

Water-Soluble Mn(III)-Porphyrins with High Relaxivity and Photosensitization

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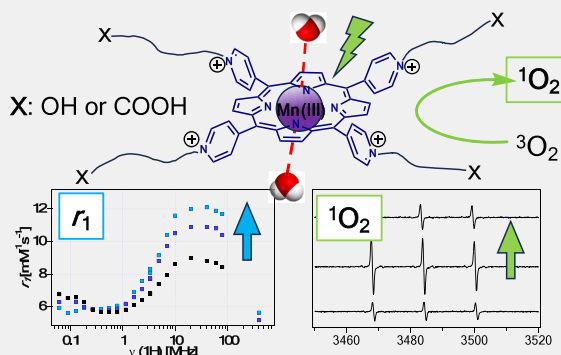
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ABSTRACT: Three water-soluble Mn(III)-porphyrin complexes with cationic pyridyl side groups bearing COOH- or OH-terminated carbon chains in the meta or para positions have been synthesized as probes for both magnetic resonance imaging (MRI) and photodynamic therapy (PDT). The complexes **Mn-1**, **Mn-2**, and **Mn-3** are highly water-soluble, and their relaxivities range between 10 and 15 mM⁻¹ s⁻¹, at 20–80 MHz and 298 K, 2–3 times higher than that of commercial Gd(III)-based agents. The complexes containing carboxylate (**Mn-2**) or alcoholic (**Mn-3**) side chains in the para position are endowed with higher relaxivities and have also shown efficient photoinduced DNA cleavage and singlet oxygen (¹O₂) generation. **Mn-3** with stronger photoinduced DNA cleavage has also revealed stabilizing and binding activities for G4 DNA, at a similar level as the known G4 binder **Mn-TMPyP4**. Nevertheless, the G4-binding activity of **Mn-3** was nonspecific. Preliminary tests evidenced photocytotoxicity of **Mn-3** on HeLa cells without a significant effect in the absence of light. Altogether, these results underline the potential of such water-soluble Mn(III)-porphyrins for the development of multimodal approaches combining MRI and PDT.

KEYWORDS: MRI contrast agent, Mn(III)-porphyrin complex, relaxivity, ROS generation, photodynamic therapy



INTRODUCTION

The design of efficient imaging probes, such as near-infrared (NIR) fluorophores, radioactive molecules, or paramagnetic metal complexes, has a pivotal role in the development of highly sensitive and functional in vivo imaging approaches based on optical, nuclear, or magnetic resonance imaging (MRI) modalities. Among these, MRI, which provides deep tissue images with excellent resolution, is regarded as a desirable, noninvasive technique that does not involve ionizing radiation hazards. To achieve a more effective contrast enhancement in MR images, paramagnetic contrast agents (MRI-CAs) are often injected into the body prior to the examination.

All MRI-CAs in current clinical use are Gd(III) complexes. Due to its seven unpaired electrons ($S = 7/2$) and slow electron spin relaxation, Gd(III) greatly enhances the relaxation process of water protons in the surrounding tissue.^{1,2} However, Gd(III) is toxic in its free, noncomplexed form, and there has been increasing concern related to toxic side effects of Gd(III)-based MRI agents, especially in patients with chronic kidney diseases (CKD).^{3–6} These include (i) rare, but serious pathologies, such as contrast-induced nephropathy (CIN)^{7,8} and nephrogenic systemic fibrosis (NSF),⁹ and (ii) the accumulation of Gd(III) in the brain of patients who received repeated Gd(III)-chelate injections.^{10–15} To avoid

these potential risks of Gd(III) complexes, Mn(II/III)-based contrast agents have recently attracted attention and are generally considered as sufficiently safe imaging probes.^{16–19} Although Mn(III) contains only four unpaired electrons ($S = 2$), one less than Mn(II) ($S = 5/2$), some Mn(III) complexes such as porphyrins are endowed with elevated relaxivity at typical clinical magnetic fields (>1.5 T), where the efficacy of Mn(II) or Gd(III) chelates tend to decrease. In Mn(III)-porphyrin complexes, it is known that the metal is coordinated with an octahedral geometry involving planar coordination of the four equatorial pyrrolic nitrogens and two additional axial ligands.¹¹ In aqueous solutions, water molecules can occupy these axial positions. Mn(III)-porphyrins have long been investigated for their water proton relaxation efficacy, with a recent revival of this field.^{20–31} Given the hydrophobic nature of porphyrins and their tendency to aggregate in aqueous solution, one of the key challenges in this field involves improvement of water-solubility. Lately, Mn-porphyrins have

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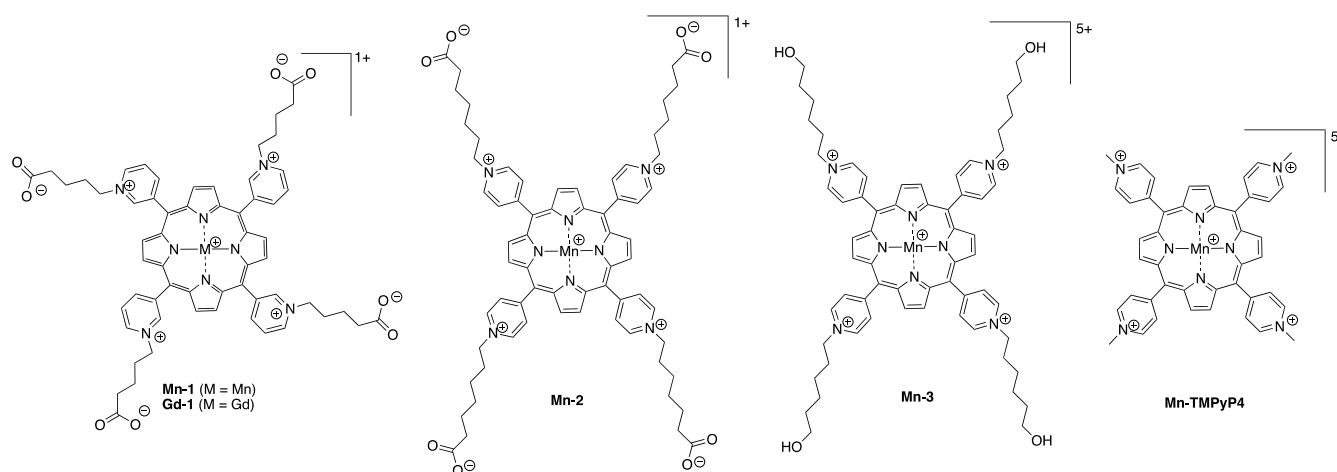


Figure 1. Structures of Gd(III)-porphyrin complex (**Gd-1**, previously reported⁴³) and Mn(III)-porphyrin complexes (**Mn-1**, **Mn-2**, **Mn-3**, and **Mn-TMPyP4**).

been also explored as potential redox-sensitive MRI probes relying on the different relaxivity of the reduced Mn(II) and the oxidized Mn(III) forms.^{30,32,33} In addition, some fluorinated derivatives have been studied as ¹⁹F MRI probes.³⁴ Furthermore, porphyrins are also well-known in the field of photodynamic therapy (PDT) as efficient photosensitizers (PSs).³⁵ Many clinically approved PDT-PSs belong to the family of porphyrins.³⁶ The combination of photosensitizing and MRI contrast enhancement capabilities within a single molecular entity has been the subject of many studies, and again, porphyrin derivatives are among the most explored systems in this field.^{37–42}

Recently, we have reported a Gd(III)-porphyrin complex (**Gd-1** in Figure 1) that offers high relaxivity as well as ROS generation and subsequent DNA cleavage under visible light irradiation.⁴³ While this complex was stable in a neutral buffer at physiological pH thanks to the supposed participation of the pending COO[−] functions in Gd(III) coordination, undesired decomplexation was observed under lower pH (<5.5). This low stability is likely due to the large ionic radius of Gd(III), leading to a less stable out-of-plane coordination by the porphyrin nitrogens and to the weak binding of the distant carboxylates. Mn(III)-complexation by porphyrins, on the other hand, is known to be extremely stable, reducing the risk of free metal leakage. Therefore, we have investigated the Mn(III) complex of the same porphyrin chelator, **Mn-1**, as well as two additional water-soluble Mn(III)-porphyrins, **Mn-2** and **Mn-3** (Figure 1), with a cationic charge in the para positions. A specific objective of this study was to test the influence of the side chains attached to the porphyrin core, which bear either a negatively charged COO[−] or a neutral OH terminal group. Field-dependent proton relaxivities, characterizing the efficacy of the complexes as potential MRI probes, were compared for these different systems. In addition, the photosensitivity⁴⁴ of porphyrins **2** and **3** and of their Mn(III)-complexes (**Mn-2** and **Mn-3**) has been studied by photo DNA cleavage, and ¹O₂ generation as monitored by ESR in the presence of a spin-trapping agent. Further, their binding to oncogene-related guanine quadruplex (G4) DNA has been investigated by a FRET assay. Finally, photocytotoxicity tests were performed on HeLa cells. For comparison, in these studies, we have also included the known G4-binding

molecule, **TMPyP4** and its Mn(III) complex as reference compounds (Figure 1).

EXPERIMENTAL SECTION

General

All reagents were purchased from Sigma-Aldrich Co. LLC (Merck KGaA, Darmstadt, Germany) unless described otherwise and purified as described when needed. Solvents were purchased from Acros Organic (Thermo Fischer Scientific, Inc., Geel, Belgium), and HPLC-grade solvents were purchased from Sigma-Aldrich Co. LLC (Merck KGaA), and dried by a solvent system (Innovative Technology Inc., Brooksville, FL) or distilled when needed. All water used was from Millipore purification system. Column chromatography and analytical TLC were performed, respectively, on SILICYCLE SilicaFlash F60 (230–400 mesh with pore size 60 Å) and silica gel 60 F254 TLC (Merck KGaA). Flash chromatographic purifications were performed on a Büchi Reveleris X2 flash purification system (Büchi Labortechnik AG, Flawil, Switzerland) using Büchi Reveleris C18 columns (PN 145171532) for reverse phase separation. NMR spectra were recorded at room temperature on a Bruker AV300 300 MHz spectrometer or a Bruker AV400 400 MHz spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany). The HR-MS data were obtained using a Bruker Solarix-FTICR 9.4 T MS instrument (Bruker Daltonics, Bremen, Germany). FT-IR spectra were recorded on a JASCO 4100 FT-IR spectrometer equipped with an ATR Pro One instrument (JASCO, Inc., Tokyo, Japan). The UV–vis spectra were measured on a Varian Cary-500 UV–vis spectrophotometer (Agilent, Santa Clara, CA) using a cuvette with 1 cm optical path (Quartz Cell, Type F15–UV-10, transmission 85.5%, GL Sciences Inc., Tokyo, Japan).

Synthesis

Detailed procedures for the synthesis of new compounds (**3**, **Mn-1–3**) and the reference compound (**Mn-TMPyP4**) including their synthetic intermediates are described in SI with available spectroscopic data.

Relaxometric Measurements

¹H NMRD profiles of aqueous solutions of **Mn-1**, **Mn-2**, **Mn-3**, and **Mn-TMPyP4** in 0.1 M HEPES buffer (pH 7.4) were measured at 25 and 37 °C on a Stelar SMARtracer fast field cycling NMR relaxometer (0.00024–0.24 T, 0.01–10 MHz ¹H Larmor frequency) (Stelar s.r.l, Mede, Italy) and a Bruker WP80 NMR electromagnet adapted to variable-field measurements (0.47–1.88 T, 20–80 MHz), and controlled by the SMARtracer PC-NMR console. The temperature was controlled by a VTC91 control unit and maintained by a gas flow. The temperature was determined according to a previous calibration with a Pt resistance temperature probe. The relaxivity at 400 MHz was determined on a Bruker Advanced NMR spectrometer.

152 ¹⁷O NMR Studies

153 Variable-temperature ¹⁷O chemical shifts and transverse relaxation
154 rates were measured on aqueous solutions of **Mn-1** and **Mn-TMPyP4**
155 on a Bruker Avance 400 MHz spectrometer using a 10 mm BBFO
156 probe (9.4 T, 54.2 MHz) in the temperature range 5–75 °C. The
157 temperature was calculated according to published calibration
158 routines with ethylene glycol and MeOH.⁴⁵ Acidified water
159 (HClO₄, pH 3.3) was used as a diamagnetic reference. Transverse
160 ¹⁷O relaxation times were obtained by the Carl–Purcell–Meiboom–
161 Gill spin–echo technique. To eliminate susceptibility corrections to
162 the chemical shifts, the sample was placed in a glass sphere fixed in a
163 10 mm NMR tube. To improve sensitivity, H₂¹⁷O (10% H₂¹⁷O,
164 CortecNet) was added to achieve ~1% ¹⁷O content in the sample.
165 The pH of the samples was 7.0 (0.1 M HEPES buffer), and the
166 complex concentrations were 0.975 mmol·kg^{−1} and 2.45 mmol·kg^{−1}
167 for **Mn-1** and **Mn-TMPyP4**, respectively. The equations used for the
168 analysis have been included in SI (section 2).

169 DNA Cleavage Tests

170 Photoirradiation was carried out using a green LED light source
171 (λ_{max} = 527 nm). A Mupid–exu gel electrophoresis apparatus
172 (Takara Bio, Kyoto, Japan) was used for agarose gel electrophoresis.
173 DNA cleavage studies were performed using supercoiled, covalently
174 closed circular pBR 322 DNA (New England Biolabs, Ipswich, MA).
175 Typically, a mixture of pBR 322 DNA (10 μL, 25 μg·mL^{−1} in Tris-
176 HCl (10 mM)-EDTA (1 mM) buffer, pH = 8), 40 mM NADH (5
177 μL) or H₂O (5 μL) and 12.8 μM porphyrin aqueous solution (5 μL)
178 was placed in a 0.5 mL Eppendorf tube. The tubes were placed with a
179 distance of 2.5 cm from the green LED and exposed to the light for 0
180 or 4 h. Each resulting mixture (15 μL) was subjected to gel
181 electrophoresis (1% agarose gel, in a 0.5x TBE buffer system at 100 V
182 for 80 min). The gel was stained with ethidium bromide (1 μg·mL^{−1})
183 and was visualized on an ETX–35.M UV transilluminator (λ_{ex} = 312
184 nm, Vilber–Lourmat, Collégien, France) and photographed. The
185 obtained images were analyzed using ImageJ software to quantify the
186 amount of relaxed circular DNA (form II) relative to the intact DNA
187 (form I).

188 ¹O₂ Generation by the ESR Spin-Trapping Method

189 ¹O₂ generation was measured by ESR using 2,2,6,6-tetramethyl-4-
190 piperidone (4-oxo-TEMP, Acros, 98%) as a spin-trapping reagent.
191 ESR spectra were recorded on a Bruker spectrometer (Bruker BioSpin
192 GmbH) equipped with a microwave bridge X-band ER 082C. Light
193 irradiation and ESR measurements were carried out on each sample in
194 a disposable capillary with a 50 μL volume (cat. no. 120800,
195 Blaumann, Wertheim, Germany), which was placed inside an ESR
196 tube with a 4 mm thin wall precision quartz, 250 mm in length (cat.
197 no. ZS67442, Wilmad, Vineland, NJ). As a standard sample, a known
198 ¹O₂-generating compound, Rose Bengal, was used. To an aliquot of
199 porphyrin or Rose Bengal in D₂O (0.5 mM, 20 μL) were added 0.5 M
200 4-oxo-TEMP (4 μL, in D₂O), 0.3 M phosphate buffer (10 μL, pH 7, in
201 D₂O), and D₂O (16 μL) and mixed well under aerobic conditions.
202 Each mixed solution was irradiated by green LED light (λ_{max} = 527
203 nm, PN 31867, Lumitronix, Hechingen, Germany) at a distance of 3
204 cm for 0, 1, 2, 5, and 10 min, introduced into a capillary, placed inside
205 an ESR tube, and immediately subjected to the ESR measurements.
206 The generation of ¹O₂ was detected by the ESR signal corresponding
207 to 4-oxo-TEMPO. Time-dependent data are shown in SI (section 5).

208 G4 Stabilization Assay by FRET

209 The G4 DNA (c-MYC, KIT or Rf1-2) labeled with FAM (5' end)
210 and TAMRA (3' end) was denatured at 95 °C for 5 min at the
211 concentration of 0.2 μM and subsequently incubated at room
212 temperature to form the G4 structure. Each G4 DNA was mixed with
213 each compound with concentrations of 0.25, 0.5, 1, 2, and 4 μM. The
214 melting temperatures at each concentration were recorded on a
215 LightCycler 480 II (Roche, Basel, Switzerland). FRET detection was
216 carried out upon a temperature change from 37 to 95 °C with a ramp
217 speed of 1 °C per minute.

G4 Binding Assay

One picomole of the single-stranded oligonucleotide ³²P-labeled G4
DNA (parallel-type G4 T₆G₂₄) or non-G4 T₆[GA]₁₂ was heat-
denatured and reannealed in the presence of 50 mM KCl and 40%
PEG200. DNA was incubated with each porphyrin (0.1 and 1 nmol
per reaction, or at the final concentration of 10 and 100 μM,
respectively) at room temperature for 10 min in 40 mM HEPES-
KOH (pH 7.6) containing 50 mM KCl, 1 mM EDTA, 10% glycerol,
and 1 mM DTT. The complexes and free DNA were separated in 1%
agarose gels in the presence of 0.1 M MES (pH 6.1) and 0.1 M
histidine and detected by radiography on Hybond N+ filter (Cytiva,
Tokyo, Japan). The detailed data are shown in SI (section 7).

Photocytotoxicity

A photocytotoxicity test was performed on HeLa cells and under
irradiation from a green LED (527 nm) using an MTT assay. The
details are described in SI (section 8).

Cytotoxicity

MCF-7, U2OS, or HeLa cells were precultured for 1 day in a 96-well
plate (1 × 10³ per well). The compounds were added at 2, 20, or 200
μM and incubated for an additional 2 days. Each culture medium was
replaced with 1/10-diluted WST-1 reagent (Roche, Basel, Switzer-
land), the cells were treated for 2 h, and then the absorbances at 450
and 595 nm were monitored by iMark (Bio-Rad, Hercules, CA). The
OD₄₅₀ values were subtracted by OD₅₉₅. As a control experiment,
samples without ligands were set as 100 and the relative values were
calculated.

RESULTS AND DISCUSSION

Synthesis of Water-Soluble Mn(III)-Porphyrins **Mn-1**, **Mn-2**,
and **Mn-3**

Water-soluble Mn(III)-porphyrin complex (**Mn-1**, Figure 1)
was synthesized in a similar way to its analogous Gd(III)-
chelate (**Gd-1**, Figure 1), which was recently reported.⁴³
Porphyrin **1** was prepared as previously reported and subjected
to the complexation reaction using MnCl₂ followed by
oxidation with air oxygen to provide a Mn(III)-complex
(**Mn-1**) (Scheme S1). The obtained complex was purified by
reversed phase HPLC and characterized by HR-ESI-MS
(Figure S2), which confirmed the 1-to-1 stoichiometry of the
Mn(III)-porphyrin complexation.

Figure 2 shows the UV–vis spectra of the free porphyrin
ligand **1** and its Mn(III) complex **Mn-1** in comparison to the
previously reported Gd(III) analogue **Gd-1**.⁴³ The Soret band
of free ligand **1** has an absorption maximum at 418 nm, which
shifted to 428 nm upon complexation with Gd(III) (**Gd-1**). It
further red-shifted to 461 nm, with a smaller intensity, upon
Mn(III) complexation (**Mn-1**), in full accordance with typical

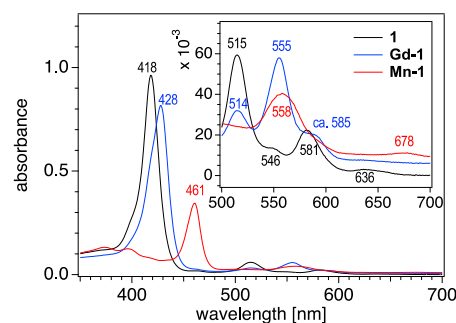


Figure 2. UV–vis spectra of porphyrin ligand **1** and its Mn(III)-complex (**Mn-1**) and Gd(III)-complex (**Gd-1**) at 12.5 μM in HEPES buffer (1 M, pH 7.4) at 25 °C.

absorption characteristics of Mn(III)-porphyrin complexes.⁴⁶ The position of this absorption band also confirms the oxidation of Mn(II) to the Mn(III) state.³⁴ Q-bands with four peaks in free **1** (maxima at 515, 546, 581, and 636 nm) changed upon metal complexation; the Gd(III)-complex (**Gd-1**) has three peaks (at 514, 555, and ca. 585 nm), indicating sitting atop (SAT) coordination structure, while the Mn(III)-complex (**Mn-1**) shows two bands (at 558 and 678 nm), suggesting higher symmetry in the structure. This can be explained by the planar metal coordination by the porphyrin in **Mn-1** that is related to the smaller ionic radius of Mn(III) in comparison to that of Gd(III), leading to a highly stable and inert chelate. In addition to the four pyrrole nitrogens coordinated in the porphyrin plane, two water molecules complete the coordination sphere in axial positions, above and below the porphyrin plane.

Two other Mn(III)-porphyrin complexes (**Mn-2** and **Mn-3**, Figure 1) with substitution at the para position of the pyridyl groups were synthesized. It was expected that COOH and OH groups, respectively, in the anchors of **Mn-2** and **Mn-3**, would help to increase water-solubility to provide the higher biocompatibility of the molecules. Porphyrin **2** was synthesized as previously reported.⁴³ Porphyrin **3** was synthesized by N-alkylation of pyridylporphyrin **S1** with bromoalkyl alcohol (Schemes S3). As a control molecule, the Mn(III) complex of **TMPyP4** (**Mn-TMPyP4**) was prepared. These porphyrins, compounds **2**, **3**, and **TMPyP4**, formed complexes with Mn(III) in a 1-to-1 stoichiometry as confirmed by HR-ESI-MS (Figures S5, S13, and S19). All porphyrins and their corresponding Mn(III) complexes were thoroughly soluble in water. As shown in Figure 3a–c, changes in UV–vis absorption spectra of porphyrin upon Mn(III) complexation were observed in a similar manner as in the case of **Mn-1** (Figure 2), showing the red shift and decreased intensity of the Soret band and reduced peak numbers in Q-bands indicating the higher symmetry in the Mn(III) complex structure. These situations were in line with the previously reported Mn(III) complexes.⁴⁶

A cyclic voltammetry study has been carried out on **Mn-1** (pH 6.9) and **Mn-TMPyP4** (pH 7.2) in 0.01 M HEPES buffer and yielded $E^\circ = -0.220$ V and -0.192 V (vs Ag/AgCl) for the midpoint potential of the Mn(II)/Mn(III) redox couple of the two complexes, respectively (see SI section 3 for details). The latter value is in good agreement with literature data.⁴⁷

Relaxivity and ¹⁷O NMR Studies of Mn(III)-Porphyrins

The efficacy of a paramagnetic complex to act as MRI-CAs is typically characterized by its relaxivity, which is the paramagnetic enhancement of the longitudinal water proton relaxation rate referred to as the 1 mM concentration of the probe. The capability of Mn(III)-porphyrin complexes to enhance MRI contrast has been evaluated by recording ¹H nuclear magnetic relaxation dispersion (NMRD) profiles at two different temperatures (25 and 37 °C), between 0.05 and 400 MHz, in HEPES buffer (1 M, pH 7.4). The ¹H NMRD profiles of **Mn-1**, **Mn-2**, **Mn-3**, and **Mn-TMPyP4** are depicted in Figure 4.

The shapes of these NMRD curves all presented a peak centered around 40–60 MHz, which was characteristic of Mn(III)-porphyrins, and the absolute relaxivity values fell also into the typical range reported for soluble, small-molecule Mn(III)-porphyrin complexes containing two inner sphere water molecules. Considerably decreased relaxivities have been

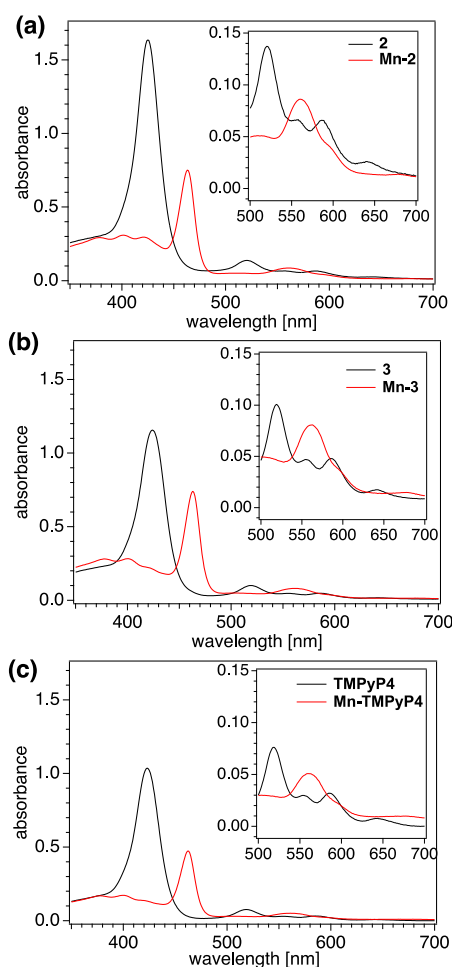


Figure 3. UV vis spectra of water-soluble cationic porphyrins **2** (a), **3** (b), and **TMPyP4** (c) and their Mn(III)-complexes **Mn-2**, **Mn-3**, and **Mn-TMPyP4** (12.5 μ M in HEPES buffer (1 M, pH 7.4) at 25 °C).

reported for more hydrophobic Mn(III)-porphyrins which undergo extensive aggregation in aqueous solutions.^{22,34} This clearly shows the importance of the charged groups appended on the porphyrin ring in ligands **1**, **2**, **3**, and **TMPyP4**, which maintain water-solubility. Indeed, aggregation was assumed to limit the exchange between the bound and solvent water molecules, which is very important for an efficient transfer of the paramagnetic relaxation effect of Mn(III) to the bulk.²² We note that in contrast to **Gd-1**, the carboxylate functions do not coordinate to the central metal ion in **Mn-1**, leaving space for the coordination of two inner sphere water molecules. This difference is related to the larger size and higher coordination number of Gd(III), implying an out-of-plane position and the coordination by the carboxylate functions of this metal ion. On the other hand, Mn(III) fits perfectly into the porphyrin plane.

At all magnetic fields and at both temperatures, the relaxivities are very similar for **Mn-1** and **Mn-TMPyP4**. On the other hand, above ~ 5 MHz, **Mn-2** and **Mn-3** have considerably higher relaxivities. It is generally accepted that the analysis of the NMRD curves of Mn(III)-porphyrins is problematic in terms of the existing paramagnetic relaxation theory to extract the parameters characterizing the various microscopic and dynamic processes, such as water-exchange, rotational motion, or electron spin relaxation. These problems are largely related to the unusual role of zero field splitting

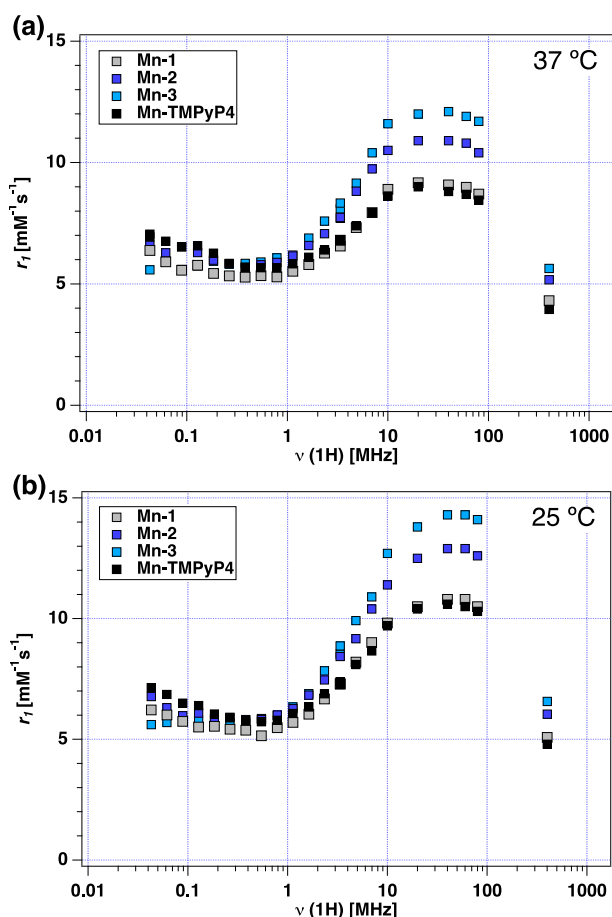


Figure 4. ^1H NMRD profiles of Mn-1–3 and Mn-TMPyP4 (1 mM in 0.1 M HEPES buffer (pH 7.4)) recorded at 37 °C (a) and 25 °C (b).

similar low field relaxivities for all four systems suggest that their electron relaxation differences are not very important and cannot account for the different high-field values. Indeed, Bryant et al. have previously compared Mn(III)-porphyrin complexes endowed with different electron spin relaxation originating from the presence or the absence of bromine atoms at the β -pyrrole positions, leading to different delocalization of the electron spin density into the ligand.²¹ They have demonstrated that electron spin relaxation affects both low- and high-field relaxivities. We can also exclude the effect of water exchange to account for the different proton relaxivities of Mn-2 and Mn-3 versus Mn-1 and Mn-TMPyP4. The rate of water exchange has been previously assessed by ^{17}O NMR studies on various Mn(III) porphyrins bearing positive or negative charges and has been shown to be independent of the porphyrin charge. The measured rates ranged between 4 and $40 \times 10^6 \text{ s}^{-1}$ (298 K).^{50,51}

We have also assessed the water exchange rate by measuring variable temperature ^{17}O chemical shifts and transverse relaxation rates for aqueous solutions of Mn-1 and Mn-TMPyP4 at 9.4 T (Figure 5). The two complexes show very similar behavior, and the reduced chemical shifts and transverse relaxation rates are also similar to those reported for bishydrated Mn(III)-porphyrins in the literature.^{50,51} This unambiguously shows the bishydrated nature of our complexes, indicating that the carboxylate functions in Mn-1 are not coordinated to the metal ion. The ^{17}O NMR data have been analyzed according to the Swift–Connick equations (see SI for details).⁵² The rate of the water exchange obtained from the fit is $k_{\text{ex}}^{298} = (7.0 \pm 0.4) \times 10^6 \text{ s}^{-1}$ for both Mn-1 and Mn-TMPyP4, while the activation enthalpy is 30 and 22 $\text{kJ}\cdot\text{mol}^{-1}$, respectively, for the two complexes. Very similar exchange rates have been previously reported for analogous Mn(III)-porphyrins.^{50,51}

These k_{ex} values imply that the water exchange is fast enough not to limit the relaxivity for such small molecular weight complexes. This is also evidenced by the increase of the relaxivities with decreasing temperature. Therefore, the high-

(ZFS) interactions and how these affect electron spin dynamics.^{48,49} Nevertheless, we can draw several qualitative conclusions by comparing the relaxivity curves. The relatively

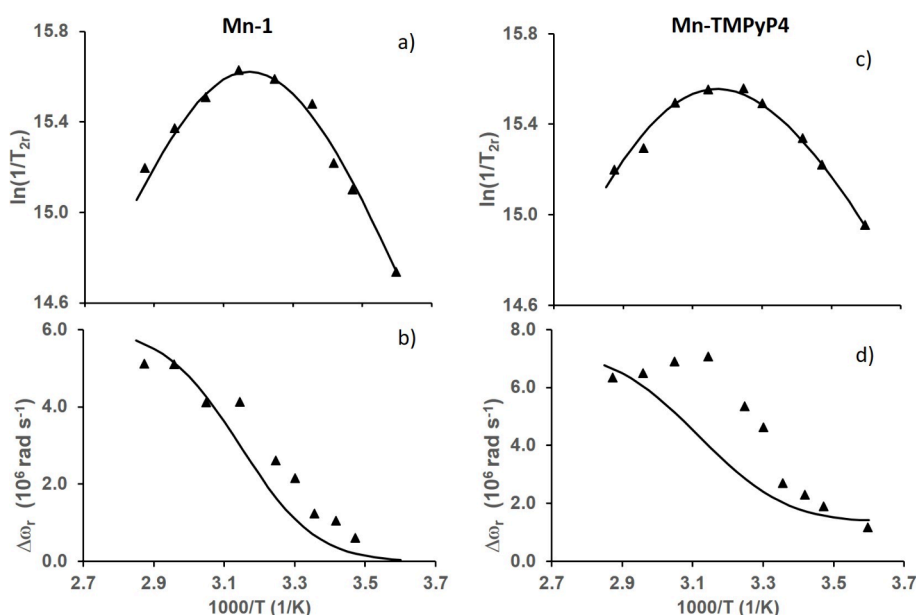


Figure 5. Variable temperature reduced ^{17}O transverse relaxation rates (a and c) and reduced chemical shifts (b and d) for Mn-1 and Mn-TMPyP4 complexes recorded at 9.4 T. The lines correspond to the fit of the data to the Swift–Connick equations (see SI section 2).

field differences in the relaxivities can be related to the different rotational motion of the chelates, as a consequence of their different size and molecular geometry. Globally, the higher relaxivities of **Mn-2** and **Mn-3** originate from their larger hydrodynamic size, related to the long (C6) pending chains introduced in the para position of the pyridine with respect to the porphyrin linkage. In **Mn-1**, the pendant chains are shorter and their orientation is different with respect to the porphyrin plane, which results in a more compact molecular structure for the complex. Interestingly, the relaxivities are practically identical at high fields for **Mn-1** and **Mn-TMPyP4**, despite their different molecular weight. One can speculate that a slightly slower electron spin relaxation of **Mn-TMPyP4** (indicated by the slightly higher relaxivities at low-field, where the effect of electronic relaxation is more important) compensates for its expectedly faster rotational motion, resulting in similar high-field relaxivities. The lower r_1 values observed for **Mn-2**, which bear charged carboxylic end-groups, versus **Mn-3** are somewhat surprising. This may be related to electrostatic attraction between the negatively charged carboxylate and the positively charged pyridine nitrogen, leading to a smaller hydrodynamic radius of the complex and thus faster rotational motion.

Beyond these differences, we can conclude that these Mn(III)-porphyrins, and in particular **Mn-2** and **Mn-3**, have very interesting relaxivities at clinically relevant magnetic fields, 2–3 times higher than that of commercially used MRI contrast agents (e.g., $r_1 = 12.0 \text{ mM}^{-1} \text{ s}^{-1}$ for **Mn-3** versus $r_1 = 3.8 \text{ mM}^{-1} \text{ s}^{-1}$ for GdDOTA, 20 MHz, 37 °C).⁵³ Their high water solubility is essential for this, since it prevents aggregation in aqueous solution, which would have negative consequences on the relaxivity.

Photoinduced DNA Cleavage

The Mn(III)-porphyrin chelates, **Mn-2** and **Mn-3**, were subjected to photo DNA cleavage tests to evaluate for their potential as photosensitizers (PSs) in PDT. A green LED with a dominant wavelength at 527 nm that overlaps with the absorption band of **Mn-2** and **Mn-3** was used. The amounts of generated nicked DNA (form II) were quantified by electrophoresis and subsequent staining with ethidium bromide. Significant DNA cleavage by **Mn-2** and **Mn-3** were observed under photoirradiation conditions (Figure 6). Especially, photoinduced DNA damage by **Mn-3** was evident,

though a stronger activity was observed with the free porphyrin **3**, presumably related to its higher absorption intensities at around 527 nm. Control experiments (1) under dark conditions with all compounds or (2) under light, but without any chemical, did not cause significant DNA cleavage, indicating that DNA cleavage occurred exclusively upon photoexcitation of the compounds.

Generation of $^1\text{O}_2$ under Visible Light Irradiation

$^1\text{O}_2$ is an important reactive oxygen species (ROS) involved in the photoinduced damages of biomolecules including DNA and often plays an essential role in PDT. The generation of $^1\text{O}_2$ by photoexcited **Mn-2** and **Mn-3** was evaluated by an ESR method using a spin-trapping reagent, 4-oxo-TEMP (see the scheme on the top of Figure 7).⁵⁴ As shown in Figures 7 and

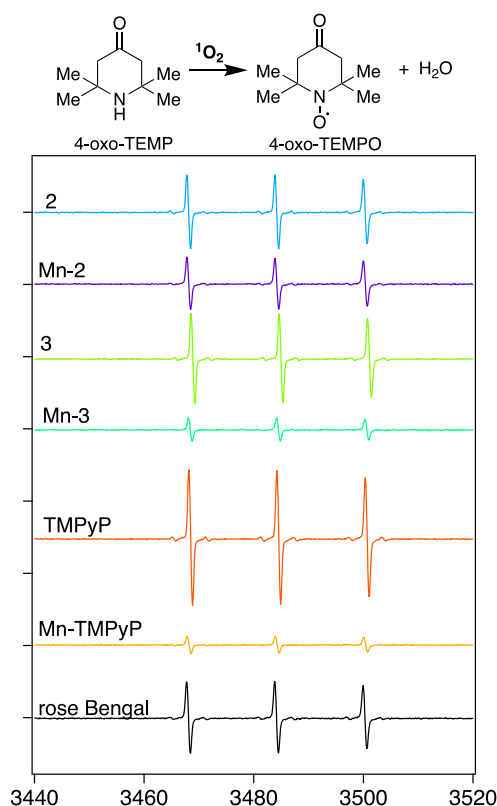


Figure 7. X-band ESR spectra for the $^1\text{O}_2$ adduct of 4-oxo-TEMP (4-oxo-TEMPO) generated by compounds **2**, **3**, and **TMPyP4** and their corresponding Mn(III)-complexes (**Mn-2**, **Mn-3**, and **Mn-TMPyP4**) solutions in D_2O (0.2 mM) in the presence of 50 mM 4-oxoTEMP. Photoirradiation: green LED (max 527 nm); distance: 3 cm; 10 min. Measurement conditions: temperature: 296 K; microwave frequency: 9.375 GHz; microwave power: 1 mW; modulation amplitude 4.00 G; modulation frequency: 100 kHz; scan time: 20.5 s. Rose Bengal was used as the standard PS for $^1\text{O}_2$ generation.

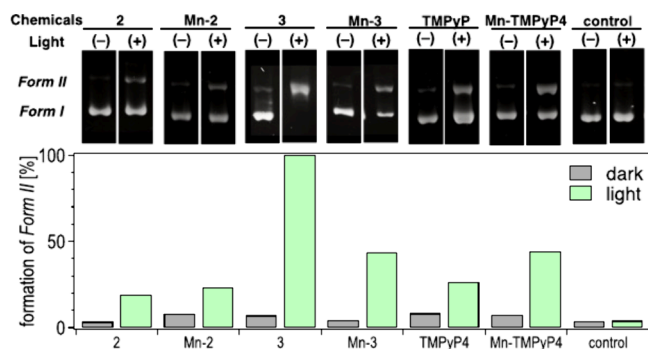


Figure 6. Photo DNA cleavage by **2**, **Mn-2**, **3**, **Mn-3**, **TMPyP4**, and **Mn-TMPyP4** under visible light irradiation. DNA: pBR322 supercoiled plasmid; photoirradiation: green LED (with dominant wavelength at 527 nm) for 4 h, 2.5 cm distance; porphyrins: 3.2 μM ; in pH 8.0 Tris-HCl-EDTA buffer. Bottom: relative amount of form II generation under photoirradiation quantified by imageJ.

S29–S35, the generation of 4-oxo-TEMPO ($^1\text{O}_2$ adduct of 4-oxo-TEMP) was observed in a time-dependent manner under irradiation from a green LED (527 nm at maximum). The $^1\text{O}_2$ generation was efficient in all Mn-porphyrin complexes (**Mn-2**, **Mn-3**, and **Mn-TMPyP4**) with slight differences, although the generation was decreased in comparison to their corresponding free porphyrins (Figure 7) in line with the data of photo DNA cleavages (Figure 6). This was presumably related to the absorption intensity of the compounds at the wavelength of the light used for photoirradiation, with a maximum at 527 nm.

The paramagnetic Mn(III) ion can also have a quenching effect on the $^1\text{O}_2$.⁵⁵ Taken together with the fact that Mn-3 revealed high r_1 relaxivity (Figure 4) and sufficient photo DNA cleaving activity (Figure 5), this Mn(III)-porphyrin complex Mn-3 can be regarded as a good candidate acting as a bifunctional molecular probe for both MRI-CA and PDT-PS.

Stabilization of G-Quadruplex (G4) DNA

TMPyP4 (Figure 1) has been known also as a G4-binding molecule due to its cationic moieties in the pyridyl side chains surrounding the rigid porphyrin skeleton.⁵⁶ G4-DNA-forming sequences are frequently present in the promoter regions of oncogenes, and a more prevalent existence of G4-DNA structures is one of the features of cancer cells, where the oncogenes are actively transcribed. We expected that the porphyrins 2 and 3 and their Mn(III)-complexes Mn-2 and Mn-3, with structures having the same cationic patterns as TMPyP4, would have similar G4-binding activity and stabilize it, and that they may serve as novel G4-DNA sensing MRI agents to detect tumor tissues. Among them, porphyrin 3 with strong photo DNA cleavage (Figure 6) and $^1\text{O}_2$ generation (Figure 7) and its complex Mn-3 with high relaxivity (Figure 4) were tested with respect to their G4 stabilizing ability.

G4 stabilization assays were conducted to monitor the G4 stabilizing effects on various G4-DNA structures. The FRET assays using three types of G4 DNAs (MYC, KIT, and Rif1-2) labeled with FAM and TAMRA at both 5' and 3' ends were performed on porphyrin 3, Mn-3, TMPyP4, and Mn-TMPyP4. Compounds 3 and Mn-3 revealed a dose-dependent increase of the melting temperature of all G4 DNAs tested (Figure 8a,b) indicative of G4-stabilizing effects, although the effects were weaker than with the control TMPyP4 (Figure 8c). Of note, the G4 stabilizing effect of Mn-TMPyP4 was significantly attenuated (Figure 8d) compared to that of the metal-free TMPyP4 (Figure 8c), whereas chelating Mn(III) to 3 did not cause a significant decrease in G4 stabilization (Figure 8b, in comparison with Figure 8a), indicating the possible dual properties of Mn-3, high relaxivity as a MRI agent and sustained G4-DNA stabilizing ability.

Binding to G4 DNA

G4-stabilizing agents interact with G4 DNA and regulate DNA transactions including DNA replication and transcription. The standard porphyrin TMPyP4, a known G4 DNA binder,⁵⁶ inhibits oncogene expression.⁵⁷ Based on the results above, G4 DNA-binding ability and selectivity were examined by an electrophoretic mobility shift assay (EMSA). To detect DNA–ligand complexes, G4 DNA (T_6G_{24}) or non-G4 DNA ($\text{T}_6(\text{GA})_{12}$) were coincubated with each compound, and the physical interaction was visualized after separation of the complexes and free DNA. As a result, Mn-3 bound not only to G4 DNA but also to non-G4 DNA at 100 μM (Figure 9, lane 13). It should be noted that a control G4-binder TMPyP4 did not clearly form complexes with G4 DNA (Figure 9, lanes 14 and 15) whereas Mn-TMPyP4 weakly binds to G4 DNA at 100 μM , indicating that Mn-3 has high DNA binding ability like Mn-TMPyP4 but poor selectivity to G4 DNA structures under these experimental conditions. Similar results were observed in EMSA in acrylamide gels (Figure S37).

Photocytotoxicity

Following the results above, we tested the effect of Mn-2, Mn-3, and Mn-TMPyP4 on the cells under visible light irradiation. A typical cancer cell line, cervical adenocarcinoma HeLa cells,

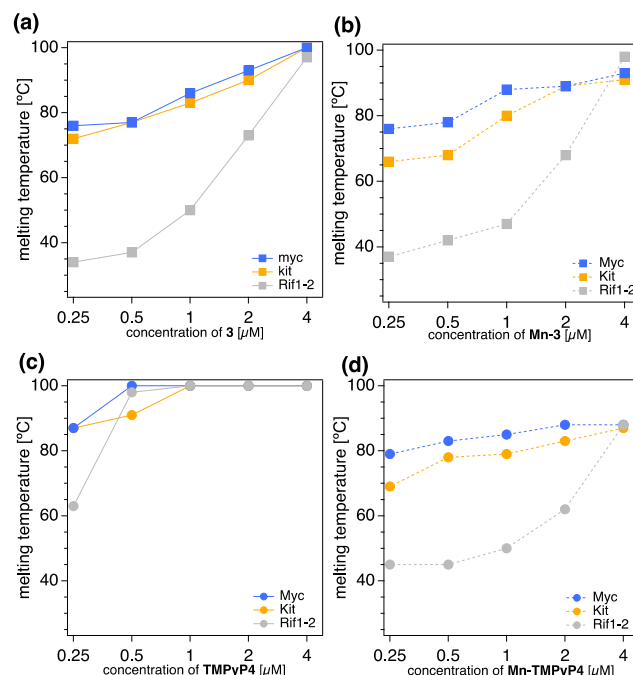


Figure 8. G4 stabilization assay of 3 (a), Mn-3 (b), TMPyP4 (c), and Mn-TMPyP4 (d) by FRET. MYC, KIT, or Rif1-2 G4 DNA labeled with FAM and TAMRA at final 0.2 μM was formed by heat denaturation and annealing and mixed with each chemical at 0.25, 0.5, 1, 2, or 4 μM . FRET was detected during the temperature shift from 37 to 100 $^{\circ}\text{C}$ at the ramp speed of 1 $^{\circ}\text{C}\cdot\text{min}^{-1}$. The melting temperatures (T_m) at each concentration are shown. T_m values for c-MYC, c-KIT, and Rif1-2 DNA without binder are 75, 65, and 30 $^{\circ}\text{C}$, respectively.

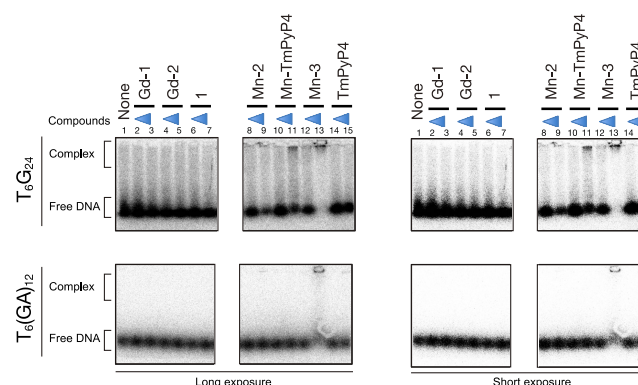


Figure 9. Binding of compounds to G4 DNA. One picomole of ^{32}P -labeled G4 DNA (parallel-type G4 T_6G_{24}) or non-G4 $\text{T}_6(\text{GA})_{12}$ was incubated with the compounds (0.1 and 1 nmol; at the final concentration of 10 and 100 μM , respectively) in HEPES buffer containing 50 mM KCl at room temperature for 10 min. The products were separated on agarose gel and detected by autoradiography.

have been used for this photocytotoxicity tests in combination with the MTT method.

As shown in Figure 10, significant photocytotoxicity was observed with all porphyrins (2, 3, and TMPyP4) and their Mn-complexes (Mn-2, Mn-3, and Mn-TMPyP4). In line with photoinduced DNA cleavage, porphyrin 3 showed the highest photocytotoxicity, presumably related to the oxidative damage of biomolecules in the cells (e.g., cell membrane and other molecules). The highest photocytotoxicity of Mn-3 among

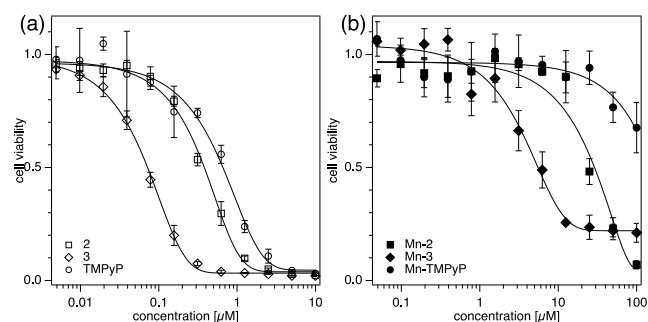


Figure 10. Photocytotoxicity of porphyrins (2, 3, and TMPyP4) (a) and their Mn(III)-complexes (Mn-2, Mn-3, and Mn-TMPyP4) (b) on HeLa cells under green LED irradiation (527 nm, 45 mW per well). Irradiation was 15 min, and relative cell viability was measured by the MTT assay.

bearing charges on pyridine nitrogens in the para position, showed slight G4-DNA-stabilizing activity, although without selectivity over B-DNAs. Combined with the promising photosensitivity data (photoinduced DNA cleavage, $^1\text{O}_2$ generation, and photocytotoxicity), Mn-3 can be considered as a potential core for the development of bimodal probes combining a good relaxation effect in MRI and therapeutic potential in PDT.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/cbmi.4c00046>.

Details of the syntheses of Mn-1, 2, Mn-2, 3, and Mn-3 including their synthetic intermediates with necessary spectroscopic data, relaxivity measurements, photoinduced DNA cleavage, ROS generation measurements by ESR, FRET assay for G4 stabilization assay, G4-binding assay, and photocytotoxicity (PDF)

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Author Contributions

N.Y.-S., H.M., E.T., and Y.Y. designed the overall project. T.N. designed detailed structures of the molecules and contributed to their synthesis and structural characterization in collaboration with C.C. and Y.Y. A.P. performed relaxivity and ^{17}O

Mn(III)-complexes was observed (Figure 10b) although the efficiency in DNA cleavage (Figure 6) and $^1\text{O}_2$ generation (Figure 7) was similar to that of Mn-TMPyP4. This might be related to the other factors such as cell internalization efficiency. Further investigation is currently under process.

Cytotoxicity

Since the safety of imaging probes is the most important and critical issue when subjected to the living animals, cytotoxicity tests were performed as an initial assay on these porphyrins. For the quantification, the effect of 3, Mn-3, TMPyP4, or Mn-TMPyP4 on cell growth were tested with three cancer cell lines (Figure S39). All porphyrins showed low toxicity ($\text{IC}_{50} > 100 \mu\text{M}$) in osteosarcoma U2OS cells and HeLa cells, while the inhibitory effect of cell growth was observed in breast cancer MCF-7 cells. The IC_{50} values of Mn-3 and Mn-TMPyP4 were summarized in Table 1.

Table 1. Summary of Cytotoxicity and Photocytotoxicity of Mn-3 and Mn-TMPyP4

complexes	$\text{IC}_{50} [\mu\text{M}]$			
	MCF-7		U2OS	
	dark	light	dark	light
Mn-3	40.4	171	178	4
Mn-TMPyP4	13.2	167	>200	>100

Furthermore, the effects of Mn-porphyrins (Mn-2, Mn-3, and Mn-TMPyP4) and nonmetal chelated 3 on cell cycle profiles were analyzed (Figure S40). While the control experiments with L2H2-6OTD, a known G4 ligand, showed increases of sub-G1 population, which correlates with apoptotic cells with fragmented DNA, experiments with other compounds did not cause significant changes.

CONCLUSION

Three water-soluble Mn(III)-porphyrin complexes, Mn-1–3, have been synthesized and investigated as potential MRI-CAS with photosensitizing capabilities. All three complexes are endowed with interesting r_1 relaxivities at clinically relevant magnetic fields, around 2–3 times higher than those of commercial Gd-based agents. The good water solubility of the Mn(III)-porphyrins is an important parameter for this, as it prevents porphyrin aggregation and subsequent relaxivity decrease often observed for more hydrophobic systems. Mn-3, with a tetracationic porphyrin structure similar to TMPyP4

620 NMR measurements in collaboration with E.T. Z.G performed
621 electrochemical measurements and analyses in collaboration
622 with E.T. N.Y.-S. and H.M. performed G4 stabilization/
623 binding activity assays and cytotoxicity tests. C.C. performed
624 photocytotoxicity tests in collaboration with Y.Y. The
625 manuscript was written with contributions of all authors. All
626 authors have given approval to the final version of the
627 manuscript.

628 Notes

629 The authors declare no competing financial interest.

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648 ■ ABBREVIATIONS

649 MRI, magnetic resonance imaging; MRI-CA, magnetic
650 resonance imaging contrast agent; PDT, photodynamic
651 therapy; PS, photosensitizer; $^1\text{O}_2$, singlet oxygen; NIR, near-
652 infrared; PET, positron emission tomography; CKD, chronic
653 kidney disease; CIN, contrast induced nephropathy; NSF,
654 nephrogenic systemic fibrosis; HPLC, high pressure liquid
655 chromatography; TLC, thin layer chromatography; NMR,
656 nuclear magnetic resonance; HRMS, high resolution mass
657 spectrometry; FTIR, Fourier transfer infrared; ESR, electro
658 spin resonance; 4-oxo-TEMP, 2,2,6,6-tetramethyl-4-piperi-
659 done; FRET, fluorescence resonance energy transfer; G4,
660 guanine quadruplex; EMSA, electrophoretic mobility shift
661 assay

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