 10-mm quartz cell. The circular dichroism (CD) spectra were measured with a JASCO J-720 WI spectropolarimeter by using 10-mm quartz cells. The ^{1}H NMR spectra were measured at 300 MHz with a JNM-LA300 spectrometer and the chemical shifts were

expressed in ppm relative to tetramethylsilane. The FAB mass spectra were recorded on a JEOL GCmate mass spectrometer. Elemental analysis was performed at the Central Laboratory of the Faculty of Science and Technology, Keio University.

Cationic water-soluble chlorophyll derivatives: 6a-,γb-,7c-Tris(2-trimethylammonioethyl)chlorin e_6 (**1**) and 6a-,γb-,7c-tris(3-methylpyridiniummethyl)chlorin e_6 (**2**) were synthesized by the method described previously.[14] 6a-,γb-,7c-Tris(2-trimethylammonioethyl)-2-(3-trimethylammonioprop-1-enyl)chlorin e_6 (**3**) was synthesized by the following method: A suspension of Eschenmoser's salt (150 mg, 0.811 mmol) in anhydrous dichloromethane (7 mL) was added to 6a-,γb-,7c-tris(2-dimethylaminoethyl)chlorin e_6[14] (60 mg, 7.3×10^{-2} mmol) in anhydrous dichloromethane (13 mL) and stirred under a nitrogen atmosphere for 84 h. The supernatant was collected by decantation and filtered; this was followed by back-extraction with distilled water. The desired product was extracted from the water layer by triethylamine-containing dichloromethane. The green-brown dichloromethane layer was washed twice with distilled water and evaporated to dryness. The residue obtained was separated by chromatography on basic alumina (Merck; Brockmann Grade V) by using chloroform/*n*-hexane (3:1) containing 2% triethylamine. The violet main band was collected and evaporated to dryness. The dark violet desired compound was dissolved in a small amount of acetone and precipitated by addition of distilled water. The precipitate was collected by centrifugation and dried over P_4O_{10} in a vacuum desiccator to give 6a-,γb-,7c-tris(2-dimethylaminoethyl)-2-(3-dimethylaminoprop-1-enyl)chlorin e_6 as dark violet crystals (15.8 mg, 1.79×10^{-2} mmol, 24.5%): ^{1}H NMR (300 MHz, $CDCl_3$): $\delta=9.69$ (s, 1H; β-H), 9.50 (s, 1H; α-H), 8.73 (s, 1H; δ-H), 7.81 (d, $^3J=16$ Hz, 1H; 2a-H), 6.83 (dt, $^3J=16$, 6.8 Hz, 1H; 2b-H), 5.32 (m, 2H; γa-CH_2), 4.84 (m, 2H; 6b-CH_2), 4.40 (m, 2H; 7-H, 8-H), 4.32 (t, $^3J=6.1$ Hz, 2H; γc-CH_2), 4.07 (m, $^3J=5.9$ Hz, 2H; 7d-CH_2), 3.79 (q, $^3J=7.8$ Hz, 2H; 4a-CH_2), 3.60 (s, 3H; 5a-CH_3), 3.54 (d, $^3J=6.8$ Hz, 2H; 2c-CH_2), 3.45 (s, 3H; 1a-CH_3), 3.30 (s, 3H; 3a-CH_3), 2.95 (t, $^3J=6.1$ Hz, 2H; 6a-CH_2), 2.57 (s, 6H; 6c-CH_3, 6c′-CH_3), 2.54 (t, $^3J=5.9$ Hz, 2H; γb-CH_2), 2.53 (m, 2H; 7b-CH_2), 2.46 (s, 6H; 2d-NCH_3, 2d′-NCH_3), 2.40 (t, $^3J=5.9$ Hz, 2H; 7c-CH_2), 2.14 (m, 2H; 7a-CH_2), 2.17 (s, 6H; γd-CH_3, γd′-CH_3), 2.12 (s, 6H; 7e-CH_3, 7e′-CH_3), 1.75 (d, $^2J=6.8$ Hz, 3H; 8a-CH_3), 1.72 (t, $^3J=7.3$ Hz, 3H; 4b-CH_3), −1.33 (brs, 1H; NH), −1.48 ppm (brs, 1H; NH); MS (FAB, glycerol): *m/z*: 868[M^++H]; elemental analysis: calcd (%) for $C_{46}H_{63}N_7O_6\cdot H_2O$: C 66.49, H 8.20, N 12.66; found: C 66.56, H 8.30, N 12.16.

Under a nitrogen atmosphere, iodomethane (2.5 mL) was added to 6a-,γb-,7c-tris(2-dimethylaminoethyl)-2-(3-dimethylaminoprop-1-enyl)chlorin e_6 (15.1 mg, 1.70×10^{-2} mmol) in anhydrous acetone (4.5 mL) and stirred for 24 h. The precipitate obtained was collected by filtration, washed with a small amount of anhydrous acetone, and dried over P_4O_{10} in a vacuum desiccator to give **3** as dark-violet crystals (23.2 mg, 1.50×10^{-2} mmol, 88.1%: ^{1}H NMR (300 MHz, [D_6]DMSO): $\delta=9.82$ (s, 1H; β-H), 9.69 (s, 1H; α-H), 9.15 (s, 1H; δ-H), 8.53 (d, $^3J=16$ Hz, 1H; 2a-H), 7.09 (dt, $^3J=15$, 7.8 Hz, 1H; 2b-H), 5.36 (m, 2H; γa-CH_2), 5.15 (m, 2H; 6b-CH_2), 4.62 (d, $^3J=7.1$ Hz, 2H; 2c-CH_2), 4.56 (m, 2H; 7-H, 8-H), 4.54 (m, 2H; γc-CH_2), 4.38 (m, 2H; 7d-CH_2), 4.08 (m, 2H; 6a-CH_2), 3.82 (q, $^3J=8.0$ Hz, 2H; 4a-CH_2), 3.64 (s, 3H; 5a-CH_3), 3.64 (m, 2H; 7c-CH_2), 3.58 (m, 2H; γb-CH_2), 3.58 (s, 3H; 1a-CH_3), 3.36 (s, 9H; 2d-NCH_3, 2d′-NCH_3, 2d″-NCH_3), 3.31 (s, 9H; 6c-CH_3, 6c′-CH_3, 6c″-CH_3), 3.29 (s, 3H; 3a-CH_3), 3.05 (s, 9H; γd-CH_3, γd′-CH_3, γd″-CH_3), 2.93 (s, 9H; 7e-CH_3, 7e′-CH_3, 7e″-CH_3), 2.73–3.14 (m, 2H; 7b-CH_2), 2.09–2.50 (m, 2H; 7a-CH_2), 1.70 (d, $^2J=7.1$ Hz, 3H; 8a-CH_3), 1.67 (t, $^3J=7.6$ Hz, 3H; 4b-CH_3), −1.39 (brs, 1H; NH), −1.61 ppm (brs, 1H; NH); UV/Vis (TE buffer at pH 7.6, 25°C): λ_{max} (ε) = 402.0 (1.17×10^5), 502.5 (8.92×10^3), 534.0 (4.42×10^3), 610.5 (4.36×10^3), 664.5 nm (2.98×10^4 $mol^{-1}m^3cm^{-1}$); elemental analysis: calcd (%) for $C_{53}H_{82}I_4N_8O_6\cdot6H_2O$: C 41.26, H 6.14, N 7.26; found: C 41.09, H 6.19, N 7.01.

DNA-unwinding assay: Typically pBR322 DNA (Form I, 0.5 μg in 33 μL (pH 7.9) of 25.9 mM Tris, 1.4 mM ethylenediaminetetraacetate (EDTA), 9.2 mM NaCl, 1.1 mM DTT, and 8.0% glycerol) was incubated with wheat-germ topoisomerase I (6.6 units) at 37°C for 60 min to afford a relaxed plasmid DNA (Form II). Certain amounts of the cationic water-soluble chlorin e_6 derivatives (2.0 μL) in different concentrations were added to the relaxed plasmid DNA topoisomers (33 μL) to give final concentrations of 0.44, 0.66, 0.76, 0.88, 1.1, 1.5, 1.9, 2.2, 2.5, 2.9, 3.8, 6.0, 8.0, 10, and 15 μM. These conditions correspond to *R* values ([chlorin]/[DNA in base pairs]) of 0.020, 0.030, 0.035, 0.040, 0.050, 0.066, 0.087, 0.099, 0.11, 0.13, 0.17, 0.27, 0.37, 0.46, and 0.68, respectively. The mixtures (35 μL) were incubated at 37°C for 60 min. The topoisomerase I assay was stopped with 10% SDS (3 μL) and extracted with TE-saturated phenol (35 μL). After mixing and centrifugation, the upper aqueous phase was reextracted with chloroform/isoamyl alcohol (24:1, 30 μL). This extraction process is known to remove the ethidium bromide (a DNA intercalator) used in the experiments. The above operations (after addition of the chlorins) were done in the dark to prevent exposure to light. A mixture of the upper phase (22 μL) and the loading buffer (5.5 mM Orange G, 30% glycerol; 5.5 μL) was loaded on a 1% agarose gel. The gel was subjected to electrophoresis at 50 V for 5.5 h in TAE buffer (5.6 Vcm^{-1}) and at room temperature. After electrophoresis, the gel was stained for 30 min in ethidium bromide (1.0 $\mu g mL^{-1}$) and excess ethidium bromide was removed by standing in distilled water for 10 min. The DNA bands were detected by UV light from a transilluminator and the fluorescence emission was visualized by a CCD camera connected with a Vilber Lourmat DP-001 FDC photodocumentation system for the Windows operating system. From the electrophoresis analysis, the DNA-unwinding angle (ϕ) can be calculated according to Equation (1),[16] where $\Delta\tau$ is the change in the writhing number and *m* is the number of dye molecules bound to one pBR322 molecule.

$$\phi\ (\text{in}\ ^\circ) = 360\,\Delta\tau/m \qquad (1)$$

Measurement of melting temperatures (T_m): The melting of a polynucleotide strand from double-stranded DNA is manifested as absorption hyperchromicity in the 260-nm region. The melting temperature (T_m) generally increases upon addition of DNA binders. The samples were prepared according to the following procedure. The concentration of the CT-DNA solution was determined by use of the extinction coefficient of $\varepsilon_{260}=1.31\times10^4\,M^{-1}\,cm^{-1}$ for CT-DNA.[31] A 2.5-mL solution of 35 μM CT-DNA in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.6 at 25°C) was mixed with various amounts of the cationic water-soluble chlorin e_6 derivatives at certain concentrations in TE buffer (pH 7.6) to give *R* values of 0–0.2 in a quartz cell with a magnetic stirrer, a Teflon stopper, and a 1-cm pathlength. The quartz cell filled with the sample solution was set in a jacketed cell compartment regulated by a JASCO ETC-505T temperature controller with heating and refrigeration capabilities. The temperature was measured by using a thermister probe attached to the ETC-505T controller and inserted into the quartz cell containing the sample solution through a hole in the Teflon stopper. The absorbance of the sample solution at 260 nm was measured and taken automatically every 10 s by a JASCO V-570 spectrophotometer equipped with the JASCO ETC-505T temperature controller while the sample solution was stirred continuously and heated gradually from 25–95°C at a speed of 2°C min^{-1}. The T_m value was taken as the temperature at the maximum point in the plot of absorbance versus 1/temperature.[32] The increase in T_m value (ΔT_m) was calculated by subtracting the T_m value at $R=0$ from the T_m value at each *R* value.

Spectral measurements: All measurements, except where specifically indicated, were performed in TE buffer. A stock solution of CT-DNA was prepared and stored in TE buffer. The visible absorption spectra were measured with a JASCO V-570 spectrophotometer at a spectral band pass of 1 nm with 0.1 nm spectral resolution. The calibration of wavelength was carried out by using a holmium oxide glass standard. CD spectra were obtained on a JASCO J-720 WI spectropolarimeter. Wavelength and intensity calibrations were performed by using a 0.060% (w/v) aqueous solution of ammonium (1*S*)-camphor-10-sulfonate (Aldrich). The CD spectra were recorded with the following instrument parameter settings: bandwidth = 2.0 nm, response time = 2.0 s, step resolution = 0.5 nm, and scan speed = 50 nm min^{-1} between 330–490 nm. In this report, the induced CD spectrum means the change in the CD spectrum of the cationic water-soluble chlorin e_6 derivatives caused by interaction with DNA. For instance, the induced CD at $R=0.01$ ($CD_{R=0.01}$) is calculated from $CD_{R=0.01}$ minus the original CD at $R=\infty$ ($CD_{R=\infty}$).

Equilibrium constants for the interaction of the cationic water-soluble chlorin e_6 derivatives with CT-DNA were determined by absorption spectrophotometric titration at 25 °C. A fixed amount of cationic chlorins in TE buffer solution was titrated with the stock solution of CT-DNA. The changes in the absorbance of the Soret band upon addition of CT-DNA were monitored at the maximum wavelength of the Soret band. The apparent equilibrium binding constant (K_{app}) between the cationic water-soluble chlorin e_6 derivatives and DNA was calculated from Equation (2), where ε_{app}, ε_1, and ε_2 correspond to $A_{observed}$/[chlorin], the extinction coefficient for the chlorin in the fully bound form in the first step (at $R=0.67$ for **1** or **2** and $R=0.50$ for **3**), and the extinction coefficient for the chlorin in the fully bound form in the second step, respectively.

$$[DNA]_{total}/(|\varepsilon_{app}-\varepsilon_1|) = \{1/(|\varepsilon_2-\varepsilon_1|)\}[DNA]_{total}+1/\{K_{app}(|\varepsilon_2-\varepsilon_1|)\} \quad (2)$$

In the plot of $[DNA]_{total}/(|\varepsilon_{app}-\varepsilon_1|)$ versus $[DNA]_{total}$, the K_{app} value is given by the ratio of the slope to the intercept.[33]

Photomodification and gel electrophoresis: The efficiency of DNA-strand photocleavage in the presence of the cationic water-soluble chlorin e_6 derivatives was determined by a supercoiled plasmid DNA assay. The sample solutions were prepared in TE buffer saturated with air. Typically, DNA stock solution (2 μL; 1.76×10^{-7} M; 7.68×10^{-4} M in base pairs) was added to the buffer solution of the cationic water-soluble chlorin e_6 derivatives (6 μL) at various concentrations. The mixture (8 μL), prepared in a clear microtube, was used for irradiation. To examine the effect of the concentration of the cationic water-soluble chlorin e_6 derivatives on DNA photocleavage, the sample solutions were prepared at various concentrations, that is, 4.40 ($R=0.011$), 8.80 (0.023), 17.6 (0.046), 25.4 (0.069), 35.2 (0.092), 44.0 (0.115), and 88.0 μM (0.229), and photoirradiated for 5 h by the method described below. All sample solutions for the study of the DNA-photocleavage mechanism were adjusted to 25.4 μM ($R=0.069$) and photoirradiated for 0–3 h. In addition, the concentrations of *N*-acetylhistidine, mannitol, and SOD in the buffer solution were adjusted to 100 mM for *N*-acetylhistidine and mannitol and 75 U mL^{-1} for SOD. The samples were prepared for the experiment in D_2O with the buffer solution made in 87.5% D_2O (12.5% H_2O). The buffer solution for the experiment in N_2 were frozen and thawed twice and then N_2 was bubbled through the solution for 30 min. The samples were prepared with this oxygen-free buffer solution and the microtubes were sealed with a polypropylene film in a chamber purged with N_2. The other samples were sealed in the air in the same manner. The microtubes containing the sample solutions prepared in these ways were placed in a thermostatically controlled holder at 25 °C and located 5.0 cm away from a light source. Photoirradiation was carried out with a mercury lamp (BHF-100–110 V, 160 W, Matsushita Electric Industrial Co., Ltd.) from which the light was passed through an interference filter (B-390, Hoya Optics) to select wavelengths (404.5 and 436.0 nm). After irradiation (0–5 h), the samples were sequentially purified with phenol and chloroform/isoamyl alcohol, then loading buffer was added and the samples were subjected to agarose gel electrophoresis. The gel was subjected to electrophoresis for 2.5 h in TAE buffer at 80 V (8.9 V cm^{-1}) and at room temperature. After electrophoresis, the gel was stained for 30 min in ethidium bromide (1.0 μg mL^{-1}) and the excess ethidium bromide was removed by standing in distilled water for 10 min. The DNA bands were detected by UV light and the fluorescence emission was visualized by a CCD camera. The densitometric quantification of the various forms of DNA (Forms I–III) was carried out with a Vilber Lourmat DP-001 FDC photodocumentation system for the Windows operating system.

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