Organic & Biomolecular Chemistry

PAPER

View Article Online
View Journal

Cite this: DOI: 10.1039/c6ob00530f

Direct subphthalocyanine conjugation to bombesin *vs.* indirect conjugation to its lipidic nanocarrier†

Yann Bernhard,[a] Elodie Gigot,[a] Victor Goncalves,[a] Mathieu Moreau,[a] Nicolas Sok,[b] Philippe Richard[a] and Richard A. Decréau*[a]

Received 9th March 2016,
Accepted 12th April 2016

DOI: 10.1039/c6ob00530f

www.rsc.org/obc

Bombesin (BBN) was covalently bound to graftable subphthalocyanine (SubPc) or to a cholesterol derivative, a component of a liposome that encapsulates non-graftable SubPc. The latter bioconjugation approach was suitable to address the stability of SubPc and was achieved by copper-free click-chemistry on the outer-face of the liposome. Liposomes were purified (FPLC) and then analyzed in size (outer diameter about 60 nm measured by DLS). *In vitro* binding studies allowed to determine the IC_{50} 13.9 nM for one component of the liposome, cholesterol, conjugated to BBN. Hence, azido- (or alkynyl-) liposomes give fluorophores with no reactive functional group available on their backbone a second chance to be (indirectly) bioconjugated (with bombesin).

Introduction

Optical Imaging (OI) of cancer models has a promising future in preclinical imaging, as it has the possibility to image non-invasively.[1] In the search for new fluorophores,[2] subphthalocyanines (SubPc) are interesting probes for OI, especially because of their domed geometry that does not favour aggregation,[3a,b] which is an interesting approach compared to other approaches developed to prevent aggregation.[4a–c] Moreover, these new probes in the field of preclinical imaging have emission wavelengths in the green-orange region of the spectrum (around 570 nm). Preliminary *in vitro* studies have shown the relevance of SubPcs (either in solution or entrapped in liposomes) that fit well in the Lavis & Raines diagram.[5] However, conjugation of probes to biomolecules is a necessary step to ensure site-selectivity. Such an approach had been previously reported for SubPc with testosterone.[6] Herein, we present two strategies to achieve the conjugation to bombesin (7-14) (BBN), a well known peptide, the receptor of which is over-expressed at the surface of tumor cells and that has been conjugated to several probes.[7a–c] One strategy consisted of entrapping SubPc in a liposome and to subsequently graft BBN to the SubPc-containing liposome to afford biovectorized liposome **1ab.** The other strategy consisted of binding BBN and SubPc covalently to afford SubPc–BBN conjugate **2ab.** Herein, these two approaches, *i.e.* direct *vs.* indirect, are developed and compared (Fig. 1).

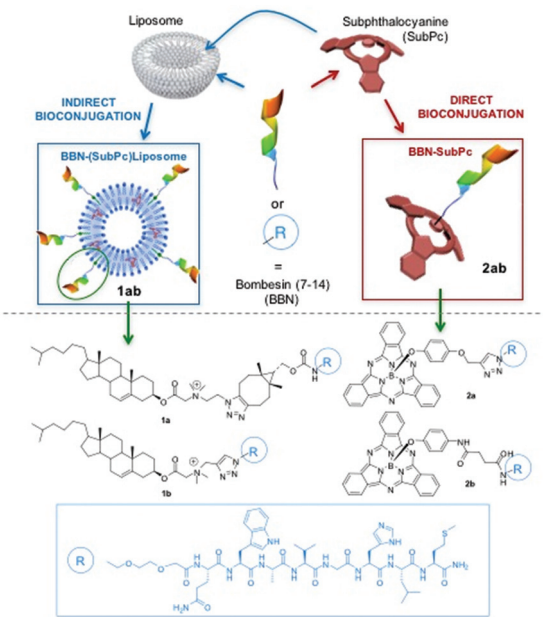


Fig. 1 General bioconjugation strategies: SubPc–BBN (direct bioconjugation) *vs.* liposome–BBN encapsulating non-graftable SubPc (indirect bioconjugation).

[a]*ICMUB Institute-Dept of Chemistry, Sciences Mirande, UMR-CNRS 6302, 9 Avenue Alain Savary, University of Burgundy Franche-Comté, France.*
E-mail: Richar.Decreau@u-bourgogne.fr
[b]*AgroSup Dijon, UMR PAM, 1 Esplanade Erasme, University Bourgogne Franche-Comté, 21078 Dijon, France*

†Electronic supplementary information (ESI) available: Experimental procedures, spectra, and crystallographic data for compound **3**. CCDC 1456530. For ESI and crystallographic data in CIF or other electronic format see DOI: 10.1039/c6ob00530f

Results and discussion

Synthesis of precursors for bioconjugation

Subphthalocyanines. The conjugation of SubPc to BBN was achieved either by peptide coupling or by click chemistry. The latter required the synthesis of alkynyl-containing SubPc **3** (Fig. 3) that was achieved by cyclotrimerization of 1,2-dicyanobenzene to afford chloro-subphthalocyanine **4**.[3a,8] SubPc was later reacted with hydroquinone to afford hydroquinone–SubPc **5**. The former was subsequently reacted with propargyl bromide to afford alkynyl-SubPc **3**. Bioconjugation by peptide coupling required the synthesis of acid-containing SubPc synthon **6** (Fig. 2) that was synthesized from the reaction of SubPcCl **4** with 4-nitrophenol to give **7**. Nitro-SubPc **7** was reduced to afford amino-SubPc **8** that reacted with succinic anhydride to afford acid-SubPc **6** (CCDC 1456530). Several techniques were used to characterize SubPcs: the ^{11}B-NMR spectrum shows a singlet (S = 3/2), and the ^{1}H-NMR spectrum shows multiplets around 8.0 and 8.8 ppm corresponding to the signals of the indole moieties and the two doublets of the phenoxy moieties. Mass spectrometry analyses give for each SubPc the corresponding molecular peak (M + H^{+}). The SubPc characteristic domed structure was observed in the alkynyl-SubPc **3** crystallographic structure (Fig. 3 and S5†). Optical properties of SubPc used for subsequent conjugation were examined by spectrometric and fluorimetric methods. The fluorescence quantum yield was in the range of that previously reported, *i.e.* 10% (Table 1).[3a,b]

Cholesterol. The conjugation of BBN to the liposome was achieved either by peptide coupling or click chemistry. Hence, cholesterol, one component of the liposome was chemically modified to allow such a grafting (Fig. 4). To achieve copper-catalyzed or copper-free click chemistries, a series of amphi-

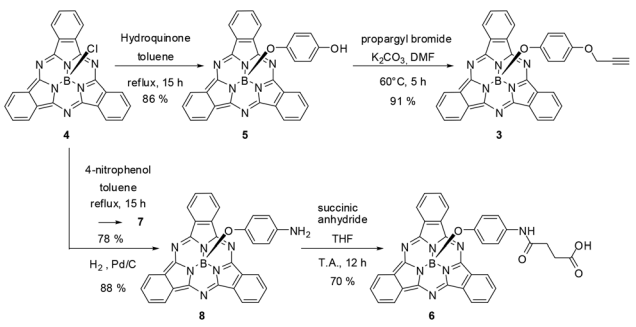


Fig. 2 Syntheses of alkynyl-subphthalocyanine **3** and succinyl-SubPc **6**.

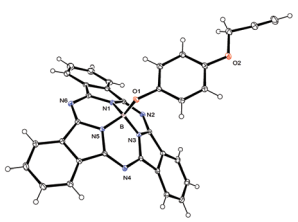


Fig. 3 X-ray diffraction structure of alkynyl-SubPc **3** (CCDC 1456530).

Table 1 Photophysical properties of functional SubPc

Compound		$\lambda_{abs}/\lambda_{em}$ (nm)	ε ($\times 10^3$ L mol^{-1} cm^{-1})	Φ_F [c]	Ref.
4	SubPc-Cl[a]	565/571	101.2	0.25	3*a*
7	SubPc-NO$_2$ [a]	563/572	56.7	0.14	3*a*
6	SubPc-COOH[b]	560/569	52.8	0.07	—
3	SubPc-CCH[a]	564/572	52.3	0.10	—

[a] In chloroform. [b] In methanol. [c] 298 K; reference: Rhodamine 6G in MeOH (Φ_F = 0.96, λ_{ex} = 488 nm); all Φ_F are corrected for changes in the refractive index.

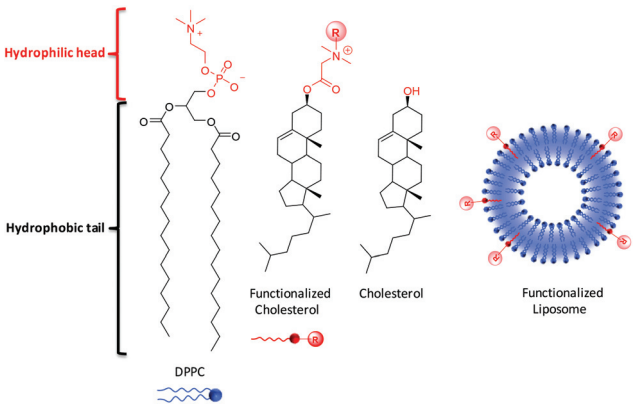


Fig. 4 Rationale for such syntheses: hydrophilic heads and hydrophobic tails found in two components of a liposome.

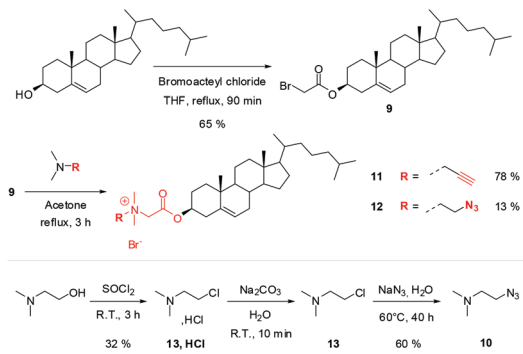


Fig. 5 Syntheses of azide- and alkynyl-cholesteryl derivatives.

philic cholesterol derivatives were synthesized as follows (Fig. 5): the reaction of cholesterol with bromoacetylbromide afforded bromoester **9**, which subsequently reacted with dimethylaminopropyne or 3-azido-dimethylpropylamine **10** to afford cholesteryl-ammonium species **11** (alkynyl) and **12** (azide), respectively. 3-Azido-dimethylpropylamine **10** was synthesized from dimethylaminoethanol that underwent chlorodehydroxylation to afford 2-chloro-dimethylethylamine hydrochloride **13**·HCl. This species was deprotonated to afford free-base species **13**, and was subsequently subjected to SN$_2$ reaction upon treatment with sodium azide, to afford cholesterol-azide **10**. Cholesterol derivatives were characterized by ^{1}H-NMR, ^{13}C-NMR and ESI-MS.

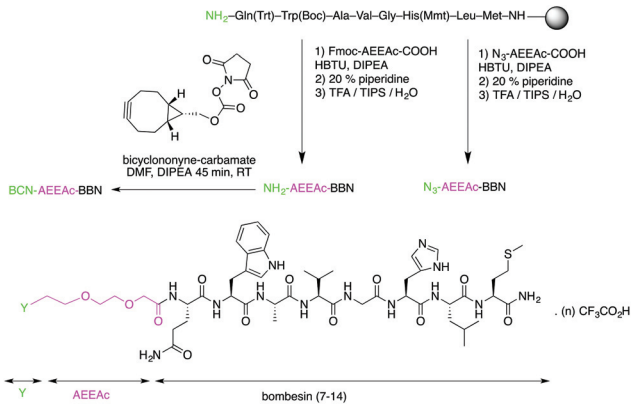


Fig. 6 Syntheses of PEG-terminated bombesin (7-14) terminated with Y = NH_2, N_3, and bi-cyclononyne, respectively.

Bombesin. The syntheses of BBN were carried out on an automated solid phase peptide synthesizer as previously described,[7b] and were modified for BBN-azide and BBN-bicyclononyne as follows (Fig. 6): BBN was elongated on a Rink amide MBHA resin by Fmoc-chemistry and its N-terminus was subsequently deprotected by treatment with 20% piperidine in DMF. Coupling of *N*-(2-(2-(2-azidoethoxy)ethoxy))acetic acid to the peptidyl-resin afforded, after cleavage and deprotection, BBN-azide with an overall 36% yield.[7c] Similarly, the amine derivative BBN-NH_2 was prepared through the acylation of BBN supported on the resin with the PEG-like spacer *N*-Fmoc (2-(2-(2-aminoethoxy)ethoxy))acetic acid, followed by the piperidine-mediated deprotection of the N-terminal Fmoc and the cleavage and deprotection of the peptide from the resin. Finally, the reaction of BBN-NH_2 with bicyclononyne-*N*-succinimide carbonate in DMF afforded BBN-bicyclononyne in 66% yield after HPLC purification.

Direct bioconjugation (probe–BBN conjugate)

SubPc-alkyne **3** was reacted with BBN-bicyclononyne in a homogeneous solution to afford SubPc–BBN bioconjugate **2a** that was purified by HPLC and obtained in 9% yield (Table 2, entry a). SubPc–BBN bioconjugate **2b** was obtained in 7% yield upon amide bond formation between carboxylic acid containing SubPc **6** and resin-bound amino-BBN (entry c). Subsequent removal of protecting groups and cleavage of SubPc–BBN from the resin was achieved to afford **2b**. Both SubPc conjugates were identified by mass spectroscopy (ESi-Q or MALDi-TOF), obtained with a >87% purity (HPLC) and were soluble in water. However, over time it appeared that they slightly degrade in solution (an HPLC-MS analysis showed the appearance of new peaks, such as oxidation products, and UV analysis shows the decay of SubPc absorbance over time), possibly because of an intramolecular reaction between SubPc and a nucleophile in BBN. Such stability issues were observed previously,[3a] hence we decided to follow the protective approach by encapsulating SubPc in liposomes.

Indirect pre/post bioconjugation (liposome–BBN conjugate)

Indirect bioconjugation to SubPc means the conjugation of BBN to a liposome (Fig. 1 and 7). Such a conjugation to a liposome[10] is made possible here by the availability of grafting

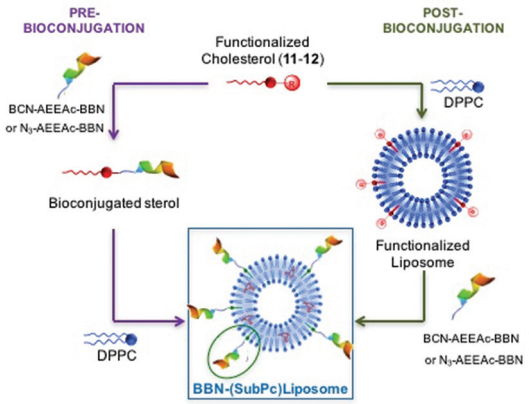
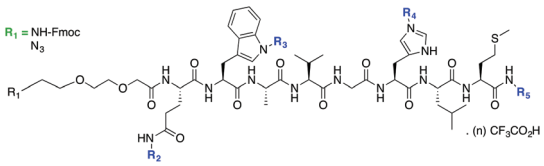


Fig. 7 Pre- and post-bioconjugation approaches for the preparation of functionalized liposomes.

Table 2 Conjugation of SubPc and cholesteryl derivatives to BBN



SubPc + BBN → SubPc–BBN (**2ab**)
Chol + BBN → Chol–BBN (**1ab**)

Entry	Bombesin	SubPc/Chol	Reaction conditions	Bioconjugate[a]	Yield
a	N_3-AEEAc-BBN	SubPc-alkyne **3**	$CuSO_4$, AscNa, DMF, RT, 90 min (R_2 = R_3 = R_4 = R_5 = H)	**2a**	9%
b	N_3-AEEAc-BBN	Cholesterol-alkyne **11**	$CuSO_4$, AscNa, DMF, RT, 4 h (R_2 = R_3 = R_4 = R_5 = H)	**1a**	31%
c	H_2N-AEEAc-BBN	SubPc-acid **6**	(1) HATU, DMF, DIPEA, RT, 90 min (2) TFA, TIPS, H_2O 95/2.5/2.5, RT, 90 min R_2 = Trt; R_3 = Boc; R_4 = Mmt; R_5 = resin	**2b**	7%
d	BCN-AEEAc-BBN	Cholesterol-azide **12**	DMF, RT, 4 h (R_2 = R_3 = R_4 = R_5 = H)	**1b**	41%

[a] See structures of conjugates **1ab** and **2ab** in Fig. 1.

functions on its outer face. At the molecular level, conjugation to a liposome means conjugation to its (alkyne- or azide-containing) cholesterol derivative component. Such a conjugation could be achieved before assembling the liposome, *i.e.* on an isolated cholesterol: this is called the indirect pre-bioconjugation. Alternatively, the conjugation of BBN could be achieved on an already formed liposome that bears hanging grafting functions: this is called the indirect post-bioconjugation. Whatever the strategy used, careful attention was given to the preparation of the liposome platform.

Indirect post-bioconjugation strategy. Clickable azido- (or alkynyl-) liposomes were prepared by the injection method,[3a,9] upon injecting organic solutions of phospholipid (DPPC), clickable cholesterol **11**/**12** (1 : 20 ratio), and the optical probe (SubPc **7**) into vigorously stirred water or PBS (Fig. 8A). The resulting liposome suspension was subsequently purified by FPLC (Fast Protein Liquid Column Chromatography, *i.e.* steric exclusion chromatography) on a HiTrap desalting column (GE-Healthcare): the fraction containing the liposome suspension was isolated at t = 1 min/4 mL (Fig. 8C) (whereas unbound SubPc and cytotoxic traces of solvents (THF, chloroform) came later). The size of the vesicles was determined by DLS (d_{DLS} = 57 nm), TEM (d_{TEM} = 60 nm). Stability of the vesicles over time was monitored by DLS: it appeared to be stable after 48 h, especially for alkynyl-liposomes (Fig. 8E). The purified suspension of vesicles (with no un-encapsulated SubPc left) was examined by UV/Vis: it showed the characteristic

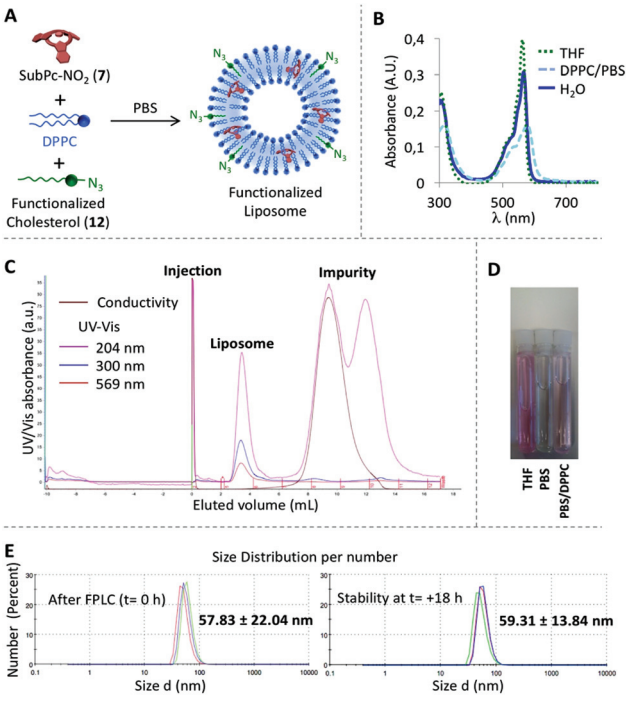


Fig. 8 (A) Schematic representation of the preparation of functionalized clickable N_3-liposomes. (B) UV/Vis spectra of SubPc **7** in THF, water or liposome. (C) FPLC profile for purification of liposome. (D) Color of solutions in THF, water or liposome. (E) DLS curves after FPLC (t_0) and stability at t = 18 h.

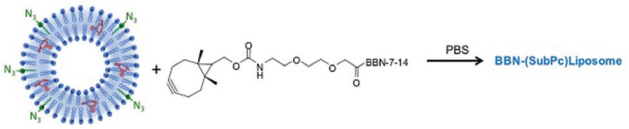


Fig. 9 Representation of a post-bioconjugation step on a N_3-liposome (containing SubPc **7**) by copper-free click chemistry with BBN–bicyclononyne.

bands of SubPc, with more resolution than in a solvent–water mixture as an indication of entrapment (Fig. 8B–D). When the preparation of liposomes was carried out upon injecting the organic solution in water, liposomes were not stable. This is unlike in buffer, where liposomes were stable.

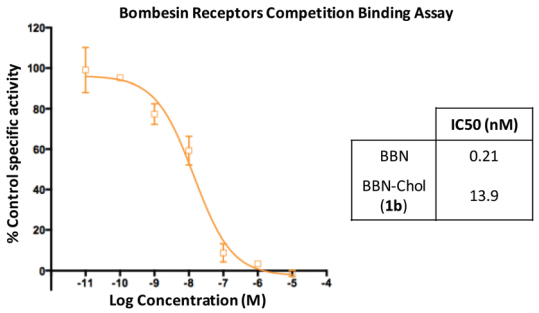
Subsequent conjugation of the functionalized liposome was carried out on a pure sample of azide-liposome (containing the azide steroid **12**). The liposome was subjected to copper-free click chemistry in the presence of BBN-cyclooctyne (Fig. 9). The progress of the reaction was monitored by MALDI-TOF analysis, from a lyophilized aliquot of the liposome suspension, until no trace of free BBN was found (t = 24 h).

The indirect pre-bioconjugation strategy (Fig. 7 and Table 1) consisted of replacing cholesterol–BBN conjugate **1a** or **1b** instead of cholesterol-azide **12** (or cholesterol-alkyne **11**) in the PBS mixture to afford the BBN-conjugated liposome.

Hence, the pre-bioconjugation strategy started from the grafting of the biomolecule to one liposome's component (*i.e.* cholesterol additive), prior to the preparation of the liposome to afford BBN–cholesterol conjugate **1ab**. The conjugation of BBN-azide with cholesteryl-alkyne **11** (1 equiv.) (*i.e.* pre-functionalization by copper-catalyzed click chemistry) was carried out in the presence of copper sulphate (1.1 equiv.) and sodium ascorbate as the reducing agents (3 equiv.) (Table 2, entry b). Alternatively BBN-bicyclononyne and cholesteryl-azide **11** were reacted without the Cu catalyst for 4 h to afford **1b** (entry d). BBN–cholesterol conjugate **1ab** was purified by semi-preparative HPLC (purity > 93%). Upon liposome formation starting from **1ab** a follow-up by DLS showed that various sizes were obtained. Also, it appeared that the targeted functionalized liposome degraded on the Hi-Trap column as a result of elution using water instead of a buffer (another buffer than PBS). When the liposome was prepared from a sterol–BBN bioconjugate subsequent DLS analyses showed the formation of large vesicles (500–1500 nm), which does not correspond to a homogeneous system as found in a post-bioconjugation approach. Hence the later appeared to be the best approach.

Validation of conjugates: affinity studies

The affinity of the cholesterol–BBN conjugate **1b** for the BBN receptors was measured on a competitive binding assay on rat cerebral cortex membranes.[11] This bioconjugate displaced the [^{125}I]-[Tyr^4]bombesin (1-14) radioligand (the K_D of which is 0.71 nM) with an IC_{50} of displacement of 13.9 nM (Fig. 10). Compared to bombesin (1-14), the reference compound, which



	IC50 (nM)
BBN	0.21
BBN-Chol (**1b**)	13.9

Fig. 10 BBN–liposome conjugates: IC_{50} binding studies for cholesterol–liposome conjugate **1b**.

has an IC_{50} of displacement of 0.28 nM, the IC_{50} found for **1b** corresponds to a 66-fold loss of inhibitory activity. Despite such a loss, the affinity may remain sufficient to consider future *in vitro* studies. Also, such a value still compares well with values reported for other studies.[12] Future *in vitro* studies will focus on the evaluation of BBN conjugates on cell over-expressing BBN receptors and subsequent imaging studies.

Conclusions

Two general approaches were chosen for the conjugation to BBN. The covalent attachment of BBN to SubPc (direct bioconjugation) through robust amide or triazole bonds was achieved in modest yields.[7] Moreover, the resulting conjugate was intrinsically unstable because of the slow reactivity of SubPc against nucleophiles and the moderate solubility in water. Hence, the alternative encapsulation approach in a liposome appeared appropriate to offer the necessary protection of SubPc against these species. Subsequent grafting of the biomolecule to the liposome encapsulating non-graftable SubPc was then considered (indirect bioconjugation). As a result one component of the liposome was chemically modified to append a function on the outer face of the nanoparticle that made click-chemistry with a BBN derivative possible. Copper-free click chemistry was preferred to copper-catalyzed click chemistry, because it is faster and the toxicity of copper is prevented. Liposomes were purified at various stages and a monitoring of the stability was achieved. The affinity of the BBN-cholesteryl derivative for bombesin receptors was still acceptable. Future studies will focus on *in vitro* cell imaging and *in vivo* tumor imaging, not before having tested (DLS, FPLC, drug release) the stability and integrity of a suspension of such liposome constructs against potentially not liposome-friendly conditions, albeit physiologically relevant to cancer (salinity, presence of various macromolecules, pH, *etc.*).

Overall, this study reinforced that liposomes offer an alternative way for fluorophores, drugs and contrast agents that are not water-soluble to be handled in aqueous physiological media, as we have shown before.[3a,9a,c] Encapsulation is also a strategy to protect sensitive molecules, such as SubPc, against exogenous reactive molecules or even water.[3a] The actual study specifically showed that azido- or alkynyl-liposomes are a convenient entry point for a bioconjugation/biovectorization approach (on the outer face of the liposome), which basically offers a second chance for fluorophores (and by extension drugs, contrast agents) with no reactive functional group available on their backbone a way of imitating (indirect) bioconjugation with a biomolecule (*i.e.* an indirect approach offered to achieve future site-specific targeting of tumors).

Experimental section

Materials

Reagents and solvents were from various suppliers. They were used as received without any further purification when not otherwise stated. Subphthalocyanines **4**, **7** and **8** were synthesized as we previously described.[3a]

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). ^{1}H NMR spectra (300 MHz) and ^{13}C NMR spectra (75 MHz) were recorded on Bruker 300 Avance III and II spectrometers, respectively. Chemical shifts are quoted in parts per million (δ) relative to tetramethylsilane, TMS (^{1}H and ^{13}C), using the residual protonated solvent (^{1}H) 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 dimethylsulfoxide 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, and m = multiplet. The identity of the compounds was established using high-resolution mass spectrometry and multinuclear NMR spectroscopy. The standard mass was obtained on an 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. DLS measurements were performed on a Shimadzu UV-2550 spectrophotometer, in a solvent of choice (DCM, THF, MeOH, DMSO, water) in glass cuvettes 1 × 1 × 3 cm (1 cm path).

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 comprising between 0.03 and 0.07. Fluorescence quantum yields (Φ_F) were determined by a comparison method, using the following equation:

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

with Std corresponds to standard (Rhodamine 6G), Φ_F and Φ_F(Std): fluorescence quantum yields, η and η(Std): refractive indices of solvents (MeOH for standard; DCM, DMF or water

for samples), Abs and Abs(Std): absorbances at the excitation wavelength (488 nm), *A* and *A*(Std): areas under the fluorescence curves.

HPLC. Compounds were analyzed on a Dionex Ultimate 3000 HPLC system. The methods used were the following: **method A:** column: Chromolith High Resolution RP-18 (5 × 4.6 mm); eluent A: CH_3CN + 0.1% TFA; eluent B: H_2O + 0.1% TFA. Flow: 3 mL min^{-1}. Equilibrate for 1.75 min; afterwards, gradient from 100% B to 100% A; duration: 5 min; keep constant for 1 min; return in 1.5 min to initial conditions. UV detectors: 214 nm, 230 nm, 254 nm, 565 nm. **Method B:** column: Kinetex 26 μm C18 100A (50 × 2.1 mm); eluent A: CH_3CN + 0.1% TFA; eluent B: H_2O + 0.1% TFA. Flow: 0.5 mL min^{-1}. Equilibrate for 1.75 min afterwards; ramp from 95% B to 5% A; duration: 5 min; keep constant for 1.5 min; return in 1.5 min to initial conditions. UV detectors: 214 nm, 230 nm, 254 nm.

X-ray diffraction (XRD) data were collected at room temperature for 24 hours using a Siemens D5000 automatic powder diffractometer, operating at 35 mA and 50 kV. The lattice parameters and the crystallite size ($\varnothing_{XRD}$) calculations were performed using the Topas software from Bruker.

Liposomes

Preparation. Liposomes were prepared by the injection method according to our reported procedure[3a] and adapted as follows. 100 μL of each of the following solutions were taken: (a) a solution of DPPC in ethanol (21 mM), (b) a solution of cholesterol or cholesteryl-ammonium in ethanol (3 mM) and (c) a solution of nitrosubphthalocyanine **7** in chloroform (0.5 mM). Overall 300 μL of solutions was quickly injected using a Hamilton syringe in 10 mL of buffer solution (PBS or NaCl) under vigorous agitation at 60 °C. The mixture was left under agitation for 2 min at the same temperature, and then cooled down to room temperature. Subsequently this suspension may be filtered through 0.2 μm filters.

Purification. The purification of the liposomes was achieved on an Äkta pure (GE). The liposome suspension (500 μL) was injected on a 1 mL loop, and elution was performed on a Hi-trap column (GE) using water as the eluent with a flow rate set at 3 mL min^{-1}. The detection wavelengths were set at 204 nm, 300 nm, and 580 nm. Upon collection of fractions, 4 mL of the suspension were collected, and subsequently lyophilized.

Characterization. DLS (Dynamic Light Scattering) hydrodynamic diameter measurements were performed on a Zeta-Nanosizer (Malvern) into 10^{-2} M NaCl solutions.

Competitive Binding Assays were carried out by Cerep.

Conjugation of liposome with BBN

To the previously prepared liposomal solution was added *N*-(2-(2-(2-(*N*-4-(((bicyclo[6.1.0]non-4-yn-9-ylmethoxy)carbonyl)amino)ethoxy)ethoxy))acetyl-bombesin (7-14)) (1.42 mg, 0.45 μmol, 3 eq.) and the solution was stirred at room temperature for 18 h. The mixture was lyophilized and subjected to MALDI-TOF MS analysis to confirm the success of the reaction. **MS MALDI-TOF:** m/z = 1801.84 $[M - Br]^+$ (calcd for $C_{93}H_{145}N_{18}O_{16}S^+$: 1802.08).

Synthesis

Cholesterol–BBN-7-14 (1a) (copper catalyzed click chemistry coupling method). A mixture of *N*-(2-(2-(2-(*N*-azidoethoxy)ethoxy))acetyl-bombesin (7-14)) (5.76 mg, 4.7 μmol), alkyne containing cholesterol derivative **11** (2.78 mg, 4.7 μmol), copper sulfate (0.83 mg, 5.2 μmol) and sodium ascorbate (2.79 mg, 14.1 μmol) in 350 μL of DMF was stirred at room temperature for 8 h. The peptide was purified by semi-preparative RP-HPLC (C18, eluent: CH_3CN + 0.1% TFA/H_2O + 0.1% TFA, gradient from 70 : 30 to 100 : 0 v/v in 40 min, Rt = 17.18 min), and pure fractions were collected and lyophilized to give the desired product (2.5 mg, 31%). MS ESI: m/z = 1620.14 $[M - Br]^+$ (calcd for $C_{83}H_{130}N_{17}O_{14}S^+$: 1620.97). HPLC (method B): Rt (min) = 5.73 (90.8% at 254 nm).

Cholesterol–BBN-7-14 (1b) (copper free click chemistry coupling method). *N*-(2-(2-(2-(*N*-4-(((Bicyclo[6.1.0]non-4-yn-9-ylmethoxy)carbonyl)amino)ethoxy)ethoxy))acetyl-bombesin (7-14)) (5.20 mg, 4.11 μmol) and cholesterol derivative **12** (2.04 mg, 3.28 μmol) were placed in 750 μL of DMF. The mixture was stirred at room temperature for 15 h. The peptide was purified by semi-preparative RP-HPLC (C18, eluent: CH_3CN + 0.1% TFA/H_2O + 0.1% TFA, gradient from 70 : 30 to 100 : 0 v/v in 40 min, Rt = 17.25 min), and pure fractions were collected and lyophilized to afford the desired product (2.3 mg, 32%). MS MALDI-TOF: m/z = 1802.26 $[M - Br]^+$ (calcd for $C_{93}H_{145}N_{18}O_{16}S^+$: 1802.08). HPLC (method B): Rt (min) = 5.81 (92.80% at 254 nm).

SubPc–BBN-7-14 (2a) (click chemistry coupling method). *N*-(2-(2-(2-(*N*-Azidoethoxy)ethoxy))acetyl-bombesin (7-14)) (15 mg, 12 μmol), SubPc-CCH **3** (6.73 mg, 12 μmol), copper sulfate (3.41 mg, 14 μmol) and sodium ascorbate (7.38, 37 μmol) were mixed together in 100 μL of DMF. The reaction mixture was stirred at room temperature for 30 min, then the solution was directly purified by semi-preparative RP-HPLC (C18, eluent: CH_3CN + 0.1% TFA/H_2O + 0.1% TFA, gradient from 35 : 65 to 65 : 35 v/v in 40 min, Rt = 27.1 min), and pure fractions were collected and lyophilized to give SubPc–BBN-7-14 conjugate **2a** as a pink solid (1.45 mg, 7%). MS MALDI-TOF: m/z = 1675.77 $[M + Na]^+$ (calcd for $C_{82}H_{93}BN_{22}O_{14}SNa^+$: 1676.62). HPLC (method A): Rt (min) = 3.07 (87.90% at 254 nm; 89.08% at 565 nm).

SubPc–BBN-7-14 (2b) (peptide coupling method). Rink Amide MBHA resin grafted (*N*-(2-(2-(2-(*N*-Fmoc)aminoethoxy)ethoxy))acetyl-(His(*N*-Mtt)-Trp(*N*-Boc)-Gln(*N*-Trt))bombesin (7-14)) (66.4 mg, 0.25 mmol g^{-1}, 16.6 μmol) was dissolved in 1 mL of DMF and stirred at room temperature for 30 min, then filtered off and washed with DMF (2 mL). The solid was re-suspended in 20% piperidine in DMF (1 mL) for 10 min (twice) to deprotect the amino group, and then washed with DMF (2 × 2 mL), DCM (2 × 2 mL), MeOH (2 mL), DCM (1 mL) and DMF (1 mL). Then SubPc-COOH **6** (15 mg, 12.5 μmol), HATU (9.47 mg, 24.9 μmol) and DIPEA (4.33 μL, 24.9 μmol) were added and the solution was made up with DMF to obtain

a total volume of 1 mL. The reaction mixture was stirred at room temperature for 90 min, and then the solid was washed with DMF (2 × 2 mL), DCM (2 × 2 mL), MeOH (2 mL), DCM (1 mL) and DMF (1 mL). The peptide was cleaved from the resin with simultaneous removal of the side-chain protecting groups by treatment with 1 mL of a TFA/TIPS/water mixture (95/2.5/2.5 v/v/v) for 90 min at room temperature. The filtrate from the cleavage mixture was concentrated, precipitated in cold Et_2O and collected by centrifugation (twice), and lyophilized to afford the crude peptide. The peptide was purified by semi-preparative RP-HPLC (C18, eluent: CH_3CN–0.1% TFA/H_2O–0.1% TFA, gradient from 30 : 70 to 60 : 40 v/v in 30 min, Rt = 20.7 min), and pure fractions were collected and lyophilized to give SubPc–BBN-7-14 conjugate **2b** as a pink solid (2.5 mg, 9%). MS ESI: m/z = 1671.62 $[M + H]^+$ (calcd for $C_{84}H_{97}BN_{21}O_{15}S^+$: 1670.73), 1692.67 $[M + Na]^+$ (calcd for $C_{84}H_{96}BN_{21}O_{15}SNa^+$: 1692.71). HPLC (method A): Rt (min) = 2.95 (88.2% at 254 nm; 92.3% at 565 nm).

***B*-(4-(3-Carboxypropanamido)phenoxy)[subphthalocyaninato] boron(III) (6).** To a solution of SubPc **8** (100 mg, 0.2 mmol) in THF (5 mL) was added succinic anhydride (30 mg, 0.3 mmol). The reaction mixture was stirred at room temperature for 12 h, and then evaporated to dryness. The resulting residue was resuspended in 5 mL of methanol and precipitated off by addition of water (20 mL), filtered off and washed with a 1 : 3 MeOH/H_2O mixture (3 × 20 mL), then subjected to silica gel column chromatography (eluent: DCM/MeOH 95 : 5) and finally dried under reduced pressure to afford compound **14** (84 mg, 70%). ^{1}H NMR (300 MHz, DMSO-d_6, 300 K): δ (ppm) = 2.40 (m, 4H); 5.24 (d, ^3J = 8.8 Hz, 2H); 6.93 (d, ^3J = 8.8 Hz, 2H); 8.00 (m, 6H); 8.84 (m, 6H); 9.59 (s, 1H); 11.55 (s broad, 1H). HR-MS ESI: m/z = 604.19160 $[M + H]^+$ (calcd for $C_{34}H_{23}BN_7O_4^+$: 604.19050); 602.17502 $[M - H]^-$ (calcd for $C_{34}H_{21}BN_7O_4^-$: 602.17485). HPLC (method A): Rt (min) = 2.94 (94.0% at 254 nm; 98.5% at 565 nm). UV-Vis (MeOH): λ_{max} (nm) ($\varepsilon \times 10^3$ L mol^{-1} cm^{-1}) = 302 (26.5), 560 (52.8).

***B*-(4-Hydroxyphenoxy)[subphthalocyaninato]boron(III) (5).** A mixture of *B*-chloro[subphthalocyaninato]boron(III) **3** (50 mg, 0.12 mmol) and hydroquinone (256 mg, 1.2 mmol) in toluene (5 mL) was heated under reflux for 15 h. Upon evaporation of the solvent, the residue was subjected to alumina gel column chromatography (eluent: DCM/MeOH 99 : 1) and then to silica gel column chromatography (eluent: DCM/MeOH 98 : 2) and dried under reduced pressure (100 mg, 86%). ^{1}H NMR (300 MHz, $CDCl_3$, 300 K): δ (ppm) = 5.32 (d, ^3J = 8.8 Hz, 2H); 6.28 (d, ^3J = 8.8 Hz, 2H); 7.92 (m, 6H); 8.86 (m, 6H). HR-MS ESI: m/z = 503.1446 $[M - H]^-$ (calcd for $C_{30}H_{16}BN_6O_2^-$: 503.1428). UV-Vis (DCM): λ_{max} (nm) ($\varepsilon \times 10^3$ L mol^{-1} cm^{-1}) = 304 (29.5), 563 (61.2).

***B*-(4-Propargyloxyphenoxy)[subphthalocyaninato]boron(III) (3).** To a solution of compound **5** (50 mg, 0.1 mmol) and potassium carbonate (25.5 mg, 0.2 mmol) in 5 mL of DMF was added propargyl bromide (10.7 μL, 0.1 mmol). The mixture was left to stand at 60 °C for 5 h, then filtered off on Clarcel® and evaporated to dryness under reduced pressure. The product was subjected to silica gel column chromatography (eluent: DCM/MeOH 98 : 2 vol) and dried under reduced pressure (49 mg, 91%). ^{1}H NMR (300 MHz, $CDCl_3$, 300 K): δ (ppm) = 2.39 (t, ^4J = 2.4 Hz, 1H); 4.42 (d, ^4J = 2.4 Hz, 2H); 5.34 (d, ^3J = 9.0 Hz, 2H); 6.37 (d, ^3J = 9.0 Hz, 2H); 7.90 (m, 6H); 8.84 (m, 6H). MS MALDI-TOF: m/z = 543.02 $[M + H]^+$ (calcd for $C_{33}H_{20}BN_6O_2$: 543.17). HR-MS ESI: m/z = 543.1752 $[M + H]^+$ (calcd for $C_{33}H_{20}BN_6O_2^+$: 543.1741). UV-Vis (DCM): λ_{max} (nm) ($\varepsilon \times 10^3$ L mol^{-1} cm^{-1}) = 302 (28.2), 564 (52.3).

Cholesteryl bromoacetate (9). To a solution of cholesterol (1 g, 2.56 mmol) in distilled THF (15 mL) was slowly added bromoacetyl chloride (654 μL, 7.77 mmol) under a nitrogen atmosphere. The reaction mixture was heated to reflux for 1.5 h, and then concentrated under vacuum. The oil obtained was dissolved in refluxing ethyl acetate, and then left at room temperature for several hours. The crystals formed were filtered off, washed with ethyl acetate and dried under reduced pressure to yield cholesteryl bromoacetate **9** as a white crystalline powder (855 mg, 65%). ^{1}H NMR (300 MHz, $CDCl_3$, 300 K): δ (ppm) = 0.68 (s, 3H); 0.86–1.70 (m, 34H); 1.75–2.07 (m, 4H); 2.36 (d, ^3J = 7.8 Hz, 2H); 3.80 (s, 2H); 4.67 (m, 1H); 5.39 (d, ^3J = 4.9 Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$, 300 K): δ (ppm) = 12.0; 18.9; 19.4; 21.2; 22.7; 22.9; 23.0; 24.4; 26.5; 27.7; 28.2; 28.4; 32.0; 32.1; 36.0; 36.3; 36.7; 37.0; 37.9; 39.7; 39.9; 42.5; 50.2; 56.3; 56.8; 76.3; 123.2; 139.3; 166.8. MS MALDI-TOF: m/z = 506.71 $[M + H]^+$ (calcd for $C_{29}H_{48}BrO_2^+$: 507.28), 530.27 $[M + Na]^+$ (calcd for $C_{29}H_{47}BrNaO_2^+$: 529.27).

Cholesteryl(prop-2-ynyl-*N*,*N*-dimethylammonium bromide) acetate (11). Cholesteryl bromoacetate **9** (250 mg, 0.493 mmol) and dimethylpropargylamine (57 μL, 0.542 mmol) in 10 mL of acetone were stirred under reflux for three hours. The white precipitate observed was filtered off, washed one time with acetone, and dried under reduced pressure to afford the ammonium derivative **11** (227 mg, 78%). ^{1}H NMR (300 MHz, $CDCl_3$, 300 K): δ (ppm) = 0.68 (s, 3H); 0.86–1.70 (m, 34H); 1.75–2.07 (m, 4H); 2.36 (m, 2H); 2.87 (t, ^4J = 2.5 Hz, 1H); 3.72 (s, 6H); 4.71 (m, 1H); 4.98 (s, 2H); 5.08 (s, 2H); 5.40 (d, ^3J = 4.9 Hz, 1H). MS ESI: m/z = 510.37 $[M - Br^-]^+$ (calcd for $C_{34}H_{56}NO_2^+$: 510.43).

Cholesteryl(2-azidoethyl-*N*,*N*-dimethylammonium bromide) acetate (12). Cholesteryl bromoacetate **9** (173 mg, 0.34 mmol) and 2-azido-*N*,*N*-dimethylethanamine **10** (74 mg, 0.65 mmol) were dissolved in 10 mL of acetone. The mixture was stirred under reflux for three hours. The white precipitate formed was filtered off, washed with acetone (20 mL), and dried under reduced pressure to afford the ammonium derivative **12** (64.3 mg, 78%). ^{1}H NMR (300 MHz, MeOD, 300 K): δ (ppm) = 0.61 (s, 3H); 0.87–2.11 (m, 40H); 2.41 (m, 2H); 3.35 (s, 6H); 3.83 (m, 2H); 3.97 (m, 2H); 4.73 (m, 1H); 5.40 (d, ^3J = 4.9 Hz, 1H). MS MALDI-TOF: m/z = 540.56 $[M - Br^-]^+$ (calcd for $C_{33}H_{57}N_4O_2^+$: 541.45).

2-Chloro-*N*,*N*-dimethylethanamine hydrochloride (13·HCl). In a round bottom flask was introduced dimethylaminoethanol (20 mL, 199 mmol), and then thionyl chloride (14.5 mL, 200 mmol) was slowly added under stirring and at

low temperature (ice bath). After completion of the addition, the viscous reaction mixture was left at room temperature for 3 h, and then 100 mL of ethanol were added. The precipitate formed was filtered off, and the filtrate was placed in an ice bath for 1 h. The crystals of chlorohydrate **13** formed were filtered off and dried under reduced pressure. The filtrate was concentrated under vacuum, and cooled in an ice bath to afford more product (9.3 g, 32%).

2-Chloro-*N*,*N*-dimethylethanamine (13). To a solution of sodium carbonate (3 g, 28.3 mmol) in water (10 mL) was added 2-chloro-*N*,*N*-dimethylethanamine hydrochloride (2 g, 13.9), and the mixture was stirred for 10 min. The product was extracted three times with diethyl ether (3 × 10 mL) and the organic layer was dried with magnesium sulfate. After evaporation, the product was obtained as a colorless oil. ^{1}H-NMR (300 MHz, $CDCl_3$, 300 K): δ (ppm) = 2.33 (s, 6H); 2.69 (t, ^{3}J = 6.7 Hz, 2H); 3.57 (t, ^{3}J = 6.7 Hz, 2H).

2-Azido-*N*,*N*-dimethylethanamine (10). A solution of 2-chloro-*N*,*N*-dimethylethanamine **13** (500 mg, 4.65 mmol) and sodium azide (670 mg, 10.31 mmol) in water (12.5 mL) was stirred at 60 °C for 40 h. After cooling the reaction mixture to room temperature, 0.5 g of potassium hydroxide was added (ice bath), and the product was extracted with diethyl ether (2 × 25 mL). The combined organic layers were dried over magnesium sulfate and concentrated under vacuum. The residue was dried under reduced pressure to afford a colorless oil (317 mg, 60%). ^{1}H NMR (300 MHz, $CDCl_3$, 300 K): δ (ppm) = 2.28 (s, 6H); 2.51 (t, ^{3}J = 6.2 Hz, 2H); 3.35 (t, ^{3}J = 6.2 Hz, 2H). ^{13}C NMR (75 MHz, $CDCl_3$, 300 K): δ (ppm) = 45.6, 49.2, 58.2.

Abbreviations

BBN	Bombesin
BBN–Chol	Bombesin–cholesterol derivative
BCN	Bicyclononyne
FPLC	Fast protein liquid chromatography
HPLC	High performance liquid chromatography
SubPc	Subphthalocyanine
DPPC	1,2-Dipalmitoyl-*sn-glycero*-3-phosphocholine
Trt	Trityl
Boc	*tert*-Butoxycarbonyl
Mmt	4,4′-Dimethoxytrityl

Acknowledgements

RAD and YB thank the Burgundy Regional Council, CRB (FABER Program), and RAD thanks CNRS for Chaire d'Excellence. This work was supported by the 3MIM agreement (CNRS, uB and CRB). MM thanks ANR (Equipex IMAPPI). We also thank France Life Imaging (FLI).

References

1 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.
2 E. Heyer, P. Lory, J. Leprince, M. Moreau, A. Romieu, M. Guardigli, A. Roda and R. Ziessel, *Angew. Chem., Int. Ed.*, 2015, **54**, 2995.
3 (*a*) Y. Bernhard, P. Winckler, R. Chassagnon, P. Richard, E. Gigot, J.-M. Perrier-Cornet and R. A. Decreau, *Chem. Commun.*, 2014, **50**, 13975; (*b*) Y. Bernhard, P. Winckler, J.-M. Perrier-Cornet and R. A. Decréau, *Dalton Trans.*, 2015, **44**, 3200.
4 (*a*) R. Decreau, M. Julliard and M. Chanon, *Synlett*, 1998, **4**, 375; (*b*) Y. Ooyama, S. Yoshikawa, S. Watanabe and K. Yoshida, *Org. Biomol. Chem.*, 2007, **5**, 1260; (*c*) P.-E. Doulain, C. Goze, E. Bodio, P. Richard and R. A. Decreau, *Chem. Commun.*, 2016, **52**, 4474.
5 L. D. Lavis and R. T. Raines, *ACS Chem. Biol.*, 2008, **3**, 142.
6 K. Adachi and H. Watarai, *Anal. Chem.*, 2006, **78**, 6840.
7 (*a*) L. Ma, P. Yu, B. Veerendra, T. L. Rold, L. Retzloff, A. Prasanphanich, G. Sieckman, T. J. Hoffman, W. A. Volkert and C. J. Smith, *Mol. Imaging*, 2007, **6**, 171; (*b*) P.-E. Doulain, R. A. Decreau, C. Racoeur, V. Goncalves, L. Dubrez, A. Bettaeib, P. Le Gendre, F. Denat, C. Paul, C. Goze and E. Bodio, *Dalton Trans.*, 2015, 4874; (*c*) B. Brizet, V. Goncalves, C. Bernhard, P. D. Harvey, F. Denat and C. Goze, *Chem. – Eur. J.*, 2014, **20**, 12933.
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.
9 (*a*) *Liposomes, a practical Approach*, ed. R. R. C. New, IRL Press, 1989; (*b*) S. Batzri and E. D. Korn, *Biochim. Biophys. Acta*, 1980, **594**, 53; (*c*) R. Decréau, M.-J. Richard, P. Verrando, M. Chanon and M. Julliard, *J. Photochem. Photobiol., B*, 1999, **48**, 48; (*d*) J. Barge, R. Decreau, M. Julliard, J. C. Hubaud, A. S. Sabatier, J. J. Grobb and P. Verrando, *Exp. Dermatol.*, 2004, **13**, 33; (*e*) R. Decreau, A. Viola, M. J. Richard, A. Jeunet and M. Julliard, *J. Porphyrins Phthalocyanines*, 1998, **2**, 405.
10 (*a*) P. Vabbilisetty and X.-L. Sun, *Org. Biomol Chem.*, 2014, **12**, 1237; (*b*) A. S. Gupta and H. A. von Recum, Bioconjugation Strategies: Lipids, Liposomes, Polymersomes, and Microbubbles, in *Chemistry of Bioconjugates: Synthesis, Characterization, and Biomedical Applications*, ed. R. Narain, John Wiley & Sons, Inc., Hoboken, NJ, USA, 2014, p. 185; (*c*) A. Accardo, G. Salsano, A. Morisco, M. Aurilio, A. Parisi, F. Maione, C. Cicala, D. Tesauro, L. Aloj, G. De Rosa and G. Morelli, *Int. J. Nanomed.*, 2012, **7**, 2007.
11 S. Guard, K. J. Watling and W. Howson, *Eur. J. Pharmacol.*, 1993, **240**, 177.
12 V. Sancho Bornez, A. Di Florio, T. W. Moody and R. T. Jensen, *Curr. Drug Delivery*, 2011, **8**, 79.

