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Interaction and photodynamic activity of cationic porphyrin derivatives bearing different patterns of charge distribution with GMP and DNA

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ABSTRACT

The interaction of amphiphilic cationic porphyrins, containing different patterns of *meso*-substitution by 4-(3-*N*,*N*,*N*-trimethylammoniumpropoxy)phenyl (A) and 4-(trifluoromethyl)phenyl (B) groups, with guanosine 5′-monophosphate (GMP) and calf thymus DNA have been studied by optical methods in phosphate buffer solution. The properties of these synthetic porphyrins were compared with those of representative standard of anionic 5,10,15,20-tetra(4-sulphonatophenyl)porphyrin ($TPPS_4{}^{4-}$) and cationic 5,10,15,20-tetra(4-*N*,*N*,*N*-trimethylammonium phenyl)porphyrin ($TMAP^{4+}$). Stable complexes with GMP were found for cationic porphyrins, except for monocationic $AB_3{}^+$. The binding constant ($K_{GMP} \sim 10^4\ M^{-1}$) follows the order: $A_3B^{3+} \sim ABAB^{2+} > A_4{}^{4+} \sim TMAP^{4+}$. Also, interaction with DNA was observed for all evaluated cationic porphyrins. For these related cationic porphyrins, the binding constant ($K_{DNA} \sim 10^5\ M^{-1}$) increases with the number of cationic charges. On the other hand, the photodynamic activity of porphyrins was analyzed in solution of GMP and DNA. Monocationic $AB_3{}^+$ is a less effective sensitizer to oxidize GMP in comparison with the other cationic porphyrins, in agreement with the lack of detected interaction with this nucleotide. The electrophoretic analysis of DNA indicates that photocleavage takes place when the samples are exposed to photoexcited tricationic and tetracationic porphyrins. In the presence of sodium azide the DNA decomposition was diminished. Also, reduction in the DNA photocleavage was observed under anoxic condition, indicating that oxygen is essential for DNA photocleavage sensitized by these cationic porphyrins. In addition, an increase in DNA degradation was not observed in deuteriated water. Therefore, an important contribution of type I photoreaction processes could be occurring in the DNA photodamage sensitized by these cationic porphyrins. These results provide a better understanding of the characteristics needed for sensitizers to produce efficient DNA photocleavage.

1. Introduction

In the last years, positively charged porphyrins have attracted considerable attention because of their remarkable ability as phototherapeutic agents. In particular, cationic porphyrin derivatives have been proposed for the treatment and control of microorganisms by photodynamic inactivation (PDI). This methodology is based on the administration of a photosensitizer, which is preferentially accumulated in the microbial cells. Subsequent irradiation with visible light, in the presence of oxygen, specifically produces cell damages that inactivate the microorganisms [1–3].

Porphyrins containing cationic groups are able to interact with DNA bases, inducing DNA lesions upon photoactivation [4,5]. Thus, the binding of cationic sensitizers to DNA is of considerable interest. In general, three binding models have been described for the interaction of cationic porphyrins with DNA, which involve intercalation, outside groove binding and outside binding with porphyrins self-stacking [6]. Intercalative binding of *meso*-tetrakis(4-*N*-methylpyridyl)porphyrin leads to a large red shift (>10 nm) and a extensive hypochromicity of the *Soret* band [7].

Cationic porphyrins can act as bifunctional compounds, which form strong complexes with DNA and induce photodynamic or chemical modifications in DNA structure [8,9]. The DNA complexes involving cationic porphyrins are presumably stabilized by electrostatic interaction between the positively charged substituents on the macrocycle periphery and the negatively charged phosphate oxygen atoms of DNA. When a DNA-bound porphyrin is photoexcited, cleavage of DNA and nucleotides can be initiated by mainly two oxidative mechanisms. In the type I photochemical reaction, the photosensitizer interacts with an adjacent base pair to produce free radicals through electron- or H-transfer. On the other hand, in type II reactions the energy of photosensitizer triplet state is transferred to molecular oxygen ground state forming singlet molecular oxygen, $O_2(^1\Delta_g)$ [10,11]. Type II photosensitization, in

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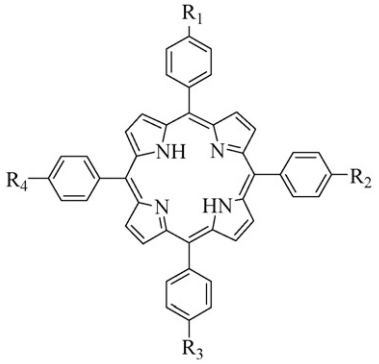
contrast to type I, does not require the binding of the porphyrin to DNA because $O_2(^1\Delta_g)$ can diffuse in the solution. In the DNA chain, guanine residues have shown to be the most easily oxidized [12–15]. Both mechanisms can occur simultaneously and the ratio between two processes depends mainly on the sensitizer, substrate and the nature of the medium.

In previous works, we have investigated the photodynamic activity of cationic porphyrin derivatives in different biomimetic media and *in vitro* as sensitizers to eradicate Gram-negative bacteria [16,17]. Porphyrins bearing three cationic charges and a highly lipophilic trifluoromethyl group showed to be active photosensitizers to inactivate *Escherichia coli* cells in liquid suspension. Previous studies about mechanistic aspects conclude that TMPyP-dependent PDI of *E. coli* is primarily dependent on genomic DNA photodamage rather than on protein or membrane malfunctions [18]. However, TMPyP is structurally different from the porphyrins used in the present investigation and the results are difficult to extrapolate. The efficiency of cationic porphyrin derivatives could be influenced by their DNA binding. Therefore, it is important to know if these cationic porphyrins can bind to DNA and produce chain cleavage after photoexcitation. Also, there is a great interest in the interaction of molecules with nucleic acid by its possible applications in biotechnology. These molecules could be used in DNA *footprinting*, in the design of telomerase inhibitors, in stabilizing DNA/RNA hybrids and in the development of materials for selective cleavage of DNA and RNA. Other interesting applications are the use of the photodynamic effect over the nucleic acids as an unspecific endonuclease. In living organisms, the complex formation with DNA can interrupt the specific base recognition of several enzymes for DNA repair, replication and transcription.

In this paper, we have evaluated the interaction of amphiphilic cationic porphyrins, with different patterns of *meso*-substitution by 4-(3-*N*,*N*,*N*-trimethylammoniumpropoxy)phenyl (A) and 4-(trifluoromethyl)phenyl (B) groups (Scheme 1), with guanosine 5′-monophosphate (GMP) and calf thymus DNA. In these porphyrins, the cationic centers are isolated from the porphyrin ring by a propoxy bridge. Thus, the charges have minimal influence on the electronic density of the tetrapyrrolic macrocycle. This design allows keeping the consistency of the photophysical properties of the sensitizer similar to those of *meso*-tetraphenylporphyrin.



A_4^{4+}	R_1=R_2=R_3=R_4:-$O(CH_2)_3N^+(CH_3)_3I^-$	
A_3B^{3+}	R_1=R_2=R_3:-$O(CH_2)_3N^+(CH_3)_3I^-$	R_4:-CF_3
$ABAB^{2+}$	R_1=R_3:-$O(CH_2)_3N^+(CH_3)_3I^-$	R_2=R_4:-CF_3
AB_3^{+}	R_1:-$O(CH_2)_3N^+(CH_3)_3I^-$	R_2=R_3=R_4:-CF_3
$TMAP^{+4}$	R_1=R_2=R_3=R_4:-$N^+(CH_3)_3OTos^-$	
$TPPS_4^{4-}$	R_1=R_2=R_3=R_4:-$SO_3^-Na^+$	

Scheme 1. Molecular structures of porphyrins.

Also, this chain provides a higher mobility of the charge on the porphyrin, which could facilitate the interaction with biomolecules. The cationic charges in these porphyrins are combined with a highly lipophilic trifluoromethyl group, which increases the amphiphilic character of the structure. The behavior of these cationic porphyrins were compared with those of a standard active tetracationic photosensitizer, 5,10,15,20-tetra(4-*N*,*N*,*N*-trimethylammoniumphenyl)porphyrin ($TMAP^{4+}$) and an anionic model 5,10,15,20-tetra(4-sulphonatophenyl) porphyrin ($TPPS_4^{4-}$) (Scheme 1). Photodynamic efficiencies of these sensitizers were analyzed in the presence of GMP or DNA. Guanine is believed to be the site of $O_2(^1\Delta_g)$ attack in the photodynamic decomposition of DNA. Thus, complexes of DNA with efficient photosensitizers, such as TMPyP, produce photosensitized cleavage of DNA. The photodamage can be initiated by electron- and/or energy-transfer between the excited porphyrin to an near base pair or by $O_2(^1\Delta_g)$ formed in close proximity to DNA [19]. Therefore, in order to obtain mechanistic evidences photocleavage of DNA was studied in the presence of sodium azide, under anaerobic conditions and in deuteriated water. This investigation provides further understanding of the characteristics needed for sensitizers to produce efficient DNA photocleavage. In addition, the results are compared with the activity of these cationic porphyrins to inactivate bacteria by PDI.

2. Materials and methods

2.1. General

Absorption spectra were recorded on a Shimadzu UV-2401PC spectrometer. Spectra were recorded using 1 cm path length quartz cuvettes at $25.0 \pm 0.5\,^{\circ}C$. The light source used was a Novamat 130 AF slide projector equipped with a 150 W lamp. The light was filtered through a 2.5-cm glass cuvette filled with water to absorb heat. A wavelength range between 350 and 800 nm was selected by optical filters. The light intensity at the treatment site was 90 mW/cm^2 (Radiometer Laser Mate-Q). Electrophoresis was performed on a IBI MP-1015 Horizontal Gel Electrophoresis Unit Shelton Scientific and the electric field was generated by power supply PS251-2 (Sigma–Aldrich Techware).

All the chemicals from Aldrich (Milwaukee, WI, USA) were used without further purification. Calf thymus double-stranded DNA was purchased from Sigma (St. Louis, MO, USA). Solvents (GR grade) from Merck were distilled. Ultrapure water was obtained from Labconco (Kansas, MO, USA) equipment model 90901-01.

2.2. Sensitizers

5-[4-(3-*N*,*N*,*N*-Trimethylammoniumpropoxy)phenyl]-10,15,20-tris(4-trifluoromethyl phenyl) porphyrin (AB_3^+), 5,15-di[4-(3-*N*,*N*,*N*-trimethylammoniumpropoxy)phenyl]-10,20-di(4-trifluoromethylphenyl) porphyrin iodide ($ABAB^{2+}$), 5,10,15-tris[4-(3-*N*,*N*,*N*-trimethylammonium propoxy)phenyl]-20-(4-trifluoromethylphenyl)porphyrin iodide (A_3B^{3+}) and 5,10,15,20-tetrakis[4-(3-*N*,*N*,*N*-trimethylammoniumpropoxy)phenyl]porphyrin iodide (A_4^{4+}) were synthesized as previously described [20]. 5,10,15,20-Tetra(4-*N*,*N*,*N*-trimethylammoniumphenyl)porphyrin *p*-tosylate ($TMAP^{4+}$) and 5,10,15,20-tetra(4-sulphonatophenyl)porphyrin ($TPPS_4^{4-}$) sodium salt were purchased from Aldrich. A porphyrin stock solution ($\sim 5.0 \times 10^{-4}$ M) was prepared by dissolution in 1 mL of water. The sensitizers concentrations were checked by spectroscopy, taking into account the value of molecular extinction coefficients (ε) [20,21].

2.3. Porphyrin binding to GMP or DNA

Stock solution of GMP (10 mM) was prepared by weighing and dilution in water. The concentration of calf thymus double-stranded DNA stock solution (2.7 mM) calculated in base pairs, was determined spectrophotometrically using molar absorptivity $\varepsilon_{260} = 1.31 \times 10^4\ M^{-1}\ cm^{-1}$ [4]. Absorbance titrations were conduced by adding concentrated stock solution of GMP or DNA directly to a cuvette containing porphyrin solution (2 mL, ~2 μM) in PBS. The apparent binding constants (K_{GMP}) for porphyrin–GMP complex were calculated from the absorbance changes at the *Soret* maximum (ΔA) assuming a 1:1 stoichiometry and that the nucleotide concentration is always significantly larger than the porphyrin concentration using Eq. (1), where $(\Delta A)_\infty$ represents the extrapolated absorbance change at [GMP] → ∞ [4,22].

$$\frac{1}{\Delta A} = \frac{1}{(\Delta A)_\infty} + \frac{1}{(\Delta A)_\infty K\,[\mathrm{GMP}]} \quad (1)$$

A plot of $1/\Delta A$ *vs.* 1/[GMP] was used to calculate the value of K_{GMP} from the ratio of the intercept to the slope. In similar way, the K_{DNA} values were determined from Eq. (1) but using DNA concentration instead GMP [5].

2.4. Steady-state photolysis of GMP and DNA

Solutions of GMP (116 μM in DMF/water 10% (v/v), 2 mL) and photosensitizer ($A^{420} = 0.3$) were irradiated in quartz cuvettes with visible light of 90 mW/cm^2. The disappearance of GMP was monitored by decrease in the absorption peak at 272 nm [4]. Similar studies were carried out in presence of 100 mM sodium azide. Where appropriate, oxygen was removed from solution by the use of argon purging.

A mixture of 1.3×10^{-4} M calf thymus DNA and 20 μM sensitizer in 1 mL of TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.6) was irradiated with visible light (90 mW/cm^2) for different times. A sample of 15 μL was taken each time and gently mixed whit 3 μL of Loading Buffer 6× (bromophenol blue 0.25% (w/v), sucrose 40% (w/v), acetic acid 1.15%, Tris 40 mM, EDTA 1 mM). DNA was analyzed by electrophoresis 1% agarose gel in TBE buffer (90 mM Tris–HCl, 90 mM boric acid and 2 mM EDTA, pH 8) at 2.9 V/cm for 2 h. Ethidium bromide (1 mg/mL) was incorporated into the agarose gel. The photographs were analyzed using ImageJ (National Institute of Health, USA) software to quantify the amount of DNA remaining after different treatments.

The inhibition assay of DNA photocleavage was performed in the presence of sodium azide (100 mM), which was added to DNA solution in TE prior to illumination. Studies in absence of oxygen were performed as described above but displacing the oxygen with argon in the tubes for 10 min before irradiation and maintaining argon atmosphere during the illumination.

3. Results and discussion

3.1. Porphyrin spectroscopic properties

The absorption spectrum of cationic porphyrins in DMF shows the typical *Soret* and *Q*-bands characteristic of free-base porphyrin derivatives with λ_{max} at about 420, 515, 550, 590 and 650 nm. The relative intensities of the *Q*-bands for these porphyrins show an *etio*-type spectrum ($\varepsilon_{VI} > \varepsilon_{III} > \varepsilon_{II} > \varepsilon_{I}$) [23]. The spectra of porphyrins were also analyzed in different media. The absorption maxima at *Soret* band are summarized in Table 1. Sharp absorption bands were obtained in organic solvents indicating that these porphyrins are mainly non-aggregated in these media. The solvatochromic effect on the location of *Soret* absorption bands shows a slight blue shift (~4 nm in all cases) upon solubilization in methanol with respect to a non-hydrogen bond donor, DMF. In water, the spectra of A_3B^{3+} and A^{4+} remain very similar to those obtained in organic solvents with a sharp *Soret* band and absorption maxima matching those in methanol (Table 1). However, a broadening of the *Soret* band is observed for AB_3^{+} and $ABAB^{2+}$, indicating that partial aggregation occurs in water, as is typical for many porphyrin derivatives with only one or two cationic groups [16,24].

Table 1
Position (nm) of *Soret* absorption bands and molecular extinction coefficients ($\varepsilon \times 10^{-5}\ M^{-1}\ cm^{-1}$, between parentheses) of porphyrins in different media

Porphyrin	DMF	Methanol	Water	PBS
AB_3^{+}	418 (1.71)	414 (1.62)	420 (0.72)	422 (0.75)
$ABAB^{2+}$	419 (1.67)	415 (1.42)	424 (0.57)	439 (0.50)
A_3B^{3+}	420 (1.69)	416 (1.71)	417 (1.15)	421 (0.96)
A_4^{4+}	421 (1.64)	417 (1.56)	418 (1.02)	419 (0.52)

Two generalized aggregate structures, *H*-aggregates and *J*-aggregates, have been recognized for water-soluble porphyrin [25]. The formation of *J*-aggregates can take place in water and the formation increases upon addition of phosphate buffer [4]. *J*-aggregates were found for porphyrins containing charged nitrogen atoms in the *meso*-phenyl substituents and the formation is characterized by a red shifted *Soret* band. In our cases, this effect is mainly significant for porphyrin $ABAB^{2+}$. The spectra of this dicationic porphyrin in PBS show two bands, a shoulder at 423 nm due to the monomer absorption and a maximum at 439 nm. The last band, which presents a bathochromic shift of ~20 nm (Table 1), can be assigned to the formation of a *J*-aggregate in this saline medium.

3.2. Interaction of porphyrin with GMP

Titration of cationic porphyrins with GMP in PBS produces the spectral changes in the *Soret* band. Typical results for A_3B^{+3} are shown in Fig. 1. The extensive hypochromicity (24–50%, Table 2) of *Soret* band observed for $ABAB^{2+}$, A_3B^{3+}, A_4^{4+} and $TMAP^{4+}$ indicates that these porphyrins bind to GMP. It is assumed that the electronic structure of the porphyrin is not greatly affected by

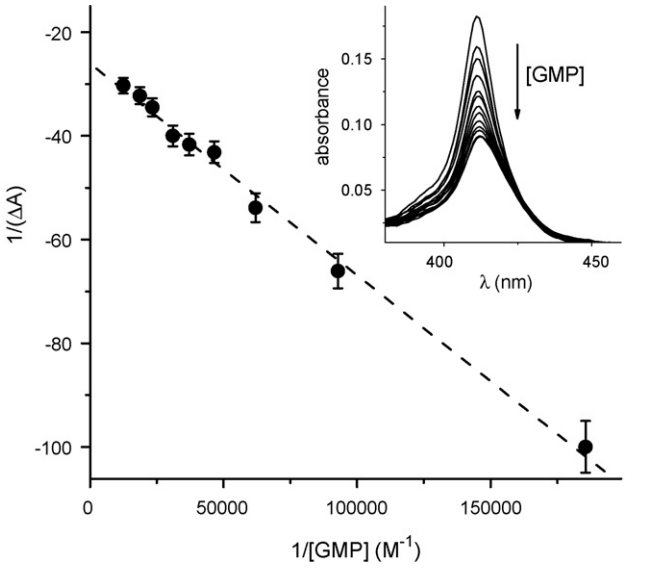


Fig. 1. Variation of $1/(\Delta A)$ vs. 1/[GMP] for spectral titration of A_3B^{+3} with GMP in PBS, λ_{max} = 411 nm. Dashed line: linear fit by Eq. (1). Inset: absorption spectra of A_3B^{+3} in PBS at different GMP concentrations (5.4, 10.7, 16.1, 21.5, 26.8, 32.2, 42.9, 53.6, 80.2 μM). Values represent mean ± standard deviation of three separate experiments.

Table 2
Position of *Soret* band for complexes, bathochromic shift ($\Delta\lambda$), hypochromic effect (h), apparent binding constant (K_{GMP}) of porphyrins with GMP in PBS and singlet molecular oxygen quantum yield (Φ_Δ).

Porphyrin	*Soret* band (nm)	$\Delta\lambda$ (nm)	h (%)	K_{GMP} (M^{-1})	Φ_Δ
AB_3^+	422[a]	0	–	N[a]	0.41[b]
$ABAB^{2+}$	426	0	24	$(5.9 \pm 0.3) \times 10^4$	0.53[b]
A_3B^{3+}	420	−3	30	$(6.4 \pm 0.2) \times 10^4$	0.53[b]
A_4^{4+}	418	4	33	$(1.2 \pm 0.1) \times 10^4$	0.51[b]
$TMAP^{4+}$	411	1	50	$(1.4 \pm 0.1) \times 10^4$	0.65[b]
$TPPS_4^{4-}$	413[a]	–	–	N[a]	0.71[c]

[a] Absorption spectroscopy gives no evidence of binding.
[b] From Ref. [17] in DMF.
[c] From Ref. [27] in water.

interaction with GMP because the positively charged groups are separated by a propylene bridge from the chromophore. Spectral analysis indicates that these porphyrins have a high affinity for GMP. Apparent binding constants (K_{GMP}, Table 2) of tetracationic porphyrins, A_4^{4+} and $TMAP^{4+}$, were calculated giving values of $\sim 1 \times 10^4\ M^{-1}$. These values are comparable with those previously reported for the interaction of tetracationic TMPyP and 5,10,15,20-tetrakis(α-trimethylammonium-*p*-tolyl)porphyrin tetrabromide ($K_{GMP} \sim 7 \times 10^3\ M^{-1}$) [4]. Also, a value of $2433\ M^{-1}$ was reported for the interaction of TMPyP with dGMP [22]. In the present study, higher values of K_{GMP} were found for dicationic and tricationic porphyrins ($K_{GMP} \sim 6 \times 10^4\ M^{-1}$). These results not only suggest that the number of charge is important for an effective interaction but also, the presence of a high lipophilic trifluoromethyl group, which enhances the amphiphilic character of the macrocycle, could help a more effective interaction.

In contrast, no significant changes were observed for AB_3^+ in the presence of GMP, either because they do not bind to nucleotide or because the binding does not perturb the porphyrin chromophore. Also, negligible spectral change was obtained with $TPPS^{4-}$, indicating that there is no binding interaction between the two anionic species.

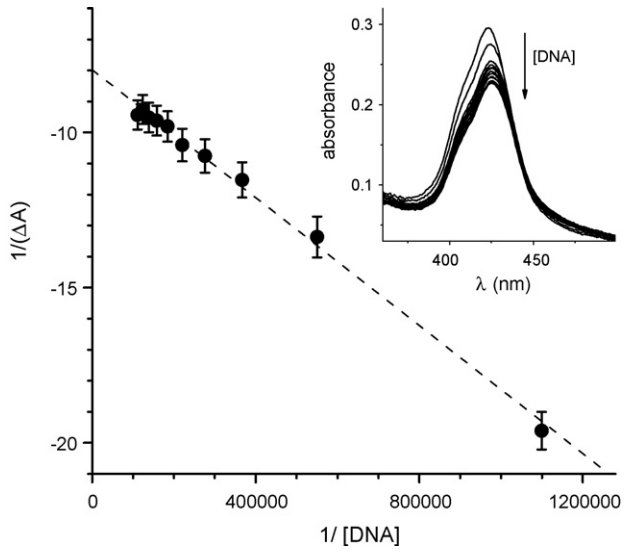


Fig. 2. Variation of $1/(\Delta A)$ vs. $1/[DNA]$ for spectral titration of A_3B^{+3} with DNA in PBS, $\lambda_{max} = 423$ nm. Dashed line: linear fit by Eq. (1). Inset: absorption spectra of A_3B^{+3} in PBS at different DNA concentrations (0.9, 1.8, 2.7, 3.6, 4.5, 5.4, 6.3, 7.2, 8.1, 9.0 μM). Values represent mean ± standard deviation of three separate experiments.

3.3. Binding of porphyrin with calf thymus DNA

In a similar way, the affinity of these porphyrins for calf thymus double-stranded DNA was analyzed by spectral titration. While the *Q*-bands of the cationic porphyrins do not shift significantly upon addition of DNA, the *Soret* band exhibited changes allowing quantification of porphyrin–DNA binding. Representative results are shown in Fig. 2 for A_3B^{+3} in PBS. It was assumed that spectral perturbations upon addition of DNA are due to association of the porphyrin with the DNA matrix. This interaction is characterized by a red shift of the *Soret* maximum of ∼4 nm and by a large hypochromicity (20–42%). The large hypochromicity suggests that porphyrin π electrons are perturbed by the association with DNA. Also, as observed above for GMP, the addition of DNA does not perturb the spectrum of $TPPS_4^{4-}$ indicating a negligible interaction with nucleotides possibly by electrostatic repulsion with negative charges of phosphate groups.

Absorption spectra were analyzed to obtain the values of apparent binding constant of porphyrin–DNA (K_{DNA}), which are summarized in Table 3. From these results several comparisons can be performed. First, the results for the two tetracationic symmetric porphyrins are significantly different. A higher value of K_{DNA} ($40 \times 10^5\ M^{-1}$) was found for A_4^{4+} with respect to $TMAP^{4+}$ ($7.5 \times 10^5\ M^{-1}$). The K_{DNA} for $TMAP^{4+}$ is comparable with those previously reported for other tetracationic porphyrin derivatives with charges directly attached to the tetrapyrrolic macrocycle. For example, values of 1.3×10^6 and $4.35 \times 10^5\ M^{-1}$ have been calculated for TMPyP [4,26]. In sensitizers, such as A_4^{4+}, the cationic centers are isolated from porphyrin ring by a propylene bridge and therefore the positive charges are localized on ammonium groups increasing the electrostatic interaction with the nucleotide.

For porphyrins with different number of charges and patterns of substitution, the apparent binding constants increase with the number of cationic charges on the macrocycle periphery ($K_{DNA} \sim 0.6–40 \times 10^5\ M^{-1}$). Although, the K_{DNA} value for A_3B^{+3} ($4.4 \times 10^5\ M^{-1}$) is about one order of magnitude lower than that for A_4^{4+}, it is quite comparable with those of other tetracationic porphyrins, such as $TMAP^{4+}$ and TMPyP, in spite of A_3B^{3+} has three cationic groups. A similar K_{DNA} value was reported for a tricationic porphyrin with a perfluorinated alkyl chain ($-COC_7F_{15}$) [8]. The amphiphilic structure of these porphyrins bears a highly lipophilic fluorinated group, which could increase the binding with DNA.

Although, the values of K_{DNA} for $ABAB^{+2}$ is slightly lower than those reported for the interaction of DNA with several tri- and tetraporphyrins, it is quite similar to those reported in the literature for *trans*-dicationic porphyrins derivatives [26]. The result obtained with AB_3^+ is lower because this monocationic porphyrin is lack of positive charges compared with the other sensitizers consequently it has weaker electrostatic interaction with DNA.

Table 3
Position of *Soret* band for complexes, bathochromic shift ($\Delta\lambda$), hypochromic effect (h) and apparent binding constant (K_{DNA}) of porphyrins with calf thymus DNA in PBS.

Porphyrin	*Soret* band (nm)	$\Delta\lambda$ (nm)	h (%)	K_{DNA} (M^{-1})	Cell survival[a]
AB_3^+	425	3	26	$(0.6 \pm 0.1) \times 10^5$	5.9 ± 0.4
$ABAB^{2+}$	442	3	20	$(1.8 \pm 0.6) \times 10^5$	5.3 ± 0.5
A_3B^{3+}	426	5	27	$(4.4 \pm 0.4) \times 10^5$	2.1 ± 0.4
A_4^{4+}	425	6	36	$(40 \pm 4) \times 10^5$	2.7 ± 0.3
$TMAP^{4+}$	417	5	42	$(7.5 \pm 0.3) \times 10^5$	2.3 ± 0.3
$TPPS_4^{4-}$	–	–	–	N[b]	6.1 ± 0.4

[a] Photoinactivation of *Escherichia coli* cells incubated with 1 μM of sensitizer for 30 min at 37 °C in dark and irradiated for 10 min with visible light (90 mW/cm²) from Ref. [17] (log CFU/mL, control 6.1 ± 0.5).
[b] Absorption spectroscopy gives no evidence of binding.

3.4. Sensitized decomposition of GMP

All sensitizers evaluated in this work present a high efficiency in the quantum yield of $O_2(^1\Delta_g)$ production (Φ_Δ) (Table 2) [17,27]. However, the values of Φ_Δ can significantly change according to the medium, diminishing when the sensitizer is partially aggregated. Thus, there are limitations to predict photodynamic activity of porphyrins in different media even though in the presence of substrates those have affinity for theses sensitizers.

The nucleotide GMP was chosen as a model for the compounds of biological interest that would be potential targets of porphyrin photosensitization and because guanine is believed to be the site of $^1O_2(^1\Delta_g)$ attack in the photodynamic decomposition of DNA [19,28]. Photosensitization of GMP was carried out in DMF/water (10%, v/v) because this is an appropriate medium to dissolve all porphyrins in monomeric form. Continuous irradiation of porphyrin with visible light in air-saturated condition leads to GMP decomposition as evidenced by the formation of a broad absorption band above 300 nm (Fig. 3A, inset). It is not possible to calculate the decomposition rate constant of GMP from these absorption data because of the overlap of the spectra of GMP with its degradations products. However, photosensitized decomposition of GMP can be monitored in PBS from decrease of the GMP peak at 272 nm as shown in Fig. 3A. As can be observed, AB_3^{+} sensitizes GMP decomposition slower than the other cationic porphyrins, probably due to that this monocationic porphyrin does not interact with the nucleotide but also because it has lower $O_2(^1\Delta_g)$ production (Table 2). However, since cationic porphyrins bind to GMP by electrostatic attraction, an electron-transfer pathway may also be contributing to its decomposition under these conditions [29]. In fact type I photosensitization mechanism leads to one-electron oxidation of nucleobase with a high preference for the guanine base that exhibits the lowest ionization potential among DNA components. This leads to degradation products such as 8-oxo-7,8-dihydroguanine (8-oxoGua) and related open imidazole ring compound, namely 2,6-diamino-4-hydroxy-5-formamidopyrimidine that are both issued from the same precursor [14]. Type II mechanism which is usually the prevalent acting mechanism for porphyrin derivatives is operating through the generation of $O_2(^1\Delta_g)$. The only DNA component target for the reaction of $O_2(^1\Delta_g)$ is guanine and as a result 8-oxoGua is formed predominantly both in isolated and cellular DNA [15]. Thus, the present results show that these cationic porphyrin can photodecompose GMP in aerobic condition, conducing to a possible photodamage in the DNA chain.

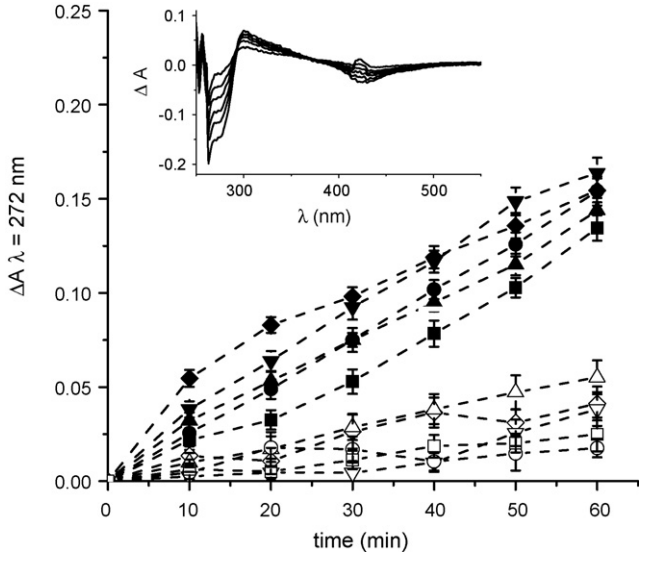


Fig. 3. Photosensitized decomposition of GMP (100 μM) sensitized by AB_3^{+1} (■), $ABAB^{+2}$ (●), A_3B^{+3} (▲), A_4^{4+} (▼) and $TMAP^{+4}$ (♦) in air-saturated solution of DMF/water (10%, v/v) and AB_3^{+1} (□), $ABAB^{+2}$ (○), A_3B^{+3} (△), A_4^{4+} (▽) and $TMAP^{+4}$ (◊) in presence of 100 mM NaN_3, from decrease of GMP absorption peak at 272 nm. Inset: different absorption spectra of GMP in the presence of A_3B^{+3} (absorbance = 0.3 at 420 nm) irradiated with visible light (90 mW/cm^2) for 10 min intervals. Values represent mean ± standard deviation of three separate experiments. PBS (A).

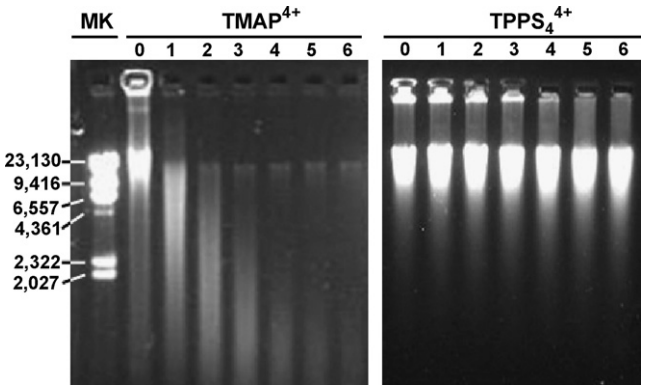


Fig. 4. Agarose gel electrophoresis of calf thymus DNA samples in TE buffer (pH 7.6) irradiated by 0, 1, 2, 3, 4, 5 and 6 h with visible light (90 mW/cm^2) in the presence of $TMAP^{4+}$ or $TPPS^{4-}$ (20 μM). MK: Lamda-Hin III DNA weight maker.

When these experiments were performed under oxygen-free conditions, these porphyrins even produce photosensitized decomposition of GMP. In the present cases, the changes in the absorbance at 272 nm reach values of ~0.1 after 60 min of irradiation, which are comparable to those found in air-saturated solutions. Similar behavior was observed before using tetracationic porphyrin derivatives as sensitizers [4]. On the other hand, photooxidation of this substrate is protected in the presence of sodium azide. In all cases, a concentration of 100 mM NaN_3 produces only a variation in the absorbance at 272 nm of ~0.05 after 60 min of irradiation (Fig. 3B). Therefore, alternative type I photoreaction pathway can be occurring, which involves a direct interaction between the excited photosensitizer and the substrate. Since these cationic compounds have a high binding affinity for GMP, an electron-transfer pathway may also be contributing together with type II photoprocess to their decomposition in this medium.

3.5. Photocleavage of calf thymus DNA

Photoinduced damage of calf thymus DNA sensitized by these porphyrins was studied in PBS solution. Samples of DNA and photosensitizer were irradiated with visible light under aerobic condition for different times. The DNA integrity was analyzed by agarose gel electrophoresis. Typical results are shown in Figs. 4 and 5. In all cases, no cleavage occurs without irradiation, indicating that cleavage of DNA take place by photodynamic action of the porphyrins. DNA photodamage is observed by the

Table 4
Photocleavage of DNA (%) (fragments between 9000 and 23,000 base pairs) sensitized by porphyrins after different irradiation times with visible light (90 mW/cm^2)

Porphyrin	1 h	2 h	3 h	4 h	5 h	6 h
$TMAP^{4+}$	59.3	94.0	99.3	99.7	99.6	99.8
$TTPS_4^{4-}$	0.7	1.1	1.3	2.0	1.2	7.5
AB_3^{+}	0.5	0.7	0.6	0.8	1.0	1.2
$ABAB^{2+}$	0.9	1.0	1.2	3.0	6.0	25.0
A_3B^{3+}	1.3	1.6	7.0	18.0	28.0	44.0
A_4^{4+}	1.0	1.5	2.0	18.0	29.0	32.0

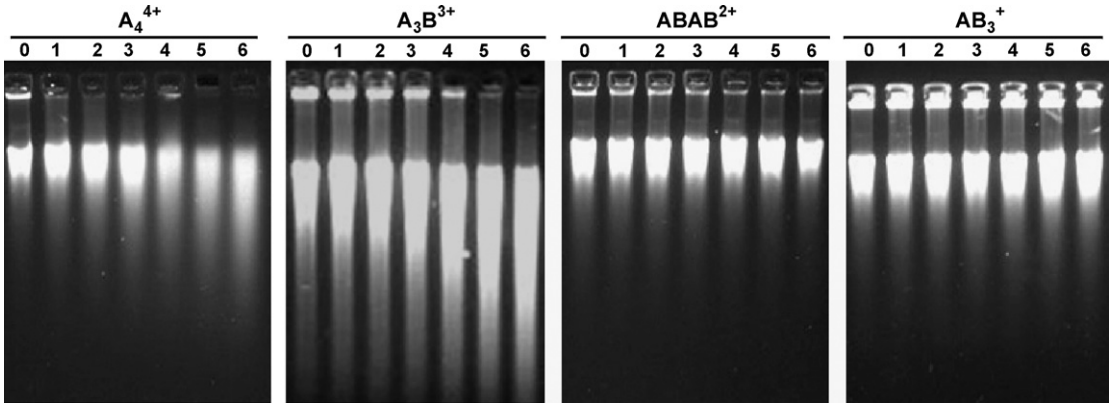


Fig. 5. Agarose gel electrophoresis of calf thymus DNA samples in TE buffer (pH 7.6) irradiated by 0, 1, 2, 3, 4, 5 and 6 h with visible light (90 mW/cm^2) in the presence of A_4^{4+}, A_3B^{3+}, $ABAB^{2+}$ and AB_3^{+} (20 μM).

appearance of a remarkable smearing after electrophoresis. The comparison of the DNA cleaving efficiencies of these porphyrins is shown in Table 4. The results show that $TMAP^{4+}$ produces a marked reduction (59.3%), in DNA band even after 1 h irradiation. On the other hand, $TPPS_4^{4-}$ does not cause any significant cleavage of DNA even after exposure to light for an extended period of 5 h. The electrophoretic results for cationic porphyrins (AB_3^{+}, $ABAB^{2+}$, A_3B^{3+} and A_4^{4+}) are summarized in Fig. 5. As can be observed, the photocleavage effect on DNA is detected when the samples are sensitized by A_4^{4+} and A_3B^{+3} after 3 h irradiation, while a slightly modified pattern for DNA with respect to control was found with mono- or dicationic sensitizers. Under these conditions the photocleavage ability of these related cationic porphyrins appears to have a relationship with their apparent binding constants to DNA, except for the tetracationic porphyrin. Even though A_4^{4+} has 10 times larger K_{DNA} than A_3B^{3+}, it presents a similar activity to decompose DNA in comparison with tricationic sensitizer. Also, A_4^{4+} is not as effective as $TMAP^{4+}$ to photocleavage DNA (Table 4).

To evaluate the photodynamic process, the DNA samples were irradiated in the presence of sodium azide, in absence of oxygen and in deuteriated water. Sodium azide is a quencher of $O_2(^1\Delta_g)$ but it also can deactivate compounds in their triplet excited state, thus preventing both type I and type II photoprocesses. In the presence of sodium azide (NaN_3, 100 μM), the cleavage of DNA introduced by the porphyrins were partially inhibited. Representative results are shown in Fig. 6, lines 4 and 8, after different periods of irradiation

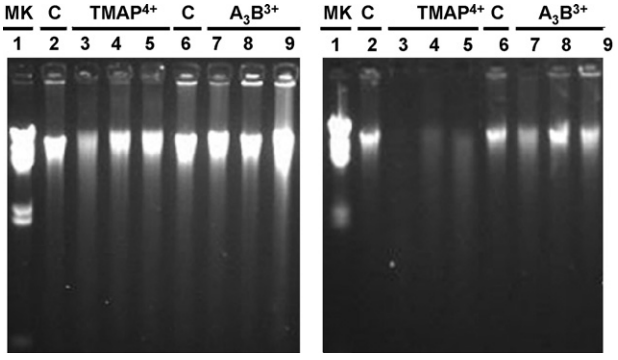


Fig. 6. Agarose gel electrophoresis of calf thymus DNA samples in TE buffer (pH 7.6) irradiated by 2 h (left) and 4 h (right) with visible light (90 mW/cm^2) in the presence of $TMAP^{4+}$ and A_3B^{3+} (20 μM). Line 1: MK Lamda-Hin III DNA weight maker DNA, lines 2 and 6: DNA control without porphyrin, lines 3 and 7: DNA in the presence of porphyrin (20 μM), lines 4 and 8: DNA in the presence of porphyrin (20 μM) and sodium azide (100 mM), lines 5 and 9: DNA in the presence of porphyrin (20 μM) and argon atmosphere.

for samples sensitized by $TMAP^{4+}$ and A_3B^{+3}. In particular for tricationic porphyrin, the protection produced by azide ion is mainly detected after longer irradiation (4 h) where the photodamage in absence of quencher is appreciable (Fig. 7). Under this condition, azide ion produces a complete DNA photoprotection for samples sensitized by A_3B^{+3}, while it reduces a 20% photocleavage using $TMAP^{4+}$.

Similar behavior is found in atmosphere of argon (Fig. 6). In anoxic condition DNA photoprotection is clearly observed after 2 h irradiation for samples treated with $TMAP^{4+}$ and A_3B^{+3}. However, partial DNA decomposition is observed for samples sensitized by these porphyrins after longer irradiation (4 h). In both cases, the argon atmosphere produces a protection of ~20% in the DNA decomposition (Fig. 7). Under these conditions, a possible electron- or proton-transfer could be producing a slow photodynamic effect on DNA.

In addition, experiments were carried out in deuteriated water in order to increase the lifetime of $O_2(^1\Delta_g)$. However, very similar behavior was obtained comparing the DNA photodamage sensitized by A_3B^{+3} or $TMAP^{4+}$ in deuteriated water with that in water (Fig. 7). Therefore, these results also evidence a small contribution of type II photosensitization in the DNA photocleavage sensitized by these cationic porphyrins.

In previous studies, we have evaluated the photodynamic activity of these photosensitizers *in vitro* on a typical Gram-negative bacterium *E. coli* [17]. The results show that the tricationic A_3B^{3+} and both tetracationic, A_4^{4+} and $TMAP^{4+}$, are effective photosensitizers to inactivate *E. coli* cellular suspensions in PBS. Examples of these results are summarized in Table 3. As can be seen, photosensitization of cells by $ABAB^{2+}$ and AB_3^{+} produce a low reduction in the cellular viability (<1 log), whereas a negligible effect was found for an anionic sensitizer, $TPPS_4^{4-}$. In all cases, photoinactivation of *E. coli* cells is considerably reduced under anoxic condition, indicating that an oxygen atmosphere is required for the mechanism of bacterial photodynamic inactivation [17]. The presence of oxygen is essential for the generation of $O_2(^1\Delta_g)$ through the type II photosensitization mechanism that involves a triplet energy-transfer reaction. However, oxygen plays also a major in type I mechanism by adding to neutral biochemical radicals and preventing recombination to occur between initially pair of radical ion and radical cation. Oxygen is also essential for the formation of superoxide anion radical that can occur as the result of the reaction of molecular oxygen with the radical anion of the photosensitizer.

According to the present results, the potency of photosensitizers to inactive *E. coli* cells is accompanied by high binding to DNA. This is the case for both tetracationic porphyrins, which present a high

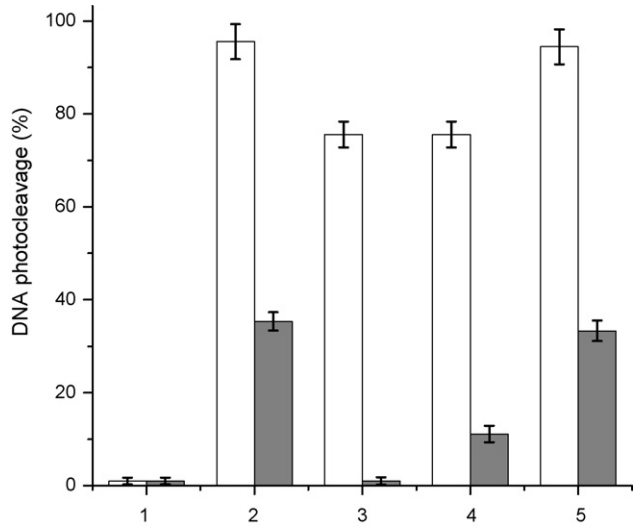


Fig. 7. Photocleavage of DNA (%) (fragments between 9000 and 23,000 base pairs) sensitized by $TMAP^{+4}$ (white bars) and A_3B^{+3} (grey bars) porphyrins after 4 h irradiation with visible light (90 mW/cm^2). Column 1: DNA control without porphyrin, column 2: DNA in the presence of porphyrin (20 μM), column 3: DNA in the presence of porphyrin (20 μM) and sodium azide (100 mM), column 4: DNA in the presence of porphyrin (20 μM) and argon atmosphere, column 5: DNA in the presence of porphyrin (20 μM) in deuteriated water.

interaction with double-stranded DNA and they produce efficient DNA photocleavage after irradiation mainly under aerobic condition and in absence of $O_2(^1\Delta_g)$ quencher. However, even though A_3B^{3+} is moderately active to photodecompose DNA in comparison with $TMAP^{4+}$, this tricationic porphyrin is very effective to inactive *E. coli* cells by PDI.

4. Conclusions

Cationic *meso*-tetraphenyl porphyrins *para*-substituted with $-O(CH_2)_3N^+(CH_3)_3$ and $-CF_3$ groups form complexes with GMP ($K_{GMP} \sim 10^4\ M^{-1}$) when the macrocycle contains at least two positive charges. Higher K_{GMP} values were obtained for those amphiphilic porphyrins, A_3B^{3+} and $ABAB^{2+}$, with asymmetric charge distribution in the periphery. However, this difference in the interaction with GMP does not appear to be highly significant in the sensitized decomposition of the nucleotide. In the other hand, the binding with double-stranded DNA increases with the number of cationic charges. Photocleavage of DNA was only detected for samples sensitized by tricationic and tetracationic porphyrins but the effect was higher in the presence of $TMAP^{4+}$. Although, $O_2(^1\Delta_g)$ mediation could be involved in DNA photocleavage, the binding affinity of the sensitizer to DNA is an important factor for an efficient decomposition. Photoprotection of DNA cleavage was found in the presence of sodium azide, which considerably reduces the extent of damage within DNA. Protection is almost complete for DNA sensitized by A_3B^{3+}, even after 4 h of irradiation. Moreover, rather large amount of damages are still produced when experiments are performed under argon atmosphere, which are not expected for a singlet oxygen-driven chemistry. In addition, porphyrin with four negative charges ($TPPS_4{}^{4-}$), which does not bind to DNA but exhibits a very good of Φ_Δ, does not produce significant DNA damage. On the other hand, an increase in DNA degradation is not observed in deuteriated water, in spite of $O_2(^1\Delta_g)$ lifetime increases in this medium. Therefore, an important contribution of type I photoreaction processes, which requires direct interaction between sensitizer and substrate, could be occurring in the DNA photodamage sensitized by these cationic porphyrins.

The high activity in DNA photocleavage showed by these tetracationic porphyrins correlates with an efficient PDI of *E. coli* cells, indicating that DNA photodamage could be involved in the cellular inactivation as previously suggested for TMPyP [18]. However, this is not necessarily the case of A_3B^{3+}, where in spite of this sensitizer is not as active as $TMAP^{4+}$ to photocleavage DNA, it is highly effective for the treatment of *E. coli* by PDI. Further investigations of photosensitization *in vitro* are presently in progress in our laboratory to establish mechanistic aspects of *E. coli* photoinactivation sensitized by these cationic porphyrins.

Acknowledgments

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