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The quest for biocompatible phthalocyanines for molecular imaging: Photophysics, relaxometry and cytotoxicity studies

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ABSTRACT

Water soluble phthalocyanines bearing either four PEG500 or four choline substituents in the macrocyclic structure, as well as their Zn(II) and Mn(III) complexes were synthesized. The metal-free and Zn(II) complexes present relatively high fluorescence quantum yields (up to 0.30), while the Mn(III) complexes show no fluorescence as a consequence of rapid non-radiative deactivation of the Mn(III) phthalocyanine excited states through low-lying metal based or charge-transfer states.

The effect of DMSO on the aggregation of the phthalocyanines was studied. It was not possible to obtain the Mn(II) complexes by reduction of the corresponding Mn(III) complexes due to the presence of electron donating substituents at the periphery of the phthalocyanines. The ^{1}H NMRD plots of the PEG500 and choline substituted Mn(III)-phthalocyanine complexes are typical of self-aggregated Mn(III) systems with r_1 relaxivities of 4.0 and 5.7 mM^{-1} s^{-1} at 20 MHz and 25 °C. The Mn(III)-phthalocyanine-PEG_4 complex shows no significant cytotoxicity to HeLa cell cultures after 2 h of incubation up to 2 mM concentration. After 24 h of cell exposure to the compound, significant toxicity was observed for all the concentrations tested with IC50 of 1.105 mM.

1. Introduction

Since the accidental discovery of phthalocyanines in the beginning of the twentieth century [1], much effort has been done to modulate the properties of their derivatives to achieve ideal molecules for a variety of applications, such as catalysis [2,3,4], chemical sensing [5,6,7], light-harvesting modules for energy transfer [8,9,10,11,12] and dye sensitized solar cells [13,14,15], non-linear optics [16,17,18,19,20,21,22,23], photodynamic therapy (PDT) [24,25,26,27], theranostics and molecular imaging [28,29,30,31]. Indeed phthalocyanines and their derivatives are chemically and thermally stable compounds that have exceptional optical and electrical properties, particularly their high molar extinction coefficients, absorbance and emission in the deep red region of the electromagnetic spectrum, and long lifetimes of excited states with quite high quantum yields [32,33].

Phthalocyanines, due to their planar structure, readily aggregate by π–π stacking, normally making them virtually insoluble in water [28,34]. This seriously limits their biomedical applications. Also, aggregation strongly decreases their fluorescence quantum yields and singlet oxygen quantum yields, limiting their applications as optical imaging and PDT agents, respectively. Many strategies have been developed to overcome this drawback, including the use of surfactants to create a micro-heterogeneous environment [35,36] and the use of dendrimers as substituents on the phthalocyanine macrocycle, which sterically inhibit molecular aggregation to improve water solubility [24,37]. Although the strategies describe above can, to some extent, improve solubility and decrease aggregation of phthalocyanine systems, only the introduction of hydrophilic moieties in the phthalocyanine structure allows an adequate solubility in water [33,38]. Generally the moieties used are negatively charged, such as sulfonate or carboxylate, positively charged like quaternized amines, or non-ionic, e.g. carbohydrates, PEG and glycerol [33,38,39,40,41,42]. Zinc(II) phthalocyanines with high water solubility can be of great utility in PDT and optical imaging applications [41,42], while Mn(III)-phthalocyanines, especially

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those with bulky and electron-withdrawing substituents, show a strongly red shifted band, sometimes moving into the NIR region, the therapeutic window, where in vivo light transmission is increased, making them interesting as optical imaging probes for biomedical applications [43,44].

Contrast in magnetic resonance imaging (MRI) is largely determined by differences in relaxation times (T_1 and T_2) of water protons in tissues. MRI contrast agents (CA) are clinically used for contrast enhancement, *e.g.* for detection and delineation of tumors [45]. Clinical MRI CAs are stable, water soluble complexes of metal ions with several unpaired electrons and long electron spin relaxation times, such as Mn(II) (five unpaired electrons) and Gd(III) (seven unpaired electrons), which efficiently increase the nuclear spin relaxation rates ($1/T_1$ and $1/T_2$) of the nearby water protons of the tissues where they are localized [45,46,47]. However, the release of free Gd^{3+} from some Gd-based MRI CAs has recently been linked with a medical condition known as nephrogenic systemic fibrosis, a rare but potentially harmful side effect observed in some patients with severe renal disease or following liver transplant [48]. Because of this, much emphasis has recently been focused on alternative approaches based on non-lanthanide metals, in particular manganese, for T_{1w} imaging [49]. Water soluble and air stable Mn(III)-porphyrins have been extensively studied as tumor targeted MRI CAs because, in addition to good nuclear spin relaxation efficiency (given by their proton relaxivities, *i.e.*, the relaxation rate enhancement per mM of metal ion) of the water protons [50–52], they localize selectively in tumors and provide enhanced *in vivo* animal image contrast of these pathologies [53,54]. Much of the work so far has concentrated on the use of Mn(III) and Mn(II) derivatives of tetraphenyl porphyrins [50–54]. More recent studies include monomeric or dimeric Mn(III) porphyrins [52,55,56], polymeric Mn(III) porphyrins, as well as Mn(III)TPPS$_4$ (TPPS4 = tetrasulfonated porphyrin derivatives) which behave as pO_2 responsive [57] and Zn^{2+} responsive MRI CAs [58,59].

So far, only one Mn(II)-phthalocyanine (tetrasulfonated manganese(II) phthalocyanine, $[Mn(II)PcS_4]^{4-}$) has been studied as a CA, both in solution and in MRI of tumor-bearing mice, and shows preferential retention and contrast in the tumor [60,61]. With the objective of developing new water soluble phthalocyanines and metallophthalocyanines with potential for molecular imaging applications, we report the synthesis of water soluble phthalocyanines, bearing either four PEG500 or four choline substituents in the macrocyclic structure, and the preparation of their corresponding Zn(II) and Mn(III) complexes. Since anionic manganese phthalocyanines have been already proposed for studies as CA for MRI, we have decided to concentrate on the two other most common types of water soluble phthalocyanines, with neutral (PEG500) and cationic (choline) substituents. We describe their photophysical characteristics, proton relaxivity, and cytotoxicity, evaluated with epithelial HeLa cells of the Mn(III)-phthalocyanine complexes.

2. Results and discussion

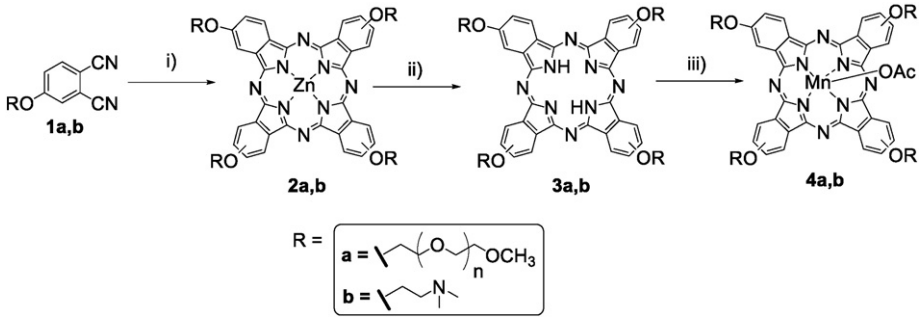
2.1. Synthesis of phthalocyanine complexes

The strategy adopted for the synthesis of biocompatible phthalocyanines **2**–**4** is depicted in Scheme 1. Phthalonitriles were synthesized by applying small changes to established methods for phthalonitrile modification [42,62,63], in particular, using ultrasounds as heating source, which considerably reduces reaction times.

The condensation reaction of both PEGylated- (**1a**) and dimethylaminoethoxy- (**1b**) substituted phthalonitriles, to give the corresponding zinc(II) phthalocyanines (**2a**–**2b**), was carried out in a DMAE/*n*-butanol (mixture DMAE = dimethylaminoethanol), following a procedure previously developed by some of us [39,40]. It should be emphasized that, using this methodology, we could obtain **2b** in a yield three times greater (50%) than that reported in the literature [62]. This might be due the different strategy adopted by us, in which a DMAE/*n*-butanol mixture was used as solvent, instead of *n*-pentanol. This mixture allows the solubilization of the reagents, while the macrocycle formed has a somewhat lower solubility, due to the presence of a long alkyl chain alcohol, especially when the phthalocyanine possesses quite polar substituents [39], thereby reducing possible macrocycle decomposition during the reaction.

The new compounds **2a**–**2b** were unequivocally characterized by means of ^{1}H-NMR, where **2a** showed its characteristic PEG peaks between 4.20 and 3.33 ppm, along with the phthalocyanine characteristic aromatic protons appearing at 7.68–7.66 ppm and 7.29–7.18 ppm. Mass spectrometry was also performed, and showed the characteristic polydispersed m/z peaks from PEGylated 2327.064, 2371.093, 2415.117, 2459.149, 2503.176 (polydisperse distribution). The data for compound **2b** is in agreement with the literature [62].

Our goal in preparing Mn(III) phthalocyanines led us to promote the demetallation of compounds **2a**–**2b** for subsequent manganese insertion (see Scheme 1). We developed this strategy because attempts at the direct condensation of the corresponding phthalonitriles with manganese(II) acetate only gave the Mn(III) phthalocyanines in very low yields, and also required quite difficult purification procedures. In contrast, the strategy of metal removal followed by metal insertion provides a very amenable methodology [64–66] to obtain the desired phthalocyanine metal complexes. Consequently, after preparing the Zn(II) phthalocyanines **2a**–**2b**, we carried out the demetallation procedure, by boiling the compounds in pyridine in presence of pyridinium-hydrochloride salt [66], following the course of reaction by UV/Vis spectroscopy to obtain free base phthalocyanines **3a**–**3b** in 68% and 79% yields, respectively. It should be noted that, using our strategy, we obtained **3b** in 47.5% overall yield, starting from phthalonitrile **1b**, while the same compound was reported before in 14% yield [62]. Subsequent complexation of the metal free phthalocyanines with manganese(II) acetate was carried out under air atmosphere, using



Scheme 1. Synthesis of the hydrophilic phthalocyanines **2**–**4**. Reagents and conditions: i) DMAE, *n*-butanol, Zn(II) acetate dihydrate, 120 °C, 24 h; ii) pyridine-HCl, pyridine, 120 °C, 17 h; iii) Mn(II) acetate tetrahydrate, DMF, 80 °C, 8 h.