ChemComm

COMMUNICATION

View Article Online
View Journal | View Issue

Cite this: *Chem. Commun.*, 2014, **50**, 13975

Received 16th July 2014,
Accepted 15th September 2014

DOI: 10.1039/c4cc05503a

www.rsc.org/chemcomm

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

Yann Bernhard,[a] Pascale Winckler,[b] Remi Chassagnon,[c] Philippe Richard,[a] Élodie Gigot,[a] Jean-Marie Perrier-Cornet[b] and Richard A. Decréau*[a]

Water-soluble disulfonato-subphthalocyanines (SubPcs) or hydrophobic nano-encapsulated SubPcs are efficient probes for the fluorescence imaging of cells. 20 nm large liposomes (TEM and DLS) incorporated about 13% SubPc. Moreover, some of these fluorophores were found to be 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 associated with known methods (MRI and 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 are activatable, *i.e.* which could be switched ON and OFF reversibly (smart-probes). This study presents a family of fluorescent molecules exhibiting such properties, subphthalocyanines (SubPcs), which have never been examined as optical probes in OI so far. They are 14-π electron aromatic macrocycles having C_3 symmetry that combine three aza-bridged isoindole units around a 4-coordinate boron atom. SubPcs have a convex π surface, which makes them peculiar fluorophores, 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 that helps prevent aggregation, but more importantly provides a convenient handle for SubPc monofunctionalization. There are numerous reports on the fluorescence of SubPcs (emission at around 570 nm, fluorescence quantum yield Φ_F = 0.25 and above depending on the nature of alpha and beta-substituents),[3] but surprisingly none have been examined in the context of OI so far. SubPcs have been widely reported in photovoltaics,[3,4] whereas reports on their use in biomedical research are scarce (PDT on bacteria).[5–7] 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 and 2), and four new facets: water-solubility, entrapment in a 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 increase water solubility of the probes and/or to get pH-induced fluorescence.

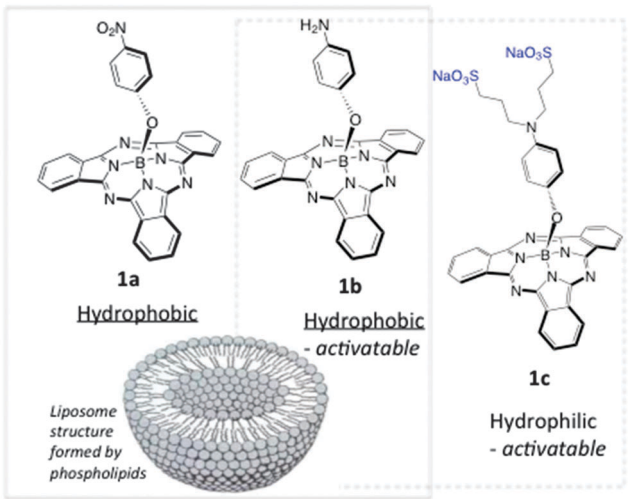

Fig. 1 Top (left to right): general structure of hydrophobic/hydrophilic activatable SubPcs **1a**, **1b** and **1c**. Bottom left: incorporation of hydrophobic SubPc in the liposome structure.

[a] *Institut de Chimie Moléculaire de l'Université de Bourgogne (ICMUB), UMR 6302 CNRS – Université de Bourgogne, BP 47870, F-21078, Dijon Cedex, France. E-mail: Richard.Decreau@u-bourgogne.fr*
[b] *Université de Bourgogne, AgroSup Dijon, Dimacell Imaging Ressource Center, UMR A 02.102 PAM, F-21000 Dijon, France*
[c] *Laboratoire Interdisciplinaire Carnot Bourgogne, UMR CNRS 6303 – Université de Bourgogne, F-21078 Dijon, France*

† Electronic supplementary information (ESI) available: Experimental procedures, spectroscopic details, crystallographic data, additional figures and references. CCDC 1014064. For the ESI and crystallographic data in CIF or other electronic format see DOI: 10.1039/c4cc05503a

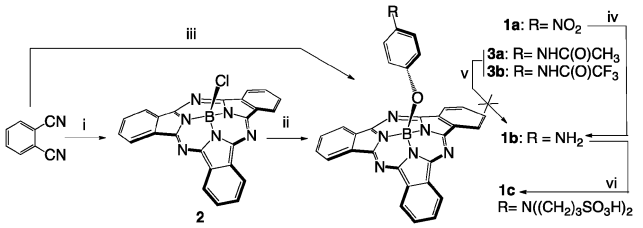

Fig. 2 Syntheses of **1a–c**. (i) BCl_3, *p*-xylene, Ar atm, reflux, 9% (ii) excess of *p*-nitrophenol, toluene, reflux, 78% for **1a** (iii) (a) BCl_3, *p*-xylene, Ar atm, reflux, (b) *evaporation* (c) excess of *para*-substituted phenol, toluene, reflux: 29% for **1a**, 16% for **3a** and 19% for **3b**;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

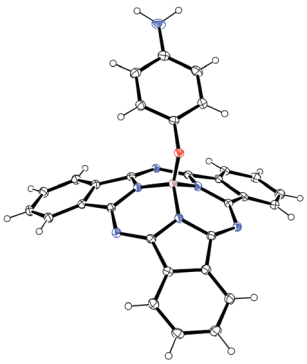

Fig. 3 X-Ray diffraction of **1b** (CCDC 1014064) showing the characteristic domed structure of subphthalocyanines.

Synthesis. Cyclotrimerization (30 min/150 °C) of dry dicyanobenzene in the presence of 1 equiv. of boron trichloride (under an 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 SubPcs **1a**, **3a**, and **3b**, respectively, in 16–29% overall yield. SubPc **1a** was subsequently reduced under a 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** and **3b** (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, 72 h) led to a statistical mixture that was examined by analytical HPLC (Fig. S1, ESI†) and appeared to contain the starting SubPc **1b** and 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), and then by semi-preparative reverse-phase chromatography (Dionex, C18, using a CH_3CN/H_2O gradient, with 0.1% TFA), affording **1c** in modest yield (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, ESI†). The ^{1}H-NMR spectrum of the SubPcs shows the classical multiplet at around 8 and 8.8 ppm corresponding to the indole moieties and the 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, ESI†). Mass spectroscopy analysis (MALDI-TOF or ESI-Q) shows the molecular isotopic in all cases, and sometimes a signal corresponding to the cleavage of the B–O bond, ($C_{24}H_{12}BN_6^+$: 395.12) (Fig. S7 and S8, ESI†). X-ray diffraction studies showed the classical domed structure in **1b** (Fig. 3; Fig. S9 and Table S1, ESI;† CCDC 1014064), *i.e.* a convex π surface.

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 the phospholipid and PBS as the medium.[9–13] Subsequent purification on Hitrap was achieved (R_t = 8 min) affording a pure suspension of liposomes, free of unbound SubPc fluorophores. Subsequent characterization of the construct based on size and the 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

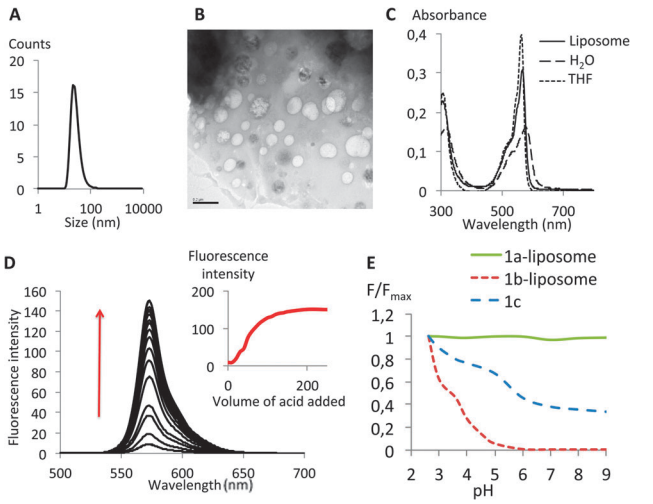

Fig. 4 Characterization and properties of **1a**-containing nano-vesicles: (A) DLS; (B) TEM and (C) absorption in THF, water/THF (97 : 3 vol), and liposome; (D) fluorescence of **1b** in DMF with addition of H_2SO_4 (inset: fluorescence max as a function of volume (μL) of 0.1% H_2SO_4 solution added)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

deposition on a grid and drying (see ESI†)). These values concurred with those found for small unilamellar vesicles (SUVs) prepared by the injection method.[9] Finally, the stability of the liposome suspension over time was monitored by DLS, and results showed that the liposomes were reasonably stable, *i.e.* the hydrodynamic diameter was found to be 40 nm (size doubled after 24 h at RT, under agitation). The optical properties of free/nano-encapsulated SubPc **1a** are reminiscent of those of porphyrin/porphyrinoid species, *i.e.* a Soret band (303–315 nm) and a Q band (562–566 nm) (Fig. 4C; Tables S2 and S3, ESI†). 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; Fig. S10 and Table S3, ESI†) gives an evidence of the location of SubPcs within the bilayer (as shown for other probes): the UV/Vis spectra of SubPcs display 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 an aqueous environment. The pure suspension of **1a**-containing-liposomes (*i.e.* after purification on Hitrap) was analyzed by UV/Vis spectroscopy, which indicates an average 13% encapsulation in 20 nm liposome vesicles (Fig. S11, ESI†).

Activation. Interestingly, hydrophobic species **1b** turned out to be non-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 and E, Fig. S11–S13 and Tables S2 and S3, ESI†). 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 turns 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 hopping 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 5 Å, 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, ESI†). 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** were observed by UV/Vis spectroscopy upon gradual addition of acid in different quantities (Fig. S11, ESI†). 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 and Table S3, ESI†). 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 and 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 which 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 pK_a values of primary aniline groups.

Water-solubility. Unlike **1a** and **1b**, alkylsulfonato-containing SubPc species **1c** is totally water-soluble. The UV/Vis spectrum of **1c** displays sharp absorption bands (Fig. S15, ESI†) comparable with that of **1a** and **1b** in organic solvents (Fig. 4C; Fig. S11 and S13, ESI†). Fluorescence studies with **1c** (Fig. S16, ESI†) 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 and S2, ESI†). The same activation processes may be invoked with tertiary amine **1c** (Fig. 4E and Fig. S17, ESI†), although the ICT and/or PET events may be affected going from tertiary aniline (in **1c**) to primary aniline (in **1b**). Moreover, even if protonation-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 pK_a value of 6.5. This protonation was examined with respect to the pK_a 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 pK_a 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, pK_a obtained for **1c** is 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 (1 h) with B16–F10 melanoma cells (in de-supplemented RPMI). Neither compound **1a** nor **1c** was found to be cytotoxic against B16 cells (upon incubation of solutions up to 10 μM for 1 h, followed by the MTT test, *i.e.* addition of 0.5 mM MTT, followed by incubation for 2 h, subsequent removal of the 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 have been classically proposed for the cell membrane crossing of free molecules (*i.e.*, molecules not incorporated in liposomes, such as water-soluble species **1c**), one being a passive diffusion process. Optical imaging studies were subsequently achieved by fluorescence imaging 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 were not incubated with a solution of SubPc

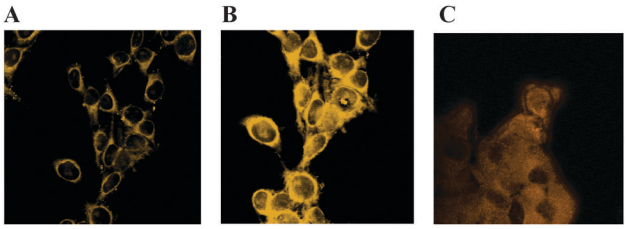

Fig. 5 Fluorescence microscopy images of B16–F10 melanoma cells upon incubation for 1 h with fluorescent SubPc probes and fixation (methanol): (A) biphotonic images of cells incubated with a liposomal suspension of **1a**; (B) biphotonic images of cells incubated with a solution of **1c** in RPMI (exc.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

(Fig. 5A–C; Fig. S18 and S19, ESI,† *i.e.* transmission, superposition and fluorescence images of B16 cells). Both strategies, nano-encapsulation and formation of water-soluble SubPcs turned out to be equally efficient for the imaging of B16 cells by fluorescence microscopy.

In summary this is the first successful report on the use of subphthalocyanines as fluorophores for the *in vitro* fluorescence imaging of cells (achieved by confocal and biphoton microscopy). Concentrations used were relevant for imaging (10 μM), with no cytotoxicity toward 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** and **1b** and (b) increasing water-solubility by the introduction of alkyl sulfonate chains; in the case of bis-sulfonated species **1c** fluorescence was observable in pure water or buffers. Species **1c** is one of the few water-soluble SubPcs reported to date besides the ammonium and carboxylate ones.[19–23] Note that the synthesis of SubPcs **1a–c** was readily achieved. Species **1b** and **1c** can 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** was not as obvious as that of **1b** because the amount of (quaternized) protonated species was already substantial. The pK_a values reported in this study are not quite relevant to biological pH values 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 to as low as 6.5, while in lysosomes, pH could be as low as 3.5.[24] Hence, we currently performed the necessary structural optimizations of SubPcs to fine-tune their pK_a, by addressing the *meta vs. para* position of the amine and the length of their alkyl groups. Overall, this set of studies has shown that SubPcs are efficient probes for molecular fluorescence imaging, and they may fit in favorably in the Lavis and Raines diagram (*i.e.* with a brightness $\Phi_F \times \varepsilon = 8 \times 10^3$ M^{-1} cm^{-1} and the wavelength of maximum absorption λ_{max} = 570 nm, which brings SubPc **1a** in the range of other known fluorophores, such as propidium, SNARF-1, and FMN).[25]

RAD, YB thank the Burgundy Regional Council, CRB (FABER Program), RAD thanks CNRS for Chaire d'Excellence (CdE). FABER and CdE fundings were used to fully set up a cell culture lab at ICMUB. This work was supported by the *3MIM* agreement (CNRS, uB and CRB). Julien Boudon, Nadine Millot and Jérémy Paris (uB, ICB Institute) for use and training on DLS. Lionel Apetoh (INSERM, U866) for a generous gift of B16F10 cells.

Notes and references

1 S. L. Pimlott and A. Sutherland, *Chem. Soc. Rev.*, 2011, **40**, 149–162.
2 E. M. C. Hillman, C. B. Amoozegar, T. Wang, A. F. H. McCaslin, M. B. Bouchard, J. Mansfield and R. M. Levenson, *Philos. Trans. R. Soc., A*, 2011, **369**, 4620–4643.
3 C. G. Claessens, D. Gonzalez-Rodriguez and T. Torres, *Chem. Rev.*, 2002, **102**, 835–853.
4 C. G. Claessens, D. Gonzalez-Rodriguez, M. S. Rodriguez-Morgade, A. Medina and T. Torres, *Chem. Rev.*, 2014, **114**, 2192–2277.
5 H. Xu, X.-J. Jiang, E. Y. M. Chan, W.-P. Fong and D. K. P. Ng, *Org. Biomol. Chem.*, 2007, **5**, 3987–3992.
6 (*a*) M. B. Spesia and E. N. Durantini, *Dyes Pigm.*, 2008, **77**, 229–237; (*b*) M. Ince, O. Seven and B. Dindar, *G.U. J. Sci.*, 2013, **26**, 1–10.
7 X. B. Wang, D. H. Tang and Z. Zhen, *Chin. Chem. Lett.*, 1999, **10**, 683–684.
8 C. G. Claessens, D. Gonzalez-Rodriguez, B. del Rey, T. Torres, G. Mark, H. P. Schuchmann, C. von Sonntag, J. G. MacDonald and R. S. Nohr, *Eur. J. Org. Chem.*, 2003, 2547–2551.
9 R. R. C. New, in *Liposomes, A Practical Approach*, series ed. D. Rickwood and B. D. Hames, IRL Press, Oxford, New York, 1989.
10 S. Batzri and E. D. Korn, *Biochim. Biophys. Acta*, 1980, **594**, 53–84.
11 R. Decréau, M.-J. Richard, P. Verrando, M. Chanon and M. Julliard, *J. Photochem. Photobiol., B*, 1999, **48**, 48–56.
12 J. Barge, R. Decreau, M. Julliard, J. C. Hubaud, A. S. Sabatier, J. J. Grobb and P. Verrando, *Exp. Dermatol.*, 2004, **13**, 33–44.
13 R. Decreau, A. Viola, M. J. Richard, A. Jeunet and M. Julliard, *J. Porphyrins Phthalocyanines*, 1998, **2**, 405–414.
14 Y. Z. Gu, Y. Wang and F. X. Gan, *Mater. Lett.*, 2002, **52**, 404–407.
15 J. Han and K. Burgess, *Chem. Rev.*, 2010, **10**, 2709–2728.
16 S. O. McDonnel, M. J. Hall, L. T. Allen, A. Byrne, W. M. Gallagher and D. F. O'Shea, *J. Am. Chem. Soc.*, 2005, **127**, 16360–16361.
17 J. Murtagh, D. O. Frimannson and D. F. O'Shea, *Org. Lett.*, 2009, **11**, 5386–5389.
18 *CRC Handbook of Chemistry and Physics*, ed. D. R. Lide, 2003.
19 L. Lapok, C. G. Claessens, D. Wohrle and T. Torres, *Tetrahedron Lett.*, 2009, **50**, 2041–2044.
20 H. Xu and D. K. P. Ng, *Chem. – Asian J.*, 2009, **4**, 104–110.
21 F. Dumoulin, M. Durmus, V. Ahsen and T. Nyokong, *Coord. Chem. Rev.*, 2010, **254**, 2792–2847.
22 S. V. Kudrevich, S. Gilbert and J. E. vanLier, *J. Org. Chem.*, 1996, **61**, 5706–5707.
23 S. Kudrevich, N. Brasseur, C. LaMadeleine, S. Gilbert and J. E. van Lier, *J. Med. Chem.*, 1997, **40**, 3897–3904.
24 (*a*) J. R. Griffiths, *Br. J. Cancer*, 1991, **64**, 425–427; (*b*) J. L. Wike-Hooley, J. Haveman and H. S. Reinhold, *Radiother. Oncol.*, 1984, **2**, 343–366.
25 L. D. Lavis and R. T. Raines, *ACS Chem. Biol.*, 2008, **3**, 142–155.

