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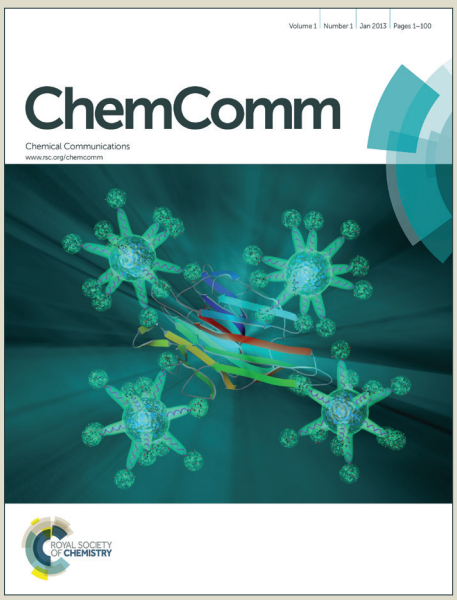

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Subphthalocyanines: addressing water-solubility, nano-encapsulation, and activation for optical imaging of B16 melanoma model.

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Water-soluble disulfonato-Subphthalocyanine (SubPc) or hydrophobic nano-encapsulated SubPc are efficient probes for the *in vitro* fluorescence imaging of cells. The 20-nm large liposome (TEM, DLS) incorporated about 13 % SubPc. Moreover, some of these fluorophores are pH activatable.

Molecular imaging allows the diagnosis of various diseases at an early stage. There is a need to develop new imaging techniques, which may eventually overcome the problems of known methods (MRI, PET).[1] Optical imaging (OI) is used in preclinical studies and seldom used in clinics. It is appealing because it is non-invasive and uses wavelengths in the optical window.[2] Hence, there is a need for new fluorophores, a) with satisfactory optical properties, and b) that could be activatable, i.e. switched ON and OFF reversibly (smart-probes). This study presents a family of fluorescent molecules which such properties, subphthalocyanine (SubPc), which has never been examined as optical probes in OI so far. They are 14-π electron aromatic macrocycles of C_3 symmetry that combine three aza-bridged isoindole units around a 4-coordinate boron atom. They have a convex π surface, which makes SubPc a peculiar fluorophore, compared to almost all known fluorophores, which are planar. We view such a structural feature as a possible asset to prevent π-π stacking (that leads to subsequent fluorescence quenching). Moreover, the apical position is another feature to help prevent aggregation, but more importantly a convenient handle for SubPc mono-functionalization. There are numerous reports on the fluorescence of SubPc (emission around 570 nm, fluorescence quantum yield Φ_F = 0.25 and up depending on the nature of alpha and beta-substituents)[3] but surprisingly none have been examined in the context of OI so far. SubPc have been widely reported in photovoltaics,[3,4] whereas reports in biomedical research are scarce (PDT on bacteria).[5,6,7] Herein, we report the use of subphthalocyanines **1a-c** as fluorescent probes for OI *in vitro*.

This study presents several aspects of fluorophores **1a-c**: the syntheses and optical properties (Fig 1,2), and four new facets: water-solubility, entrapment in lipidic nanoparticle (Np) with thorough characterization of the SubPc-containing-Np (Fig. 3), pH-induced fluorescence activation of the amino- containing probes (Fig. 3) and finally SubPc fluorescence imaging of melanoma B16 cells by both confocal and biphotonic microscopies. The nature of the R group on the apical phenoxy part was addressed in order to bring water solubility of the probes and/or to get pH-induced fluorescence

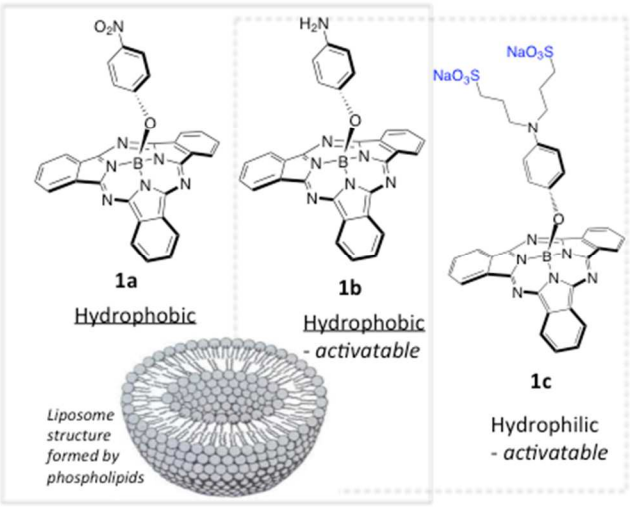

Fig 1: Top (left to right): general structure of hydrophobic/hydrophilic activatable SubPc **1a**, **1b** and **1c**. Bottom left: incorporation of hydrophobic SubPc in liposome structure.

Synthesis. Cyclotrimerization (30 min/150°C) of dry dicyanobenzene in the presence of 1 equiv. of boron trichloride (under inert/anhydrous atmosphere) led to subphthalocyanine **2** bearing an axial chlorine atom (SubPc-Cl), which could be isolated in 9% yield after chromatography (silica/ dichloromethane)(Fig. 2).[8] The former was heated in toluene in the presence of an excess of p-nitrophenol to afford phenoxy-substituted subphthalocyanine **1a** in substantial yield (78%, i.e. 7% overall yield from dicyanobenzene). Alternatively, a *one pot* approach was conducted: upon cyclotrimerization, a subsequent quick removal (distillation) of excess BCl_3 (and p-xylene) was achieved, then the crude mixture was reacted with a given phenol derivative affording SubPc **1a**, **3a**, and **3b**, respectively, in 16-29% overall yield. SubPc **1a** was subsequently reduced under H_2 atm. in the presence of activated Pd

on charcoal (2.5 equiv.), and purified by chromatography (silica, dichloromethane/MeOH) to afford SubPc-NH_2 **1b** in high yield (88%). Attempts to remove the acetyl and trifluoroacetyl protecting groups in species **3a**,**b** (upon treatment with either sodium bicarbonate or ammonia/MeOH solutions) led to substantial decomposition of SubPc, and **1a** was barely obtained. The reaction of **1b** with propane sultone (5 equiv. in DMF/50°C, 72h) led to a statistical mixture that was examined by analytical HPLC (Fig. S1) and appeared to contain the starting SubPc **1b**, the hydrophilic mono- and bis- alkylsulfonate species **1c** (each peak was subjected to MS). Under these conditions, the quaternized tris-alkylated ammonium species was not obtained. The mixture was subsequently subjected to two sets of purifications by chromatography (silica, dichloromethane/methanol gradient), then semi-preparative reverse phase (Dionex, C18, using a CH_3CN/ H_2O gradient, with 0.1% TFA), affording **1c** in modest yield (12%).

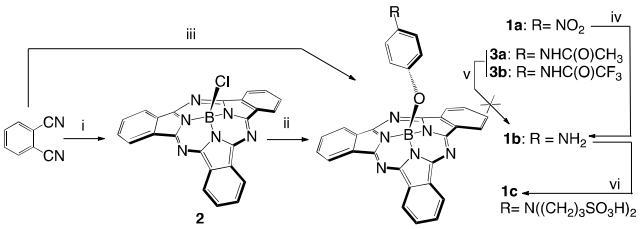

Fig. 2 Syntheses of **1a-c**. (i) BCl_3, p-xylene, Ar atm, reflux, 9 % (ii) excess of p-nitrophenol, toluene, reflux, 78 % for **1a** (iii) (a) BCl_3, p-xylene, Ar atm, reflux, (b) *evaporation* (c) excess of para-substituted phenol, toluene, reflux: 29 % for **1a**, 16 % for **3a** and 19 % for **3b**; (iv) H_2, Pd/C, 88 %; (v) MeOH, NH_3, or $NaHCO_3$; (vi) 1,3-propanesultone, DMF, 50°C, 12 %.

The ^{11}B-NMR spectrum (^{11}B : S = 3/2) of boron-containing subphthalocyanines shows a singlet, which is 30 nm upfield shifted in aryloxy-containing species **1a-c** compared to chlorine-containing species **2** (Fig. S2). The ^{1}H-NMR spectrum of the SubPcs showed the classical multiplet around 8 and 8.8 ppm corresponding to the indole moieties and shifted doublet of the phenoxy picket, and the ^{13}C-NMR spectrum of SubPcs shows aromatic carbons in the 118-158 ppm window (Fig. S3-S6). Mass spectroscopy analysis (MALDI-TOF or ESI-Q) shows in all case the molecular isotopic pic, and sometimes a signal corresponding to the cleavage of B-O bond, ($C_{24}H_{12}BN_6^+$: 395.12)(Fig.S7,S8). X-ray diffraction study showed the classical domed structure in **1b** (Fig. 3, S9, Table S1; CCDC 1014064) i.e. a convex π surface.

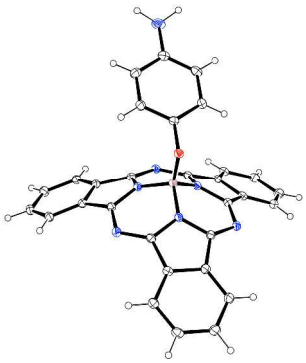

Fig. 3 X-Ray diffraction of **1b** (CCDC 1014064) showing the characteristic domed structure of Subphthalocyanines.

Liposome. Species **1a** is hydrophobic (no solubility in water whatsoever), hence it was entrapped in a lipidic nanoparticle (Np). Liposomes were prepared according to a modified version of the Batzri and Korn's injection method, using DPPC as phospholipid and PBS as a media.[9-13] Subsequent purification on Hitrap was achieved (Rt = 8 min) affording a pure suspension of liposomes, free of unbound SubPc fluorophore. Subsequent characterization of the construct in size and dye content was achieved. The size of the SubPc-containing Np was given by: a) the hydrodynamic diameter determined by Dynamic Light Scattering (DLS): d_H = 21 nm (Fig. 4A); b) which correlates with the mean diameter determined by Transmission Electron Microscopy (TEM)*:* d_{TEM} = 20 nm (Fig. 4B) (using the negative staining approach, which was carried out by mixing a drop of liposome suspension with a solution of ammonium molybdate, and subsequent deposition on a grid and drying (see SI)). Such values concur with that found for Small Unilamellar Vesicles (SUV) prepared by the injection method.[9] Finally, DLS monitored the stability of the liposome suspension over time, and showed that the liposomes were reasonably stable, i.e. the hydrodynamic diameter was found to be 40 nm (size doubled after 24h at RT, under agitation). The optical properties of free/nano-encapsulated SubPc **1a** are reminiscent of that of porphyrin / porphyrinoid species, i.e. a Soret band (303-315 nm) and a Q band (562-566 nm) (Fig. 4C; Tables S2,S3). The fluorescence quantum yield of **1a** was found to be Φ_F = 14 % (λ_{ex}= 488 nm, ref. Rhodamine 6G; λ_{em}= 570-574 nm). A comparison of the absorption and emission spectra in pure THF, water/ THF (97:3 vol.) and in liposome suspension (Fig. 4C, S10, Table S3) gives an evidence for the location of SubPc within the bilayer (as shown for other probes): the UV/Vis spectra of SubPc displays sharp peaks upon SubPc incorporation in liposomes, whereas broadened and shifted features are observed for SubPc in water, which is characteristic of aggregation of hydrophobic species in aqueous environment. The pure suspension of **1a**-containing-liposome (i.e. after purification on Hitrap) was analyzed by UV/Vis, and indicates an average 13% encapsulation in 20-nm liposome vesicle (Fig.S11).

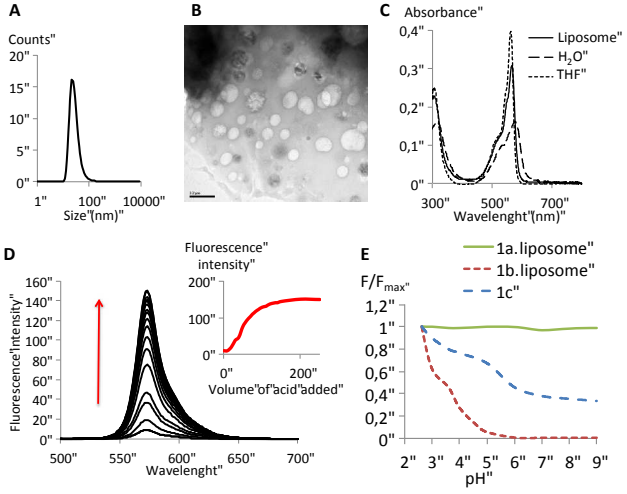

Fig. 4 Characterization and properties of **1a**-containing nano-vesicle: (A) DLS; (B) TEM and (C) absorption in THF, water/ THF (97:3 vol.), and liposome; (D) fluorescence of **1b** in DMF with addition of H_2SO_4 (insert: fluorescence max as a function of volume (µL) of 0.1 % H_2SO_4 solution added) (E) fluorescence of **1a**-**b** in liposome and **1c** solution as a function of pH (2.6 to 9 in chosen buffers, see SI).

Activation. Interestingly, hydrophobic species **1b** turned out to be not fluorescent under the conditions described for **1a**. Further fluorescence studies conducted with **1b** in various media showed a change in the fluorescence quantum yield (Φ_F) from <1% (DMF or $CHCl_3$) to 12-15% (DMF + H_2SO_4 or $CHCl_3$ + TFA) (Fig. 4C, 4E, S11-S13; Tables S2, S3). Hence, the fluorescence of this species may be switched-on upon treatment with an acid. Studies show that the phenomenon is reversible upon addition of a base. A control experiment carried out with **1a** (always ON species), showed no fluorescence change upon addition of acid. Hence, it suggests that protonation of **1b** (leading to **1b**-H^+) occurs at the amine site, and results in turning the fluorescence ON. The fluorescence switch-on occurs upon protonation of the nitrogen at acidic pH through the suppression of internal charge transfer (ICT) and/or photoinduced electron transfer (PET).[15-17] In the non-protonated form the one electron hoping from the electron-rich macrocycle may occur, which results in a formal fluorescence extinction. Although the amine is not conjugated to the cycle, ICT / PET may still occur within 5Å, resulting in fluorescence extinction, encountered in other amine-containing dyes. Moreover, parallel experiments were conducted to examine the possible protonation of the azomethine bridges in SubPc 1a (Fig.S11). Previous studies on phthalocyanines suggest a weak Brönsted basicity of the azomethine bridges, hence the weak acidic conditions employed here may not be sufficient to protonate them. The absorbance bands of SubPc **1a** was monitored by UV/Vis upon gradual addition of quantities of acid (Fig. S11). This suggests that even in pure trifluoroacetic acid, protonation of all aza-bridges may not be complete. The protonation-induced fluorescence has also been examined upon incorporation of **1b** in liposomes (Fig. S14; Table S3). At pH 7 the fluorescence emission from a suspension of nano-encapsulated **1b** in PBS buffer is low, which was expected based on our previous observations on free SubPc **1b** in solution (Fig. 4D,E). Upon lowering the pH, a gradual fluorescence emission was measured. This is because of protons diffusing through the bilayer leading to the protonation of **1b** to form **1b**-H^+. A gradient of pH was used and led to an increase in fluorescence: at 50% Φ_F, the pH was found to be 3.5 (Fig. 4D), which is consistent with average pKa values of primary aniline groups**.**

Water-solubility. Unlike **1a-b**, alkylsulfonato-containing SubPc species **1c** is totally water-soluble. UV/Vis spectrum of **1c** displays sharp absorption bands (Fig. S15) comparable with that of **1ab** in organic solvents (Fig. 4C, S11, S13). Fluorescence studies with **1c** (Fig. S16) were performed in pure water or buffer, the fluorescence quantum yield, Φ_F = 4 % (at pH < 6; phosphate-citrate buffer) was found to be lower than that of **1a** in solution but comparable with that of **1a** in liposome (Tables S1-S2). The same activation processes may be invoked with tertiary amine **1c** (Fig. 4E, S17), although the ICT and/or PET events may be affected going from tertiary aniline (in **1c**) compared to primary aniline (in **1b**). Moreover, even if protonated-induced fluorescence occurs, the fluorescence increase from a deactivated to an activated probe is not as pronounced (by 18-20 for **1b** against 3 for **1c**). The gradient of pH allows the determination of a pKa value of 6.5. This protonation has been examined with respect to the pKa of an anilinium ion, i.e. the conjugate acid of aniline. But an aniline is protonable depending on the nature of the substituents: although the pKa is within 3-4, it jumps as high as 7 with *N*-*t*Bu; 6.5 in the case of *N, N*-diethylaniline.[18] Hence, pKa obtained for **1c** are consistent with these values, considering propylsulfonate groups as ethyl groups. In fact **1c** is always partially turned ON (equilibrium between the amine/sulfonic acid form and the ammonium/ sulfonate zwitterionic form), as a result the activation threshold is not as obvious as for **1b**.

Biology. Nano-encapsulated hydrophobic SubPc **1a** and hydrophilic bis-alkylsulfonato-SubPc **1c** were subsequently incubated (1h) with B16-F10 melanoma cells (in de-supplemented RPMI). Neither compounds **1a** or **1c** were found cytotoxic against B16 cells (upon 1h incubation of solutions up to 10 □M, followed by the MTT test, i.e. addition 0.5 mM MTT, incubation for 2h, followed by removal of medium and solubilisation in DMSO). For imaging studies, the same concentrations/incubation times were used; cells were subsequently rinsed (with PBS) and fixed (with cold (-30°C) methanol left on cells for 5 min at 4 °C). It is expected that liposome incorporation into the cell results in the fusion of membranes, which ought to leave subphthalocyanine 1a in a membrane (bilayer) environment. On the other hand, several mechanisms are classically proposed for the crossing of cell membrane of free molecules (i. e. not incorporated in liposomes, such as water-soluble species 1c), one being a passive diffusion process. Optical imaging studies were subsequently achieved by fluorescence using both biphotonic and confocal microscopies (as optical and non-linear optics of SubPcs have been reported).[7,14] B16 Cells were clearly observed, using a set of filters (DAPI, FITC etc.), and upon comparison with cells which did not incubate a solution of SubPc (Fig. 5A-C, S18, S19, i.e. transmission, superposition and fluorescence images of B16 cells). Both strategies, nano-encapsulated *vs* water-soluble SubPcs turned out to be equally efficient for the imaging of B16 cells by fluorescence microscopy.

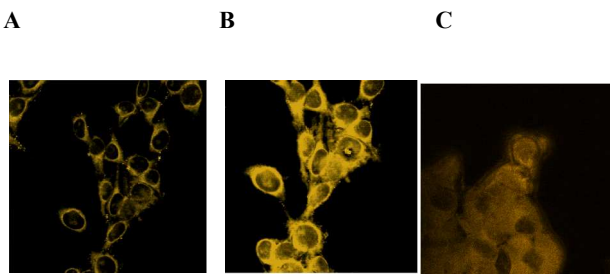

Fig. 5 Fluorescence microscopy images of B16-F10 melanoma cells upon 1h incubation of fluorescent SubPc probes and fixation (methanol): A) biphotonic images of cells incubated with a liposomal suspension of 1a; B) biphotonic images of cells incubated with a solution of 1c in RPMI (exc. 720 nm). C) confocal imaging of cells incubated with 1c (exc. 561 nm).

Conclusions

In summary this is the first, and successful report of the use of subphthalocyanines as fluorophores for the in vitro fluorescence imaging of cells (achieved by confocal and biphoton microscopy). Concentrations needed were relevant for imaging (10 □M), with no cytotoxicity on B16F10 melanoma cells. Two strategies were equally successful for the delivery to cells: a) nano-encapsulation in 20 nm SUV (DPPC) liposomes for hydrophobic species **1a,b**; b) water-solubility through the introduction of alkyl sulfonate chains: bis-sulfonated species **1c** fluorescence is observable in pure water or buffers. Species **1c** is one of the few water-soluble SubPcs reported to date beside the ammonium and carboxylate reported ones.[19-23] Note that the synthesis of SubPc **1a-c** was readily achieved. Species **1b,c** are to be considered as *pro-fluorophores*: the fluorescence could be turned on at pH 3.5 (**1b** - liposome) or even at mildly acidic pH 6.5 (**1c** - in water). However, the activation of amphoteric subphthalocyanine **1c** is not as obvious as **1b** because the

amount of (quaternized) protonated species is already substantial. The pKa values reported in this study are not quite relevant to biological pHs yet, but are somewhat getting close. Normal pH in cells is around 7.33, and cancer cells in hypoxia generate lactic acid lowering the pH as low as 6.5, while in lysosomes, pH could be as low as 3.5.[24] Hence we currently undergo the necessary structural optimizations of SubPcs to fine-tune their pKa, by addressing the meta *vs* para position of the amine, and the length of its alkyl groups. Overall, this set of studies has shown that SubPc are efficient probes for molecular fluorescence imaging, and they may fit in favorably in the Lavis and Raines diagram (i.e. with a brightness Φ_F x $\varepsilon = 8x10^3$ $M^{-1}cm^{-1}$ and a wavelength of maximum absorption λ_{max} = 570 nm, which makes SubPc **1a** in the range of other known fluorophores such as, propidium, SNARF-1, or FMN).[25]

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Notes and references

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† Electronic Supplementary Information (ESI) available: experimental procedures, spectroscopic details, crystallographic data, additional figures and references. See DOI: 10.1039/b000000x/

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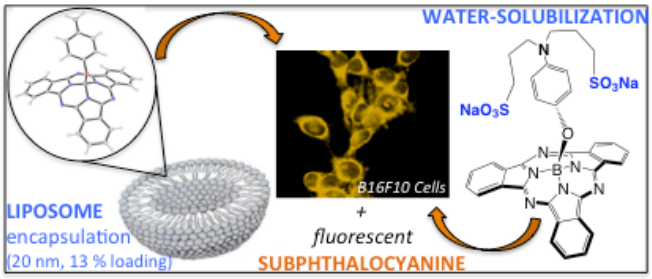
GRAPHICAL ABSTRACT



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