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Harnessing medically-relevant Metals onto water-soluble Subphthalocyanines: towards Bimodal Imaging and Theranostics

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Subphthalocyanine (SubPc), a putative fluorophore for optical imaging (OI), was conjugated to chelating ligands (DOTA, DTPA) affording water-soluble conjugates complexed with (non radioactive) metals relevant to the following medical imaging techniques / therapies: MRI (Gd), PET (Cu, Ga), SPECT (In, Ga), RIT (Cu, Y), NCT (Gd). Magneto-optical properties of ditopic Gadolinium species (and optical properties of other metal containing species) were examined (brightness ($\varepsilon \times \Phi_F$) and relaxivity R_1) and fluorescence confocal/biphoton microscopy studies were conducted.

Introduction

Metals are commonly used for biomedical applications, such as imaging or therapy.[1-3] *(i) Imaging:* paramagnetic metals, such as Gd, are used for Magnetic Resonance Imaging (MRI), the metal binds one molecule of water that relaxes.[1] Metal radionuclides, such as ^{111}In or ^{67}Ga are used in Single Photon Emission Computed Tomography (SPECT), as they emit one gamma ray upon radioactive disintegration.[2] Other radionuclides such as ^{64}Cu or ^{68}Ga are used for Positron-Emission Tomography (PET), as they emit a positron, which annihilates with an electron affording two gamma rays.[3] So far, most imaging techniques rely on MRI and radionuclide-containing species.[1-3] However, Optical Imaging (OI) is a promising non-invasive technique mostly used in preclinical studies, ophthalmic angiography and occasionally used for tumor resection.[4-6] OI is a sensitive technique, that relies on fluorophores, the efficiency of which is assessed by a range of optical and photophysical properties: absorption and emission couples that need to be biologically-relevant, Stokes shift, epsilon, fluorescence quantum yield, robustness.[4-6] In classical fluorophores, OI does not involve metals, albeit a variant luminescent lanthanides.[7] We, and others, suggested that subphthalocyanines (SubPc; aza-bridged tris-isoindole centered on metalloid Boron) might be candidates of interests.[8-11] SubPcs are well known either as synthons for the synthesis of A_3B-phthalocyanines, or in the field of photovoltaics,[12,13] but they remain quite new in the field of biomedical research.

(ii) Therapy: metal ions are also employed to achieve various therapies: more energetic β^- emitter radionuclides may be specifically used for radioimmunotherapy (RIT), such as ^{67}Cu, ^{177}Lu, or ^{90}Y (lymphomes).[14] A variant called neutron capture therapy (NCT) uses neutron bombardement of metalloid or metals, such as ^{157}Gd (Gd-NCT) to liberate high-energy Auger electrons.[15]

A popular approach consists in binding two imaging probes or an imaging probe and a therapeutic probe together, to afford a monomolecular bimodal contrast agent or a theranostic contrast agent, respectively.[16,17] Bimodal imaging allows to add the advantages of each imaging technique (increasing the accuracy, space resolution, etc. to significantly improve the medical diagnosis), while theranostics is a bimodality achieving imaging and therapy.

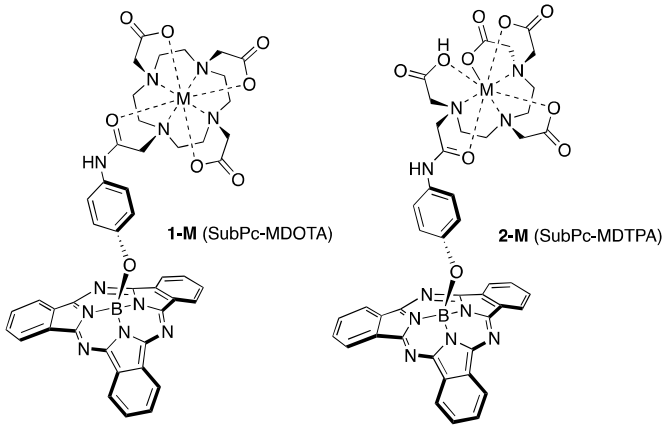

Fig. 1: SubPc-MDOTA/DTPA **1/2-M**: Subphthalocyanine appended with a chelator moity (DOTA or DTPA) complexed with medically-relevant metals: Gd, Y, In, Cu, Ga, Lu (non radioactive analogs used here).

Herein, the appending of cyclic or linear polyamino-carboxylic acid DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) and DTPA (diethylene triamine pentaacetic acid) onto SubPcs addressed two main goals: A) make SubPc water soluble, B) harness known metal complexes relevant to medical imaging/therapy (MRI, PET, SPECT, RIT, NCT) [1-3,14-15] to SubPcs affording bimodal/theranostic species **1/2-M** (Fig.1). Such strong chelators of metals (known to prevent inadventious metal release and subsequent toxicity)[18] were appended on SubPc prior to the chelation of medically relevant metals (Cu, Ga, In, Lu, Y).[1-3,14,15] Subsequent imaging properties will be addressed with the non-radioactive magneto-optical **1-Gd** species ($\varepsilon \times \Phi_F$, R_1), and fluorescence microscopy imaging will be achieved.

Results and discussion

Synthesis of water-soluble SubPcs 1/2-M. SubPc-DOTA/DTPA **1/2** were synthesized from amino-SubPc synthon **4** (Fig. 2), the synthesis of which was previously described.[11] Briefly, cyclotrimerization of dicyanobenzene with BCl_3 in p-xylene led to a subphthalocyanine bearing an axial chlorine atom (SubPc-Cl),[19] which was not isolated and was directly reacted with 4-nitrophenol. It resulted in the displacement of the chlorine atom for a distal phenoxy moiety affording SubPc-NO_2 species **3**. Compound **3** was subsequently reduced into the amine **4** by hydrogenation in the presence of palladium on charcoal. SubPc-NH_2 **4** was further acylated with commercially available tri- and tetra- protected acid DOTA and DTPA (DO3AtBu or DT4AtBu) by peptide coupling using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) as coupling agent.

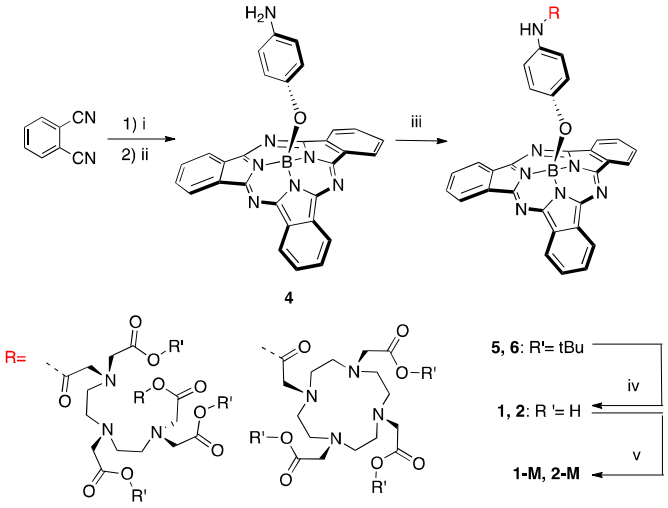

Fig 2: Syntheses of SubPc-DOTA **1** and SubPc-DTPA **2** and subsequent metallation to afford **1-M** and **2-M** (see Fig.1). Conditions: (i) BCl_3, p-xylene, reflux then 4-nitrophenol, toluene, reflux 29 % (ii) H_2, Pd/C, T.A. 88 %; (iii) DO3AtBu (tri-t-butyl-2,2',2''-(1,4,7,10-tetraazacyclododecane-1,4,7-triyl) triacetate, or DT4AtBu (3,6,9-tris(2-(t-butoxt)-2-oxoethyl)-13,13-dimethyl-11-oxo-12-oxa-3,6,9-triazatetradecan-1-oic-acid), EDC, NHS, THF, T.A. 61 % for DOTA and 50 % for DTPA (iv), TFA, T.A. 95 % for DOTA and 75 % for DTPA (v) MX_2 or MX_3, DMSO/H_2O (5:95 vol.), T.A. (see table 1 for yields)

The reaction proceeds with reasonable yield, affording SubPc **5** and **6** with 61 % and 50 %, respectively. Compounds **5** and **6** were fully characterized by conventional spectrometric (MS) and spectroscopic methods (NMR, UV-Vis, MS) (S1-S6).

The ^{1}H-NMR spectrum of SubPcDTPA-tBu_4 **6** exhibits various signals, which confirms the presence of the polyamine moieties (Fig. 3). The hydrogen atom chemical shift of the amide group was found at 9.26 ppm (**e**). The *tert*-butyl groups lead to three singlets at 1.11, 1.14 and 1.15 ppm (**h**). Peaks at 2.97-3.06 ppm (acetate, **g**) and 2.38-2.53 ppm (**f**) correspond to the aliphatic protons of the DTPA moiety. The signals of the SubPc moieties were found in the classical window downfield, i.e. 7.64 and 8.58 ppm for peripheral hydrogen atoms (**a** and **b**), and 5.09/6.80 for doublets of the apical phenoxy group (**c** and **d**), which are shielded because of the anisotropic cone effect of the SubPc macrocycle.

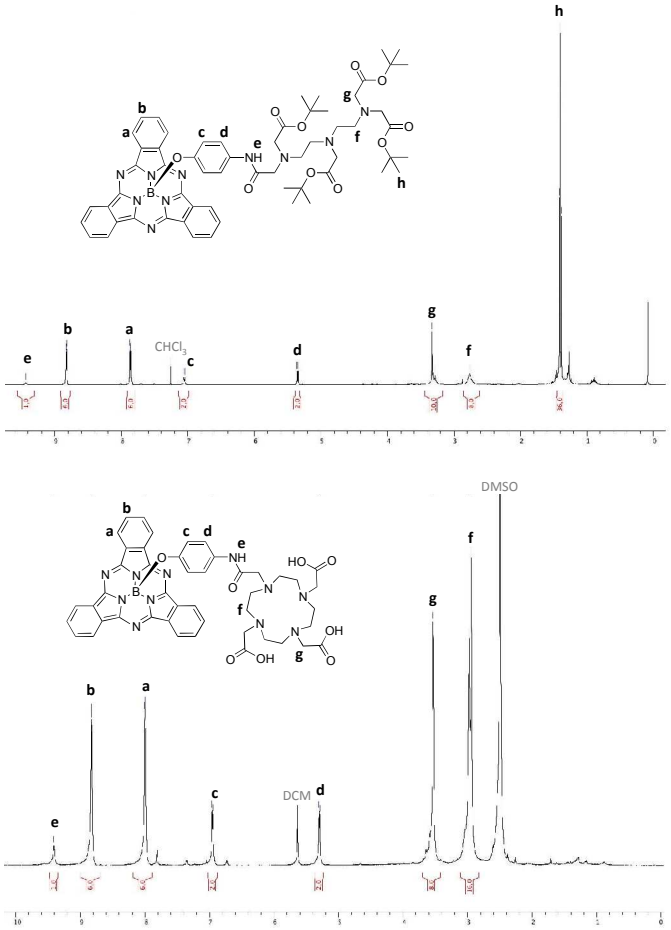

Fig. 3: Top: ^{1}H-NMR spectrum of **6** in $CDCl_3$ (300 MHz, 300 K). Bottom: ^{1}H-NMR spectrum of **1** in DMSO-d_6 (500 MHz, 400 K).

The removal of the t-Bu protective groups in **5**, **6** was achieved upon hydrolysis of the ester groups using pure trifluoroacetic acid (TFA) and room temperature conditions, to afford SubPc **1** and **2**, respectively. While esters **5** and **6** are fully soluble in dichloromethane, the deprotected acid species **1** and **2** are not. Species **1** and **2** are highly hydrophilic, and have a good solubility in methanol and water. Therefore, appending DOTA

or DTPA chelates onto SubPc appeared as an efficient strategy to achieve water-solubility of SubPc in mM ranges. Spectroscopic studies (UV-Vis) seem to indicate that SubPcDOTA **1** does not aggregate in a water-DMSO mixture (95/5 vol.) (Fig. 4): it has a sharp maximum absorption band at 569 nm, comparable to that of monomeric SubPc species in organic solvents. Compounds **1** and **2** were fully characterized by HR-MS, ^{1}H-NMR, and UV/Vis (Fig.3AB, 4A,S1-S10).[11] The ^{1}H-NMR spectrum of the DOTA-SubPc conjugate **1** is depicted in Fig. 3 (Bottom).

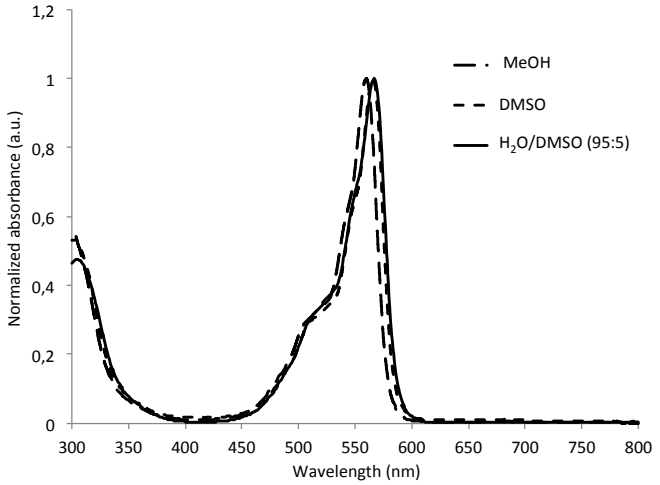

Fig. 5: Normalized UV-Vis spectrum of **1** in various solvent (MeOH, DMSO and 5 % DMSO in water).

The completion of the deprotection step affording **1-2** was verified by the absence of the *tert*-butyl singlet signals upfield (1-1.5 ppm). Moreover, the ^{1}H-NMR spectrum exhibits signals comparable to that previously described with **6**. The water-solubility of **1**/**2** decreased after complexation with metals. The metallation step and the purification were carefully optimized to anticipate future metallations with radiolabelled analogs relevant to PET/SPECT/ RIT, and also to characterize the metal complex. The procedure described below took ca. 120 minutes to achieve both the metallation and purification steps, which may still appear reasonable in light of the half-life ($t_{1/2}$) of the metal-radionuclides (that ranges from days (^{177}Lu (6.65 days), ^{67}Ga (3.26 days), ^{90}Y (3 days), ^{111}In (2.80 days), ^{67}Cu (2.57 days), to only hours (^{64}Cu (12h), ^{68}Ga (1.08h))).[1-3,14,15] Typically, SubPc-DOTA/DTPA species **1**/**2** were reacted with 1.1 equiv. of metal salt (i.e. $Gd(NO_3)_3.6H_2O$, $CuCl_2$, MCl_3 (In, Lu, Y)) at 50°C for 2h. Purification of metal complexes was achieved upon centrifugation and washing with water to remove the excess of free metals (4x10 min). However, radiolabelling are known to use a deficit of metal (up to one hundredth) rather than an excess (here), as a result the centrifugation step may not be achieved for metal radionuclides, which shortens the time of the overall protocol. All (cold) complexes were characterized by HR-MS (ESI-QTOF)(Fig. S7-S11). An example is shown Fig. 6 (Top), which

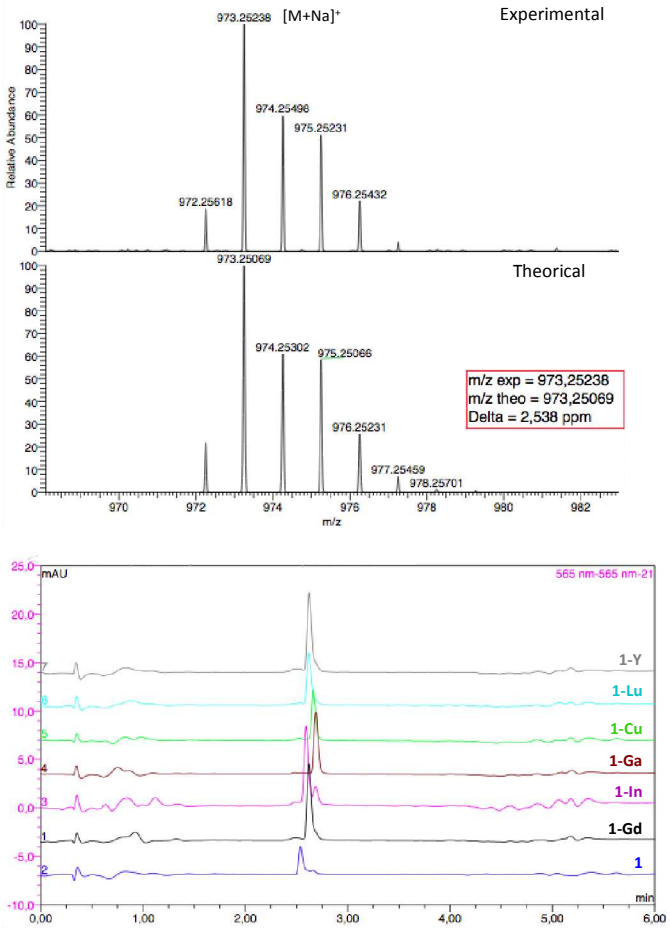

Fig. 6: Top: HR-MS spectrum of **1-Cu**. Bottom: superposition of HPLC chromatograms of ligand **1** and gadolinium, Indium, Gallium, Copper, Lutetium and Yttrium complex **1-M**.

depicts the copper complex. The $[M+Na]^+$ ion peak is observed at m/z= 973.26236, which corresponds to the theorical mass. The purity of the complexes was assessed by analytical HPLC (reverse phase C18), where the chromatogram displays only one peak (Fig. 6, bottom). DOTA/DTPA metal complexes (i.e. metal ion in a tetra/tris-carboxylate/ mono-amide / tetraamine environment) are well documented in the literature regarding the expected geometry of the metal ions coordination sphere, and the metal potential (pM) (with Gd^{3+}, In^{3+}, Y^{3+}, Lu^{3+} and Cu^{2+} ions).[18,20] Spectroscopic studies (UV-Vis) examining **1-Gd** in the presence of gadolinium complexing methyl-orange[21] did not show the presence of free metal, which is crucial for subsequent relaxivity measurements (Fig. S12).‡ **Imaging properties** of **1**/**2** and **1-M**/**2-M** were addressed through fluorescence and through relaxivity measurements and fluorescence microscopy in the case of **1-Gd** (Fig.7).

A) The fluorescence properties of SubPc-DOTA/DTPA **1**/**2** were found to be comparable with that of other parent SubPcs previously reported (Table 1).[6-8,12,13] Upon excitation at 478 nm in methanol, subsequent fluorescence emission at 565 nm was observed (Fig. 7-Top). Measurements were also performed in dimethylsulfoxyde (DMSO) or water containing DMSO (95:5). Even a lower fluorescence quantum yield (Φ_F = 0.06-0.10),

	Metallation Yield (%)	Abs maximum wavelength (λ_{max}, nm)[a]	Em maximum wavelength (λ_{em}, nm)[a]	Stokes Shift (nm)[a]	Molar extinction coefficient (ε, 10^{-3} mol^{-1}.L^{-1}.cm^{-1})[a]	Fluorescence quantum yield (Φ_F)	Brightness[a]
1	/	560	569	9	61.6	0.17[a]/0.06[b]/0.10[c]	10.472
1-Gd	82	561	570	9	52.1	0.09[a]	4.689
1-In	74	561	570	9	52.1	0.11[a]	5.731
1-Ga	51	561	570	9	60.3	0.08[a]	4.824
1-Cu	66	560	570	10	49.6	0.03[a]	1.488
1-Lu	51	559	570	11	59.8	0.10[a]	5.980
1-Y	92	559	570	11	66.2	0.10[a]	6.620
2	/	560	570	10	65.1	0.18[a]/0.07[b]/0.07[c]	11.718
2-In	81	560	570		63.2	0.10[a]	6.320

Table 1: Optical properties of SubPc-MDOTA **1-M** and SubPc-DOTA **1** in methanol[a], DMSO[b] or water/DMSO (95:5)

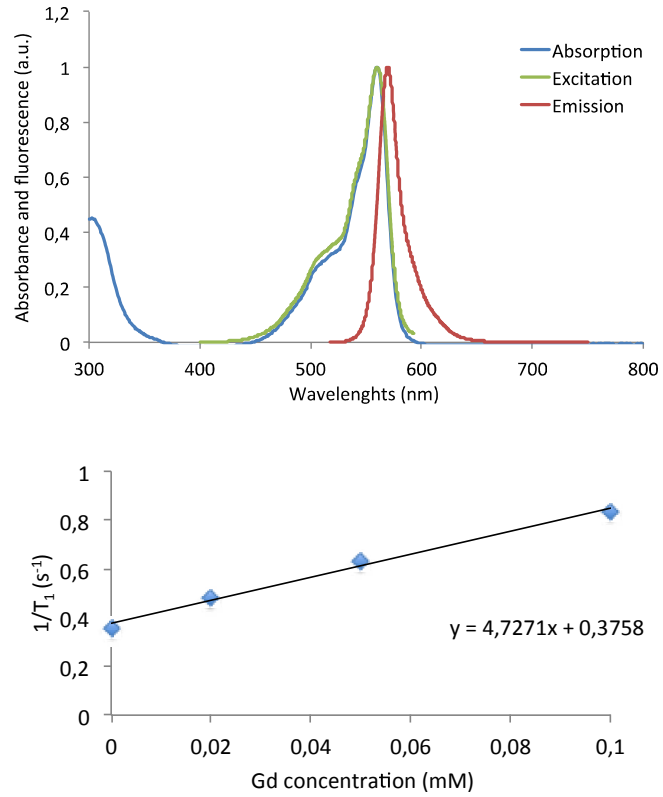

Fig. 7: Magneto-optical properties for **1** and **1-Gd**. Top: Absorbtion (blue line), emission (red line) and excitation (green line) spectrum of **1-Gd** in MeOH (298 K*)*. Middle: Relaxivity measurements of **1-Gd** solution (5 % DMSO in water). Reverse of longitudinal relaxation time as a function of concentration. Bottom: Biphotonic imaging of B16 cells incubated with probes (1h) and fixation (methanol) (exc. 720 nm). Biphotonic images of SubPc-DOTA-Gd (**1-Gd**) : D) orange channel, E) Red Channel, F) zoom (60x)

fluorescence still occurs in water. Similarly, the fluorescence quantum yield of most metallated SubPc-**M**DOTA/DTPA species (**1/2-M**) measured in methanol (Φ_F = 0.08-0.10) was about twice as less than its free-base precursor (Φ_F = 0.17-0.18), except for paramagnetic Cu^{2+} (Φ_F = 0.02), which was reported to dramatically affect the fluorescence of vicinal fluorophores by energy transfer,[22] unlike others metals.

B) The Relaxivity measurement on **1-Gd** in DMSO/water mixture (95:5) was performed at low field (20 MHz) and for three concentrations (Fig. 7-middle). It led to R_1 values up to 4.7 $mM_{Gd}^{-1}s^{-1}$, which is in the range of clinically used DOTA-Gadolinum complex (Dotarem®, 3.6 $mM_{Gd}^{-1}s^{-1}$).

C) In vitro fluorescence microscopy studies (confocal/biphoton) were carried out on B16F10 cells following an established procedure:[11, ‡] a) Incubation of probes over a period of one hour was performed, followed by rinsing cells with PBS, and cells fixation with methanol b) Probes were not cytotoxic against B16 over the incubation period (1h, MTT test); c) Preliminary stability studies were carried out in the medium where incubation took place for imaging studies (i.e. incubation in RPMI at 37°C in the dark) by monitoring the decrease of the UV/Vis absorbance. This may be accountable for 10% decomposition over 1 h (Fig. S14). Also, an MS of such a mixture after 1h and 36h incubation showed the presence of the molecular peak, which indicates that the metal was not released. It also indicates that the cleavage of the apical B-O bond was not substantial. This is reminiscent of previous studies addressing the stability of the B-O bond in various media.[23] d) Subsequent fluorescence imaging of B16 cells were achieved by biphotonic microscopy. Detection was performed at different spectral windows, and fluorescence was observed on the orange channel (Fig. 7A-B) or the red channels (Fig. 7C). Fluorescence images of B16 cells showed that cells incorporated the DOTA/DTPA-containing SubPc **1-2**, either the non metallated version **1** (Fig. 7A), or the metallated species **1-Gd** (Fig. 7B-C).

Conclusions

SubPcs are optical probes of interest for biological studies in light of the Lavis and Raines diagram, with emission wavelenghts (560 nm/570 nm) falling in the category used by biologists. Two possible apical chelates (DOTA, DTPA) were conveniently introduced affording water-soluble subphthalocyanines **1/2**, which are of interest compared to other water-soluble SubPcs.[24-28] These conjugates are versatile platforms for subsequent introduction of metals relevant to medical imaging/ therapy (affording bimodal and theranostic contrast agents), such as: Gd (MRI, GdNCT), Cu (PET, RIT), In (SPECT), Lu, Y (RIT). At such a preliminary stage, the study only focused on: a) the syntheses of water-soluble SubPcs b) an optimized and time-controlled methodology for the metallation and purification steps of **1/2-M** achieved with non-radioactive (cold) metal species transposable to future work with the "real" radionuclides, the $t_{1/2}$ of which is an issue (i.e. ^{68}Ga, $t_{1/2}$ = 68 min) (the protocol developed herein managed to remain below/within acceptable range of the half-life of the radionuclide);[3,29] c) a variant in the design of our magneto-optical probes compared to our previous system (paramagnetic nanoparticle/phthalocyanine nanohybrid),[30] leading to **1-Gd**, with competitive properties (i.e. brightness ε x Φ_F (4,700) and R_1 (4.7 $mM_{Gd}^{-1}s^{-1}$)), d) and fluorescence imaging of B16 cells was successfully achieved by confocal/biphoton microscopy (albeit MRI, PET/SPECT imaging studies are planned to address the dual imaging of **1/2-M**).

Experimental

Materials. All solvents used were analytical grade. All other reagents were commercially available and used as received (see SI).

Instrumentation. NMR and Mass Spectrometry analyses were carried out at the "Plateforme d'Analyses Chimiques et de Synthèse Moléculaire de l'Université de Bourgogne" (PACSMUB). ^1H NMR spectra (300 MHz, 500 MHz, 600 MHz), and ^{13}C NMR spectra (75 MHz), were recorded on Bruker 300 Avance III, Bruker 500 Avance III, or Bruker 600 Avance II spectrometers, respectively. Chemical shifts are quoted in parts per million (δ) relative to tetramethylsilane, TMS (^1H and ^{13}C), using the residual protonated solvent (^1H) or the deuterated solvent (^{13}C) as an internal standard (residual chloroform from deuterated chloroform chemical shift was set at 7.26 ppm and deuterated dimethylsufoxyde at 2.50 ppm). Coupling constants are reported in Hertz. The following abbreviations were used to describe spin multiplicity: s = singlet, d = doublet, t = triplet, m = multiplet. The identity of the complexes were unambiguously established using high-resolution mass spectrometry and multinuclear NMR spectroscopy. The standard mass were performed on Ultraflex II LRF 2000 (BRUKER), using dithranol or DHB as a matrix. The exact mass of the complexes was obtained on a Thermo LTQ Orbitrap XL ESI-MS. SubPc spectra (UV-Vis) were performed on a Shimadzu UV-2550 spectrophotometer, in a solvent of choice (DCM, MeOH, DMSO, water) in glass cuvettes 1x1x3 cm (1 cm path).

Synthetic Chemistry

Compound 1. (B-(4 (DO3AAM)phenoxy)[subphtalocyaninato] boron(III)) **5** (150 mg, 0.142 mmol) was stirred during 12 h at room temperature in 5 mL of trifluroacetic acid. The blue solution was evaporated and the residue was resuspended in 5 mL of dichloromethane, dispersed by ultrasonication (5 min) and further evaporated to dryness. This operation was repeated three times to remove the residual acid. The pink solid obtained was resuspended in 5 mL of dichloromethane, filtered off and washed 3 times with dichloromethane, then dried under reduced pressure (119 mg, 95 %). ^1H NMR (600 MHz, DMSO-d_6, 360 K): δ (ppm)= 2.89-3.04 (m, 16H); 3.57 (m, 8H); 5.29 (d, ^3J= 8.9 Hz, 2H); 6.96 (d, ^3J= 8.9 Hz, 2H); 8.80 (m, 6H); 8.83 (m, 6H); 9.41 (s, 1H). MS MALDI-TOF: m/z= 394.74 [M-OPhDOTAAM]$^+$ (calcd for $C_{24}H_{12}BN_6^+$: 395.12), 518.93 [M-Sub+Na]$^+$ (calcd for $C_{20}H_{29}N_5O_8Na^+$: 518.22), 890.31 [M+H]$^+$ (calcd for $C_{46}H_{45}BN_{11}O_8^+$: 890.35), 912.23 [M+Na]$^+$ (calcd for $C_{46}H_{44}BN_{11}O_8Na^+$: 912.34), 928.28 [M+K]$^+$ (calcd for $C_{46}H_{44}BN_{11}O_8K^+$: 928.31), 950.20 [M-H+Na+K]$^+$ (calcd for $C_{46}H_{43}BN_{11}O_8NaK^+$: 950.29), 972.188 [M-2H+2Na+K]$^+$ (calcd for $C_{46}H_{42}BN_{11}O_8Na_2K^+$: 972.27). HR-MS ESI: m/z= 890.35147 [M+H]$^+$ (calcd for $C_{46}H_{44}BN_{11}O_8^+$: 890.35480), 912.33251 [M+Na]$^+$ (calcd for $C_{46}H_{44}BN_{11}O_8Na^+$: 912.33674). HPLC: Rt (min)= 2.533 (97.76 % at 254 nm; 99.65 % at 565 nm). UV-Vis (MeOH), λ_{max} (nm) ($\varepsilon \times 10^3$ $L.mol^{-1}.cm^{-1}$): 303 (31.7), 560 (61.6).

Compound 2. (B-(4-(DT4AAM)phenoxy)[subphtalocyaninato] boron(III)) **6** (150 mg, 0,136 mmol) was stirred during 12 h at room temperature in 5 mL of trifluoroacetic acid. The blue solution was evaporated and the residue was resuspended in 5 mL of dichloromethane, dispersed by ultrasonication (5 min) and further evaporated to dryness. This operation was repeated three times until the residual traces of acid were removed. The pink solid obtain was resuspended again in 5 mL of dichloromethane, filtered off and washed 3 times with dichloromethane, then dried under reduced pressure (90 mg, 75 %). ^1H NMR (600 MHz, DMSO-d_6, 400 K): δ (ppm)= 2.79 (m, 8H); 3.29 (s, 2H); 3.35 (s, 2H); 3.38 (s, 6H); 5.28 (d, ^3J= 8.9 Hz, 2H); 6.95 (d, ^3J= 8.9 Hz, 2H); 8.80 (m, 6H); 8.83 (m, 6H); 9.26 (s, 1H); 11.58 (s broad, 4H). MS MALDI-TOF: m/z= 484.64 [M-Sub+H]$^+$ (calcd for $C_{20}H_{29}N_4O_{10}^+$: 485.19), 879.122 [M+H]$^+$ (calcd for $C_{44}H_{40}BN_{10}O_{10}^+$: 879.30), 901.105 [M+Na]$^+$ (calcd for $C_{44}H_{39}BN_{10}O_{10}Na^+$: 901.28), 917.08 [M+K]$^+$ (calcd for $C_{44}H_{39}BN_{10}O_{10}K^+$: 917.26). HR-MS ESI-Q: m/z= 879.29973 [M+H]$^+$ (calcd for $C_{60}H_{71}BN_{10}O_{10}Na^+$: 879.30240), 901.28059 [M+Na]$^+$ (calcd for $C_{60}H_{71}BN_{10}O_{10}Na^+$: 901.28434). HPLC: Rt (min)= 2.610 (95.2 % at 254 nm; 96.85 % at 565 nm). UV-Vis (MeOH), λ_{max} (nm) ($\varepsilon \times 10^3$ $L.mol^{-1}.cm^{-1}$): 303 (33.5), 560 (65.1).

General metallation procedure for the synthesis of Sub-MDOTA (1-M) and Sub-MDTPA (2) complexes. B-(4-(DO3AAM)phenoxy)[subphtalocyaninato]boron(III) **1** (10 mg, 0.0112 mmol) or B-(4-(DT4AAM)phenoxy)[subphtalocyaninato]boron(III) **2** (10 mg, 0.0114 mmol) were solubilized in a DMSO/water mixture (95:5 vol., 10 mL). The pH of the solution was adjusted to approximately 7 using an aqueous solution of sodium hydroxide (0.1 M). Then 1 mL of a 12.4 µM solution of the corresponding metallic salt was added (1.1 equiv.), and the reaction was left for 2 hours at 50°C. Upon cooling down to room temperature, the mixture was diluted with 20 mL of water and centrifuged. The precipitate was subsequently washed with water by centrifugation (3×30 mL). Finally, the pink residual solid was suspended in 10 mL of water and lyophilized to yield the desired subphtalocyanine.

Compound 1-Gd. B-(4-(DO3AAM-gadolinium(III))[phenoxy) subphtalocyaninato]boron(III). Metallic salt solution: $Gd(NO_3)_3$, 5 H_2O (5.37 mg, 0.0124 mmol) in 1 mL of water. (9.5 mg, 82 %). MS MALDI-TOF: m/z= 1045.35 $[M+H]^+$ (calcd for $C_{46}H_{42}BGdN_{11}O_8^+$: 1045.26), 1067.38 $[M+Na]^+$ (calcd for $C_{46}H_{41}BGdN_{11}O_8Na^+$: 1067.24), 1083.35 $[M+K]^+$ (calcd for $C_{46}H_{41}BGdN_{11}O_8K^+$: 1083.21). HR-MS ESI-Q: m/z= 1067.23979 $[M+Na]^+$ (calcd for $C_{46}H_{41}BGdN_{11}O_8Na^+$: 1067.23826). HPLC: Rt (min)= 2.620 (96.50 % at 254 nm; 96.90 % at 565 nm). UV-Vis (MeOH), λ_{max} (nm) ($\varepsilon \times 10^3$ $L.mol^{-1}.cm^{-1}$): 301 (31.7), 561 (52.1).

Compound 1-In. B-(4-(DO3AAM-indium(III))[phenoxy) subphtalocyaninato]boron(III). Metallic salt solution: $InCl_3$ (2.74 mg, 0.0124 mmol) in 1 mL of water. (8.3 mg, 74 %). MS MALDI-TOF: m/z= 607.74 $[M-Sub+H]^+$ (calcd for $C_{22}H_{31}BInN_5O_8^+$: 608.12), 1002.20 $[M+H]^+$ (calcd for $C_{46}H_{42}BInN_{11}O_8^+$: 1002.23), 1024.18 $[M+Na]^+$ (calcd for $C_{46}H_{41}BInN_{11}O_8Na^+$: 1024.22), 1040.16 $[M+K]^+$ (calcd for $C_{46}H_{41}BInN_{11}O_8K^+$: 1040.19), 1062.15 $[M-H+Na+K]^+$ (calcd for $C_{46}H_{40}BInN_{11}O_8NaK^+$: 1062.17). HR-MS ESI-Q: m/z= 1024.21812 $[M+Na]^+$ (calcd for $C_{46}H_{41}BInN_{11}O_8Na^+$: 1024.21718). HPLC: Rt (min)= 2.593 (86.2 % at 254 nm; 79.98 % at 565 nm). UV-Vis (MeOH), λ_{max} (nm) ($\varepsilon \times 10^3$ $L.mol^{-1}.cm^{-1}$): 300 (32.2), 561 (52.1).

Compound 1-Ga. B-(4-(DO3AAM-gallium(III))[phenoxy) subphtalocyaninato]boron(III). Metallic salt solution: $GaCl_3$ (44 µL of a solution in acetic acid, 0,0124 mmol) in 1 mL of water. (5.4 mg, 51 %). MS MALDI-TOF: m/z= 394.64 $[M-OPhGaDOTAAM]^+$ (calcd for $C_{24}H_{12}BN_6^+$: 395.12), 561.68 $[M-Sub+H]^+$ (calcd for $C_{22}H_{31}GaN_5O_8^+$: 562.14), 599.67 $[M-Sub+K]^+$ (calcd for $C_{22}H_{31}GaN_5O_8K^+$: 600.10), 956.42 $[M+H]^+$ (calcd for $C_{46}H_{42}BGaN_{11}O_8^+$: 956.26), 994.38 $[M+Na]^+$ (calcd for $C_{46}H_{41}BGaN_{11}O_8K^+$: 994.21). HR-MS ESI-Q: m/z= 978.24108 $[M+Na]^+$ (calcd for $C_{46}H_{41}BGaN_{11}O_8Na^+$: 978.23885). HPLC: Rt (min)= 2.690 (87.47 % at 254 nm; 98.34 % at 565 nm). UV-Vis (MeOH), λ_{max} (nm) ($\varepsilon \times 10^3$ $L.mol^{-1}.cm^{-1}$): 303 (31.1), 561 (60.3).

Compound 1-Cu. B-(4-(DO3AAM-copper(II))[phenoxy) subphtalocyaninato]boron(III). Metallic salt solution: $Cu(NO_3)_2$ (3 mg , 0.0124 mmol) in 1 mL of water. (7 mg, 66 %). MS MALDI-TOF: m/z= 952.24 $[M+2H]^+$ (calcd for $C_{46}H_{44}BCuN_{11}O_8^+$: 952.28), 974.22 $[M+H+Na]^+$ (calcd for $C_{46}H_{43}BCuN_{11}O_8Na^+$: 974.26), 990.20 $[M+H+K]^+$ (calcd for $C_{46}H_{43}BCuN_{11}O_8K^+$: 989.22). HR-MS ESI-Q: m/z= 973.25238 $[M+Na]^+$ (calcd for $C_{46}H_{41}BCuN_{11}O_8Na^+$: 973.25069). HPLC: Rt (min)= 2.667 (95.92 % at 254 nm; 90.86 % at 565 nm). UV-Vis (MeOH), λ_{max} (nm) ($\varepsilon \times 10^3$ $L.mol^{-1}.cm^{-1}$): 300 (30.0), 560 (49.6).

Compound 1-Lu. B-(4-(DO3AAM-lutetium(III))[phenoxy) subphtalocyaninato]boron(III). Metallic salt solution: $LuCl_3$, 6 H_2O (4.83 mg, 0.0124 mmol) in 1 mL of water. (5.4 mg, 51 %). MS MALDI-TOF: m/z= 1062.36 $[M+H]^+$ (calcd for $C_{46}H_{42}BLuN_{11}O_8^+$: 1062.27), 1084.38 $[M+Na]^+$ (calcd for $C_{46}H_{41}BLuN_{11}O_8Na^+$: 1084.25), 1100.33 $[M+K]^+$ (calcd for $C_{46}H_{41}BLuN_{11}O_8K^+$: 1100.23). HR-MS ESI-Q: m/z= 1084.25608 $[M+Na]^+$ (calcd for $C_{46}H_{41}BLuN_{11}O_8Na^+$: 1084.25406). HPLC: Rt (min)= 2.620 (89.19 % at 254 nm; 96.77 % at 565 nm). UV-Vis (MeOH), λ_{max} (nm) ($\varepsilon \times 10^3$ $L.mol^{-1}.cm^{-1}$): 302 (28.9), 559 (59.8).

Compound 1-Y. B-(4-(DO3AAM-yttrium(III))[phenoxy) subphtalocyaninato]boron(III). Metallic salt solution: YCl_3, 6 H_2O (3.76 mg, 0.0124 mmol) in 1 mL of water. (10 mg, 92 %). MS MALDI-TOF: m/z= 394.79 $[M-OPhLuDOTAAM]^+$ (calcd for $C_{24}H_{12}BN_6^+$: 395.12), 582.08 $[M-Sub+H]^+$ (calcd for $C_{22}H_{31}N_5O_8Y^+$: 582.12), 976.49 $[M+H]^+$ (calcd for $C_{46}H_{42}BN_{11}O_8Y^+$: 976.24), 998.48 $[M+Na]^+$ (calcd for $C_{46}H_{41}BN_{11}O_8YNa^+$: 998.22), 1014.47 $[M+K]^+$ (calcd for $C_{46}H_{41}BN_{11}O_8YK^+$: 1014.19). HR-MS ESI-Q: m/z= 998.21999 $[M+Na]^+$ (calcd for $C_{46}H_{41}BYN_{11}O_8Na^+$: 998.21911). HPLC: Rt (min)= 2.623 (87.55 % at 254 nm; 96.40 % at 565 nm). UV-Vis (MeOH), λ_{max} (nm) ($\varepsilon \times 10^3$ $L.mol^{-1}.cm^{-1}$): 303 (34.1), 560 (66.2).

Compound 2-In. B-(4-(DT4AAM-indium(III))[phenoxy) subphtalocyaninato]boron(III). Metallic salt solution: $InCl_3$, 6 H_2O (2.76 mg, 0.0125 mmol) in 1 mL of water. (7.9 mg, 81 %). MS MALDI-TOF: m/z= 991.12 $[M+H]^+$ (calcd for $C_{44}H_{37}BInN_{10}O_{10}^+$: 991.18), 1013.21 $[M+Na]^+$ (calcd for $C_{44}H_{36}BInN_{10}O_{10}Na^+$: 1013.16), 1035.19 $[M-H+2Na]^+$ (calcd for $C_{44}H_{35}BInN_{10}O_{10}Na_2^+$: 1035.15), 1051.17 $[M-H+Na+K]^+$ (calcd for $C_{44}H_{35}BInN_{10}O_{10}KNa^+$: 1051.12). HPLC: Rt (min)= 2.635 (93.2 % at 254 nm; 95.03 % at 565 nm). UV-Vis (MeOH), λ_{max} (nm) ($\varepsilon \times 10^3$ $L.mol^{-1}.cm^{-1}$): 304 (35.2), 560 (63.2).

Compound 3. (B-(4-nitrophenoxy)[subphtalocyaninato] boron(III)) BCl_3 (4 mL, 1 M solution in p-xylene) was added to dry phtalonitrile (0.5 g, 4 mmol), under an argon atmosphere. The reaction mixture was poured in a preheated oil bath (150°C), stirred and refluxed for 30 min. The solvent was removed under reduced pressure and the resulting solid was

resuspended in toluene (30 mL). Excess of 4-nitrophenol (12 mmol) was added and the mixture was heated to reflux for 15 hours. After evaporation to dryness, the residue was subjected to alumina gel column chromatography (eluent: DCM) ; the pure fraction was subsequently recrystallized in a dichloromethane/heptane mixture (1:1 vol.) by slow evaporation of dichloromethane, to afford (4-nitrophenolato)[subphtalocyaninato]boron(III) as a bronze crystalline solid (200 mg, 29 %). ^{1}H NMR (300 MHz, $CDCl_3$, 300 K): δ (ppm)= 5.39 (d, ^{3}J= 9.1 Hz, 2H); 7,66 (d, ^{3}J= 9.1 Hz, 2H); 7.91 (m, 6H); 8.86 (m, 6H). ^{13}C NMR (75 MHz, $CDCl_3$, 300 K): δ (ppm)= 118.3, 122.1, 125.0, 129.9, 130.7, 141.3, 151.2, 158.5. UV-Vis (DCM), λ_{max} (nm) (10^3 ε): 305.1 (41.3), 563.5 (56.7).

Compound 4: B-(4-aminophenoxy)[subphthalocyaninato] boron(III). A mixture of **3** (240 mg, 0,45 mmol) and activated palladium 10% on carbon (20 mg, 0.17 mmol) in a dichlormethane/methanol mixture (20 mL, 1:1) was stirred under hydrogen atmosphere during 72 hours. The mixture was filtered off on celite to remove the palladium and charcoal, then the solvent was removed under reduced pressure. The resulting solid was subjected to silica gel column chromatography (eluent: dichloromethane/methanol 99:1) to obtain (4-aminophenolato)[subphtalocyaninato]boron(III) **4** as a bronze solid (200 mg, 88 %). ^{1}H NMR (300 MHz, $CDCl_3$, 300 K): δ (ppm)= 3.62 (s, 2H); 5.25 (d, ^{3}J= 8.8 Hz, 2H); 6.16 (d, ^{3}J= 8.8 Hz, 2H); 7.87 (m, 6H); 8.83 (m, 6H,). ^{13}C NMR (75 MHz, $CDCl_3$, 300 K): δ (ppm)= 116.7, 120.2, 122.4, 129.9, 131.1, 139.2, 145.6, 151.4. MS MALDI-TOF: m/z= 394.34 $[M\text{-}OPhNH_2]^+$ (calcd for $C_{24}H_{12}BN_6^+$: 395.12), 503.54 $[M+H]^+$ (calcd for $C_{30}H_{19}BN_7O^+$: 504.17). HR-MS ESI: m/z= 504.1740 $[M+H]^+$ (calcd for $C_{30}H_{19}BN_7O^+$: 504.1744). UV-Vis (DCM), λ_{max} (nm) ($\varepsilon \times 10^3$ L.mol^{-1}.cm^{-1}): 303 (38.6), 562 (75.0).

Compound 5. (B-(4-(DO3A(tBu)AM)phenoxy)[subphtalocyaninato]boron(III)) A mixture of **4** (250 mg, 0.55 mmol), DOTA-tris(tBu)ester (286.4 mg, 0.5 mmol) and HBTU (227.4 mg, 0.6 mmol) into THF (15 mL) was stirred at room temperature during 12 h. The solvent was removed under reduced pressure, then the resulting solid was solubilized in 50 mL of dichloromethane. The organic layer was washed with an aqueous solution of citric acid (pH= 4, 3×50 mL) then with water (3×50 mL), and subsequently dried with magnesium sulfate, filtered off, and concentrated under vacuum. The product was subbjected to silica gel column chromatography (eluent: from pure DCM to DCM/MeOH 98:2) then dried under reduced pressure (320 mg, 61 %). ^{1}H NMR (300 MHz, $CDCl_3$, 300 K): δ (ppm)= 1.25 (s, 18H); 1.41 (s, 9H); 2.79 (m, 8H); 5.24 (d, ^{3}J= 8.9 Hz, 2H); 6.94 (d, ^{3}J= 8.8 Hz, 2H); 7.56 (s, 1H); 7.88 (m, 6H); 8.82 (m, 6H). MS MALDI-TOF: m/z= 394.35 $[M\text{-}OPhDOTAtBuAM]^+$ (calcd for $C_{24}H_{12}BN_6^+$: 395.12), 685.93 $[M\text{-}Sub+Na]^+$ (calcd for $C_{34}H_{57}N_5O_{8Na}^+$: 686.41), 1080.37 $[M+Na]^+$ (calcd for $C_{58}H_{68}BN_{11}O_8Na^+$: 1080.52). HR-MS ESI: m/z= 1080.51923 $[M+Na]^+$ (calcd for $C_{58}H_{68}BN_{11}O_8Na^+$: 1080.52472). UV-Vis ($CHCl_3$), λ_{max} (nm) ($\varepsilon \times 10^3$ L.mol^{-1}.cm^{-1}): 305 (34.5), 563 (67.0).

Compound 6. (B-(4-(DT4A(tBu)AM)phenoxy)[subphtalocyaninato]boron(III)) A mixture of **4** (250 mg, 0,55 mmol), DTPA-tetra(tBu)ester (308.6 mg, 0.5 mmol) and HBTU (227.4 mg, 0.6 mmol) into THF (15 mL) was stirred at room temperature during 12 h. The solvent was removed under reduced pressure, then the resulting solid was solubilized in 50 mL of dichloromethane. The organic layer was washed with an aqueous solution of citric acid (pH= 4, 3×50mL) then with water (3×50 mL), and subsequently dried with magnesium sulfate, filtered off, and concentrated under vacuum. The product was subjected to silica gel column chromatography (eluent: gradient of DCM/MeOH 100:0 - 98: 2 vol.) then dried under reduced pressure (275 mg, 50 %). ^{1}H NMR (300 MHz, $CDCl_3$, 300 K): δ (ppm)= 1.11 (s, 9H); 1.14 (s, 18H); 1.15 (s, 9H); 2.38-2.53 (m, 8H); 2.97 (s, 2H); 3.01 (s, 2H); 3.05 (s, 2H); 3.06 (s, 4H); 5.09 (d, ^{3}J= 8.9 Hz, 2H); 6.80 (d, ^{3}J= 8.9 Hz, 2H); 7.64 (m, 6H); 8.58 (m, 6H); 9.26 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$, 300 K): δ (ppm)= 27.14, 28.69, 80.59, 55.01, 79.98, 118.34, 119.24, 121.20, 128.76, 129.96, 131.30, 147.55, 150.25, 169.45, 169.6. MS MALDI-TOF: m/z= 394.34 $[M\text{-}OPhDT4AtBuAM]^+$ (calcd for $C_{24}H_{12}BN_6^+$: 395.12), 1103.58 $[M+H]^+$ (calcd for $C_{60}H_{77}BN_{10}O_{10}^+$: 1125.53). MS ESI: m/z= 1126.0 $[M+Na]^+$ (calcd for $C_{60}H_{71}BN_{10}O_{10}Na^+$: 1125.5). HR-MS ESI-Q: m/z= 1125.52961 $[M+Na]^+$ (calcd for $C_{60}H_{71}BN_{10}O_{10}Na^+$: 1125.53498). UV-Vis ($CHCl_3$), λ_{max} (nm) ($\varepsilon \times 10^3$ L.mol^{-1}.cm^{-1}): 303 (34.0), 563 (66.1).

Analytical chemistry

HPLC. Hydrophylic compounds (**1**, **2**, **1-M, 2-M**) were analyzed on Dionex Ultimate 3000, equipped with a Chromolith High Resolution RP-18 endcapped (5-4.6 mm, Merck) column. The Method used was the following: eluent A: CH_3CN + 0.1 % TFA; eluent B: H_2O + 0.1 % TFA; flow: 3 mL/min; equilibrate for 1 min 45 min afterwards; ramp from 100 % B to 100 % A; duration: 5 min; keep constant for 1 min; return in 1.5 min to initial conditions; detector: 214 nm, 230 nm, 254 nm, 565 nm.

Detection of free Gd. Detection of free gadolinium was performed using a complexometric indicator, xylenol orange (see Barge et al.).[21] The substraction of the curve of the mixture of **1-Gd** and xylenol minus the curve of **1-Gd** alone (full line) could be overlayed and found identical with that of free xylenol (red dashed line). This proves the absence of free gadolinium.

Relaxivity Studies. Longitudinal relaxation speeds were recorded with a Bruker Minispec low-field apparatus (20 MHz, 0.5 T, 40°C) and for three concentrations of **1-Gd** in 5% DMSO / 95 % water mixture (v/v) (Fig. S13).

Fluorescence Studies. Fluorescence measurements were performed on a Jasco FP-8500 spectrofluorometer equipped with a Xe source. Fluorescence quantum yields were calculated

using Rhodamine 6G in methanol as a reference (Φ_F = 0.94). Excitation was performed at 488 nm for both sample and reference. Emission spectra were recorded for an absorbance at 488 nm comprise between 0.03 and 0.07. Fluorescence quantum yields (Φ_F) were determined by the comparison method, using the following equation:

$$\phi_F = \phi_F(Std) \times \left(\frac{\eta}{\eta(Std)}\right)^2 \times \left(\frac{A}{A(Std)}\right) \times \left(\frac{1-10^{-Abs}}{1-10^{-Abs(Std)}}\right)$$

With:
Std correspond to standard (Rhodamine 6G)
Φ_F and Φ_F(Std): fluorescence quantum yields
η and η(Std): refractive indices of solvent used (MeOH for standard; DCM, MeOH, DMSO or water for samples)
Abs and Abs(Std): absorbances at excitation wavelength (488 nm)
A and A(Std): areas under the fluorescence curves

Biology

Cells (culture, incubation, fixation). B16-F10 Melanoma Cells were grown in RPMI supplemented with Foetal Calf Serum and 1% streptavidine. Cells were platted in 8 chambers polystyrene vessel (with tissue culture treated glass slide; BD Falcon Culture slides Ref 3541 18) two days before the experiment. Then the medium was removed and replaced with non-supplemented RPMI medium mixed with the solution of subphthalocyanine. The resulting concentration in SubPc was 10 μM. The time of incubation was set at 1h, then the cells were rinsed with PBS. The overall process of cell fixation was achieved as follows: after removing PBS, cold (-30°C) methanol (100 □L per well) was subsequently added, the plate was stored in the fridge (4°C) for 5 min, then the methanol was removed. Cold PBS (4°C) was added and the plate was stored in an ice-container prior to microscopy studies. PBS was removed by tilting the plate, the top part (plastic walls) of the plate was removed, and a small layer of PBS was added to cover the cells, then a μm-thin glass plate (for microscopy use) was placed on top of the PBS layered cells.

Cell Viability Assay. Cells were platted on 96-wells culture plates two days before the experiment. The percentage of cell viability was assessed by the tetrazolium colorimetric assay (MTT) as described by T. Mossman et al.[31] Immediately after incubation of the cells with **1/2-M**, cells were rinsed with PBS then culture medium was added to the wells, and cells were left at 37°C for 6h. The MTT solution was then added to the culture plate, and subsequent incubation was allowed for 2h, allowing the formation of blue formazan crystals. Then, the experiment was stopped : the medium was removed, and DMSO was added to dissolve the crystals. A purple solution was obtained, the absorbance of which was examined at 570 nm (using a Multiscan GO spectrometer). No toxicity was found at 10 □M and below.

Stability Studies. Probes were put in solution in RPMI medium and the absorption was monitored every 15 min for 60 min at absorbtion maximum (569 nm) (Fig. S14).

Biphoton Microscopy. Biphotonic images were collected on a Nikon A1-MP scanning microscope (Nikon, Japan). Imaging was carried out with a ×25 Apo LWD objective (NA: 1.1, Water Immersion, Nikon, Japan) at a scanning speed of 0.5 frame per second. An IR laser (Chameleon, Coherent) was used to provide a 780nm excitation. Fluorescence emission was collected on four detection channels (FF01-492/SP, FF03-525/50, FF01-575/25,FF01-629/56, Semrock).

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Notes and references

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Harnessing medically-relevant Metals to Subphthalocyanines: towards bimodal imaging and theranostics

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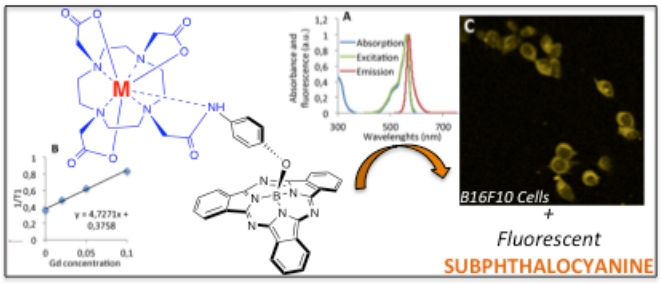

Abstract

Subphthalocyanine (SubPc), a putative fluorophore for optical imaging (OI), was conjugated to chelating ligands (DOTA, DTPA) affording water-soluble conjugates **1-2** complexed with (non radioactive) metals relevant to the following medical imaging techniques / therapies: MRI (Gd), PET (Cu, Ga), SPECT (In, Ga), RIT (Cu, Y), NCT (Gd). Magneto-optical properties of ditopic **1-Gd** species were examined (brightness (ε x Φ_F and relaxivity *RI*) and fluorescence confocal/biphoton microscopy studies were conducted.