# Impact of photosensitizing agents incorporation on relaxivities of gadolinium-loaded paramagnetic liposomes

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**Abstract**

Previously described enhancement of the relaxivities of the liposomes with gadolinium-functionalized lipids embedded in the lipid bilayer, caused by the incorporation of zinc phthalocyanine (ZnPc), opens the way to reduction of the dose of potentially harmful Gd(III) in novel theranostic liposomal formulations. The lipid bilayer water permeability and flexibility/deformability alterations were found the most likely reasons for the relaxivity increase. Here we present the relevant difference between outcomes of incorporation of ZnPc and other hydrophobic photosensitizing agents, namely Temoporfin (TP) and Verteporfin (VP), into the structure of Gd(III)-loaded liposomes. Three types of theranostic liposomes, each containing one of the photosensitizers, and liposomes with no model drug, were prepared by a thin-film hydration method and examined for particle size distributions and relaxivities $r_1$ and $r_2$ (9.4 T, 37°C). Only incorporation of ZnPc affected relaxation properties of the liposomes. It is our hypothesis that it is energetically non optimal for ZnPc not only to be located in hydrophilic regions but also (to a lesser extent) in non-polar lipid chains regions. The above may be the reason for locally incompact structure of the lipid bilayer resulting in an increase of water permeability and flexibility/deformability of the membrane, affecting relaxivity. This would not be the case for TP and VP as they mix well with less polar environment.

**Keywords**

MRI contrast agents, relaxivity, theranostics, liposomes, zinc phthalocyanine, Temoporfin, Verteporfin

## 1. Introduction

Liposomes are biocompatible carriers with plethora of applications. Amphiphilicity of lipids allows transfer of both hydrophilic and hydrophobic substances incorporated into liposomes and a wide range of available types of lipids provides a large number of possible liposomal formulations differing in physicochemical properties [1,2]. There are numerous examples of liposomes as drug carriers e.g. in cancer treatment [2–5] or as MRI contrast agents [6,7]. On the other hand, using theranostic nanoparticles, acting simultaneously as contrast agents in imaging techniques and therapeutic agents carriers, enables combination of diagnostic and treatment procedures and thus, reduction of the patients' discomfort [8,9]. All the above paved the way to creation of multimodal nanoparticles with joined diagnostic (magnetic resonance imaging contrast agent) and therapeutic (photodynamic therapy agent) function which example are the gadolinium- and photophotosensitizer-loaded liposomal systems [1,10] being the object of our research. In this work we investigate the effect of incorporation of different model photosensitizers on the $r_1$ and $r_2$ relaxivities of Gd(III)-loaded paramagnetic liposomes.

One of the main challenges in designing this type of systems is understanding interactions between the contrasting and therapeutic components. Previously described enhancement of the relaxivities [10] of the liposomes with gadolinium-functionalized lipids embedded in the lipid bilayer, caused by the incorporation of zinc phthalocyanine (ZnPc) into the bilayer, opens the way to reduction of the dose of potentially harmful Gd(III) in novel theranostic liposomal formulations [1,10]. It was shown that the relaxivity enhancement effect originates mainly from liposome membrane structure modification, not from the magnetic properties of ZnPc molecule. The results of fitting Modified Florence model [11] to the experimental data, in combination with the literature [12], indicated the lipid bilayer water permeability and flexibility/deformability alterations (that occur as a result of ZnPc incorporation) as the most likely reasons for the relaxivity increase [10]. Information on the biocompatibility, temporal stability, satisfactory therapeutic efficacy and safety of use of this kind of liposomal systems was previously published [1], as well as detailed study on the influence of ZnPc incorporation on the observed relaxivities in wide range of magnetic field strengths in similar liposomal formulations [10] . Now we present the relevant difference between outcomes of incorporation of ZnPc and other hydrophobic photosensitizing agents, namely Temoporfin (TP) and Verteporfin (VP), into the structure of Gd(III)-loaded liposomes.

## 2. Materials and methods

### *2.1. Liposomes*

The nanoparticles were composed of commercially available fatty acids derivatives of Gd(III) salt (bis(1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine)-N-N'-diethylenetriaminepentaacetic acid (gadolinium salt), Gd-DTPA2) as well as PG (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphate) and POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine) as basic phospholipids of liposomal membrane. Three model photosensitizing agents, namely zinc phthalocyanine (ZnPc), Temoporfin (TP) and Verteporfin (VP), were incorporated into the hydrophobic part of the lipid bilayer. The theranostic liposomes, with the molar ratio of components 0.05:8:2:0.05 mM (Gd-DTPA2:POPC:PG:ZnPc (Gd-ZnPc-lip) or Gd-DTPA2:POPC:PG:TP (Gd-TP-lip) or Gd-DTPA2:POPC:PG:VP (Gd-VP-lip)) and liposomes with no model drug (Gd-DTPA2:POPC:PG 0.05:8:2; Gd-lip), were prepared by a thin-film hydration method as described in [13] with the difference of TP and VP being dissolved in chloroform solutions before thin-film formation, unlike ZnPc (dissolved in N-methyl-2-pyrrolidone (NMP)). The reason for dissolving ZnPc in NMP was its low solubility in chloroform (below $10^{-8}$ mol·kg$^{-1}$) and high solubility in NMP ($6.92 \cdot 10^{-3}$ mol·kg$^{-1}$) [14]. The sizes of the liposomes were measured by dynamic light scattering (DLS) method using Malvern Panalytical Zetasizer (Zetasizer Nano Malvern Ins., UK) at 37°C (corresponding to the

temperature of human internal organs). Size distribution curve for each liposomal formulation was calculated by averaging six DLS measurements (single measurement duration: 60 s).

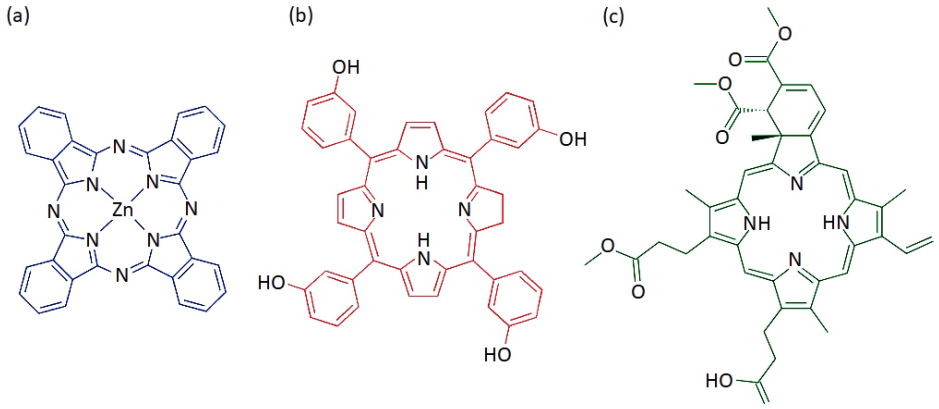


**Fig. 1** Chemical structures of the photosensitizing agents: (a) zinc phthalocyanine (ZnPc), (b) Temoporfin (TP) and (c) Verteporfin (VP).

### *2.2. NMR relaxometry*

The final gadolinium concentration in the samples was estimated using NMR relaxometry following drying the sample and retransferring to chloroform solution as previously described [13] and included in the relaxivity calculation. NMR relaxation experiments were performed at 37°C on a 9.4 T horizontal bore Agilent MRI scanner using a 40 mm diameter millipede coil for transmission and reception. Proton $T_1$ relaxation times were measured for four samples of known concentrations for each liposomal formulation using Inversion Recovery pulse sequence. The $T_2$ relaxation times of the same samples were measured using Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence. Relaxivities $r_1$ and $r_2$ were calculated as a slope of a line fitted to the relaxation rates ($R_1 = 1/T_1$; $R_2 = 1/T_2$) plotted against Gd(III) concentration.

## 3. Results

Four liposomal formulations were successfully synthesized and examined for size distributions and NMR relaxation times $T_1$ and $T_2$. Fig. 2 and the first section of Table 1 present DLS results.

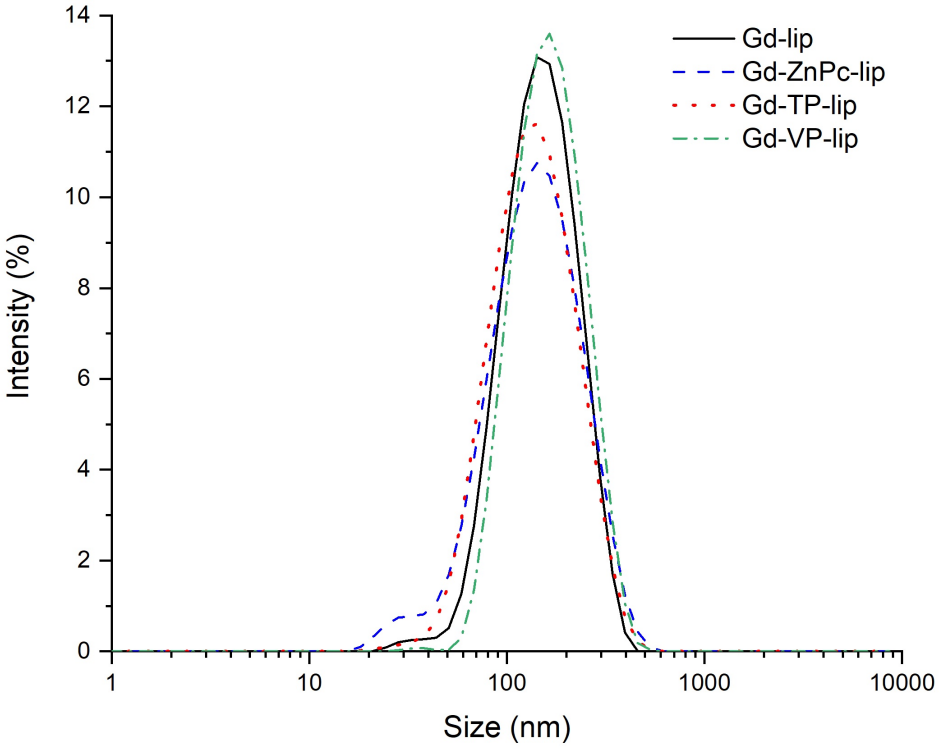


**Fig. 2** Size distributions (DLS results) of the liposomes.

**Table 1** DLS results (Z-Average, polydispersity index PDI, peak position) and relaxivities ($r_1$ and $r_2$) of Gd-lip, Gd-ZnPc-lip, Gd-TP-lip and Gd-VP-lip.

| | Gd-lip | Gd-ZnPc-lip [13] | Gd-TP-lip | Gd-VP-lip |
|---|---|---|---|---|
| Z-Average (nm) | 129.4 ± 0.4 | 117.0 ± 0.9 | 115.5 ± 0.6 | 147.1 ± 1.1 |
| PDI | 0.175 ± 0.009 | 0.235 ± 0.009 | 0.215 ± 0.014 | 0.149 ± 0.011 |
| Peak position (nm) | 142 | 142 | 142 | 164 |
| $r_1$ ($mM^{-1}s^{-1}$) | 24.09 ± 0.36 | 29.09 ± 0.25 | 23.88 ± 0.27 | 24.46 ± 0.42 |
| $r_2$ ($mM^{-1}s^{-1}$) | 153.30 ± 3.88 | 148.82 ± 1.09 | 153.55 ± 2.49 | 154.57 ± 2.10 |

Size distribution curves vary between liposomal formulations resulting in differences between Z-Average and PDI values. The main difference lies within smaller size range (below 70 nm). The main peaks are in good agreement (142 nm for Gd-lip, Gd-ZnPc-lip and Gd-TP-lip; 164 nm for Gd-VP-lip). Relaxometry results are presented in the second section of Table 1 and in Fig. 3.

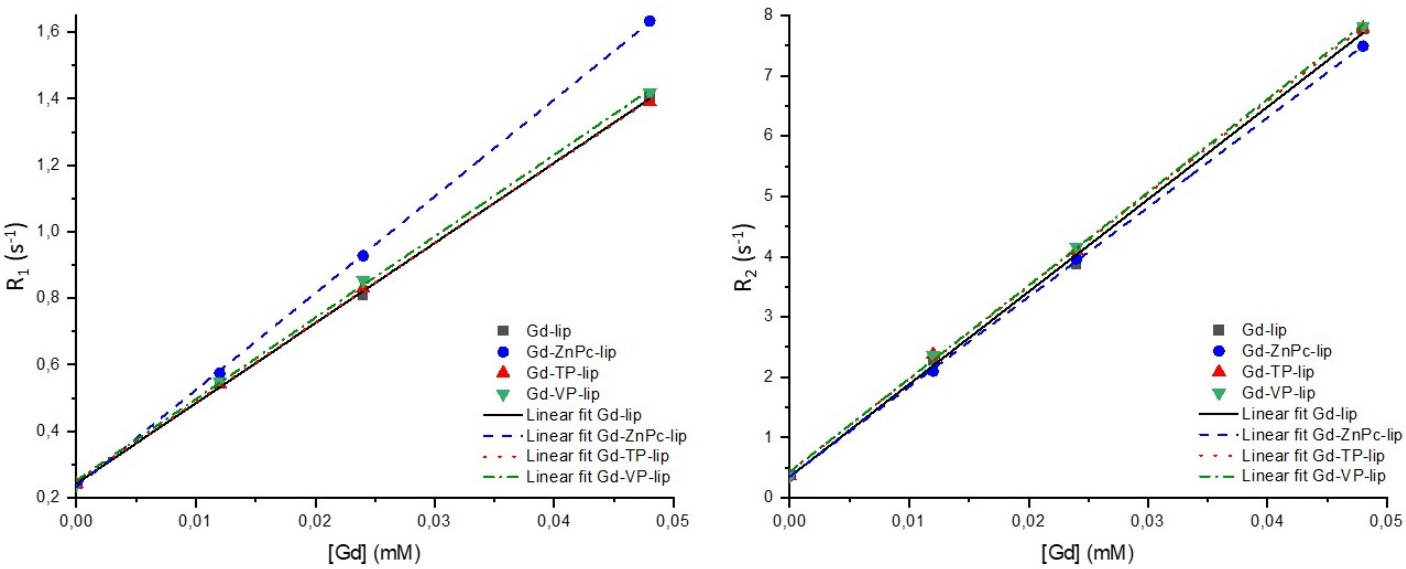


**Fig. 3** NMR relaxometry results for Gd-lip, Gd-ZnPc-lip, Gd-TP-lip and Gd-VP-lip ($R_1 = 1/T_1$; $R_2 = 1/T_2$).

Gd-ZnPc-lip exhibited visibly higher $r_1$ (29.09 ± 0.25 $mM^{-1}s^{-1}$) comparing to the other examined types of liposomes (approx. 24 $mM^{-1}s^{-1}$). The $r_2$ values ranged from 148.82 ± 1.09 (for Gd-ZnPc-lip) to 154.57 ± 2.10 $mM^{-1}s^{-1}$ (for Gd-VP-lip). The $r_2$ value differences are close to the uncertainty of the measurements, therefore are not conclusive.

**4. Discussion and conclusions**

Fraction of nanoparticles smaller than 50 nm is notably more represented in Gd-ZnPc-lip formulation than in other examined liposome types. The size of MRI contrast agents molecules is correlated with their rotational correlation time in solution and thus relaxivities in a given magnetic field strength [15,16]. The nanoparticles of size below 50 nm exhibit rotational correlation times far shorter than optimal and thus, add rather small contribution to overall relaxivities of the examined solutions. This can result in smaller measured values of both relaxivities comparing to the values that could be obtained if the size distribution was more optimal. This is another reason for recognizing the small differences between $r_2$ values as not conclusive, as they can reflect the above phenomenon more than the changed properties of the main size group of the nanoparticles. Nevertheless, it is worth emphasizing that ZnPc incorporation into Gd-loaded liposomes can result in both a decrease and an increase of $r_2$ as well as $r_1$ values and the effect varies with the liposomal formulation and the magnetic field strength [1,10]. Previously published studies [1 ,10] present incorporation of ZnPc into 12 different Gd-loaded paramagnetic liposome types. For experiments in 9.4 T at 37°C, in 10 cases of 12, ZnPc clearly affected both relaxivities, and there were 2 cases in which the effect was clear for $r_2$ but not conclusive for $r_1$. So far, there was no case in which ZnPc would not notably affect $r_1$ nor $r_2$ in 9.4 T, unlike what can be now observed for TP and VP. It was not shown that TP and VP would not affect any liposomal formulation's relaxation properties in any magnetic field strength. However, good agreement of the main peaks of the size distributions of Gd-lip, Gd-ZnPc-lip, Gd-TP-lip and Gd-VP-lip together with significant difference between $r_1$ value obtained for Gd-ZnPc-lip and the $r_1$ values of the other formulations lead to a conclusion, that the impact of ZnPc can be of unique nature comparing to the other photosensitizers.

It was previously shown that the enhancement of the relaxivity of paramagnetic liposomes caused by the incorporation of ZnPc is not due to the photosensitizer's magnetic properties but originates mainly from liposomal membrane structure modification [10]. The previous results indicate lipid bilayer water permeability and flexibility/deformability alterations as the most likely reasons for the relaxivity increase [10]. It is of interest if the above effect is due to size of the photosensitizer's particles located between the lipids of the membrane. Molecular weights of the molecules of the photosensitizing agents used in this study are 577.9 (ZnPc), 680.7 (TP) and 1437.6 (VP). ZnPc and TP particles are of similar size. ZnPc incorporation visibly affects the $r_1$ value and the nanoparticles' size distribution whereas presence of TP does not cause any significant relaxation properties alteration, at least not

ones visible in high magnetic field. Comparing to Gd-lip, Gd-TP-lip size distribution shape is slightly altered in favor of smaller particle sizes (similarly to Gd-ZnPc-lip but without clearly visible peak below 70 nm). VP particles are notably larger than those of ZnPc and they slightly shift the size distribution curve towards bigger nanoparticles' sizes, but do not cause any relaxivities changes in the presented experiments. The above observations indicate that the relaxivity enhancement is not due to increasing mass of the liposome or due to the photosensitizer's particle size itself, however, to our knowledge, the conformation of ZnPc, TP and VP in liposomal membranes and the volumes occupied in such environment have not yet been determined.

All the photosensitizers used in this study are hydrophobic. TP and VP are soluble in chloroform. ZnPc presents insolubility in water (protic solvent, polarity index: 10.2, dipole moment: 1.87) and low solubility in chloroform (aprotic solvent, polarity index: 4.1, dipole moment: 1.15) and other solvents with lower polarity index and dipole moment (e.g. toluene, dichloromethane, chlorobenzene) [14]. ZnPc dissolves well in NMP (aprotic solvent, polarity index: 6.7, dipole moment: 4.09) and other aprotic polar solvents with high dipole moment like DMPU (1,3-dimethyl-3,4,5,6-tetrahydro-2(1H)-pyrimidinone) and DMAC (dimethylacetamide) [14]. There is no simple correlation between maximum concentration of ZnPc in a given solvent and its dipole moment nor polarity index but it can be noticed that in most cases ZnPc mixes well with polar particles that do not create hydrogen bonds.

It is our hypothesis that it is energetically non optimal for ZnPc not only to be located in hydrophilic regions but also (to a lesser extent) in non-polar lipid chains regions. The above may be the reason for locally incompact structure of the lipid bilayer resulting in an increase of water permeability and flexibility/deformability of the membrane (affecting relaxivity). This would not be the case for TP and VP as they mix well with less polar environment. It should also be noted that in the past studies paramagnetic ZnPc-containing liposomes were synthesized using chloroformic solutions of ZnPc for thin-film formation (with no NMP, unlike this study) [1,10], so it was proven that the relaxation enhancement effect is not due to the use of NMP. This work is another step to understanding how contrasting efficiency of anticancer-MRI theranostics can be optimized, leading the way to reduction of the dose of potentially harmful Gd(III) in next generation of bimodal liposomes combining diagnostic and therapeutic function. Nevertheless, there is still a lot to uncover.

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**Competing Interests**

The authors declare no competing interests.

**Author Contributions**

Study conception and design: BW. Liposomes synthesis and DLS: KS and BW. NMR measurements, data analysis and manuscript drafting: BW. Critical revision: KS.

**Data Statement**

The datasets generated during the current study are available from the corresponding author on reasonable request.

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