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Smart tools and orthogonal click-like reactions onto small unilamellar vesicles

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ABSTRACT

Click-based reactions were conducted at the surface of small unilamellar vesicles (SUVs) to provide onto-vesicle chemistry with efficient innovative ready-for-use tools. For that purpose, four amphiphilic molecules were designed to insert into bilayers while presenting a reactive functional head. In this manner, a dioleylglycero-ethoxy-ethoxy-ethoxy-ethanamine (DOG-PEG_4-NH_2) was chosen as a common platform while the reactive amine head was converted into several electrophilic functions. Thus, two dioleylglycerol-based cyclooctyne anchors were prepared: cyclooct-1-yn-3-glycolic acid-based anchor (DOG-COA) and 1-fluorocyclooct-2-ynecarboxylic acid-based anchor (DOG-FCOA). The last one differed from the first one in that a fluorine atom reinforces the electrophilic properties of the unsaturated bond. In addition, a third dioleylglycerol-based triphenylphosphine (DOG-PPh_3) was synthesized for the first time. These three innovative amphiphilic anchors were designed to react with any azide-based biomolecule following copper-free Huisgen 1,4-cycloaddition and Staudinger ligation, respectively. A fourth anchor bearing a 3,4-dibromomaleimide ring (DOG-DBM) was also unprecedentedly synthesized, to be further substituted by two thiols. Model reactions conducted in solution with either model biotinyl azide or model biotinyl disulfide gave good to total conversions and excellent isolated yields. The four new anchors were inserted into SUVs whose formula is classically used in *in vivo* biology. Stability and surface overall electrostatic charge were in the expected range and constant over the study. Then, the functionalized liposomes were ligated to biotin-based reagents and the experimental conditions were finely tuned to optimize the conversion. The biotinyl liposomes were demonstrated functional and totally accessible in an affinity test based on biotin scaffold quantification. Finally, DOG-FCOA's reactivity was confronted to that of DOG-DBM in a 'one-pot' orthogonal reaction. (Biotin-S)$_2$ and TAMRA-N_3 (tetramethylcarboxyrhodamine azide) were successively conjugated to the liposome suspension in a successful manner. These data implement and reinforce the interest of bioorthogonal click-like reactions onto lipid nanoparticles.

Abbreviations: AcOH, acetic acid; CH_2Cl_2, dichloromethane; Chol, cholesterol; DCC, dicyclohexylcarbodiimide; DIEA, diisopropylethylamine; DLS, dynamic light scattering; 4-DMAP, 4-*N*,*N*-dimethylaminopyridine; DMF, *N*,*N*-dimethylformamide; DOG-PEG_4-NH_2, dioleylglycero-ethoxy-ethoxy-ethoxy-ethanamine; EDCI, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride; Et_3N, triethylamine; EtOA, acetyl acetate; HATU, 2-(1H-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate; HBTU, *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyl-uronium-hexafluoro-phosphate; HMPA, hexamethylphosphoramide; HOBt, hydroxybenzotriazole; NHS, *N*-hydroxysuccinimide; PC, L-α-phosphatidylcholine; PDI, polydispersity index; PEG, polyethylene glycol; PG, L-α-phosphatidylglycerol; PTSA, para-toluene sulfonic acid; PyBOP, benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate; SUVs, small unilamellar vesicles; TAMRA-N_3, tetramethylcarboxyrhodamine azide; TBAS, tetrabutylammonium sulfate; TCEP, *tris*(2-carboxyethyl)phosphine; THF, tetrahydrofurane; rt, room temperature.

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1. Introduction

The modification of the surface properties of liposomes by peptides, proteins or carbohydrates that convey either specificity or targeting has been extensively studied (Sankaram, 1994; Silvius and l'Heureux, 1994; Hojo et al., 1996; Boeckler et al., 1998; Schelté et al., 2000). For example, the stable anchorage of peptides into a phospholipid half-bilayer requires their branching by a lipid tail (Shahinian and Silvius, 1995; Epand, 1997) composed of at least one, or better, two alkyl chains (Martin and Papahadjopoulos, 1982; Kung and Redemann, 1986). However, it is a tedious task due to the poor water solubility of the resulting amphiphilic compounds. To overcome these difficulties, alternative strategies have been developed where the compound to be immobilized is covalently ligated to a functionalized lipid anchor already inserted in the membrane (Heeremans et al., 1992; Fleiner et al., 2001). In these strategies and until the mid-2000s, the chemical bond between anchor and either peptide, protein or carbohydrate compounds aimed at decorating the liposomes was chosen among amide bond (Kamps et al., 1996), thioether bond (Roth et al., 2004; Horak et al., 1999; Nakano et al., 2001), disulfide bridge (Zalipsky et al., 1995; Elliott and Prestwich, 2000) or imine bond (Harding et al., 1997). Later on, hydrazone and alpha-oxo hydrazone bonds were also developed and for the first time fully 'bioorthogonal' chemoselective ligations were available (Zalipsky, 1993; Chenevier et al., 2002; Bourel-Bonnet et al., 2005; Jolimaitre et al., 2007).

Then bioconjugate chemistry knew the boom of 'click chemistry' (Kolb et al., 2001; Torne et al., 2002; Rostovtsev et al., 2002). As a consequence several thousand papers and reviews can now be reached when typing these two words on a relevant research motor. Many authors now consider 'click chemistry' as a powerful tool for chemistry (Meldal and Torne, 2008), pharmaceutical sciences (Hein et al., 2008) and chemical biology (Speers et al., 2003; Boyce and Bertozzi, 2011; Beal and Jones, 2012). In this wake, in parallel to another pioneer work (Cavalli et al., 2006) and before a further work in this field (Mourtas et al., 2011), we have already proposed a Huisgen [1,3]-cyclo-addition – called 'click chemistry' by metonymy – to site-specifically immobilize a mannose-based tree onto preformed liposomes (Said Hassane et al., 2006; Frisch et al., 2010). The disadvantage of this method is the *in situ* generation of a reducing agent – Cu(I) – in excess. Moreover, the subsequent use of a water soluble Cu(I) chelator, such as bathophenanthroline disulfonate, was essential to obtain good yields (80%) in reasonable reaction times (1 h) while eliminating copper ions reputed toxic for living organisms. However, as assessed by agglutination experiments using concanavalin A, the mannose residues were perfectly accessible at the surface of the targeted vesicles. This first click chemistry onto preformed vesicles paved the way for further fully 'bioorthogonal' reactions. Nowadays, copper-free click chemistry is an outstanding and popular alternative and an increasing amount of experiments performed in the field of chemical biology (Jewett and Bertozzi, 2010; Shelbourne et al., 2012) are now conducted without copper to get a totally 'bioorthogonal' bond as the semantics puts it (Sletten and Bertozzi, 2009). As the use of this alternate route remained very rare in the field of chemical ligation involving liposomes or nanoparticles until very recently (Jolck et al., 2011; Tarallo et al., 2011; Bostic et al., 2012; Colombo et al., 2012; Koo et al., 2012; Wu et al., 2012; Emmetiere et al., 2013; Airoldi et al., 2014), we decided to implement our previous work by a further study. Here, we have designed four new anchors (Fig. 1) based on a dioleylglycero-ethoxy-ethoxy-ethoxy-ethanamine platform (DOG-PEG_4-NH_2, (**1**), whose amine has been converted into several electrophilic heads: cyclooctyne (DOG-COA, (**2**), fluorocyclooctyne (DOG-FCOA, (**3**), triphenylphosphine (DOG-PPh_3, (**4**) and dibromomaleimide (DOG-DBM, (**5**). These anchors were expected to react following copper-free Huisgen cycloaddition, Staudinger ligation and substitution respectively.

Biotin- and TAMRA-based tools were designed and synthesized (Fig. 2) to tune and monitor the experimental conditions leading to the expected decorated liposomes. The reactions could be perfectly conducted in solution and were transposed to SUVs suspensions. The biotinyl liposome adducts were proven functional in a biocompatible environment.

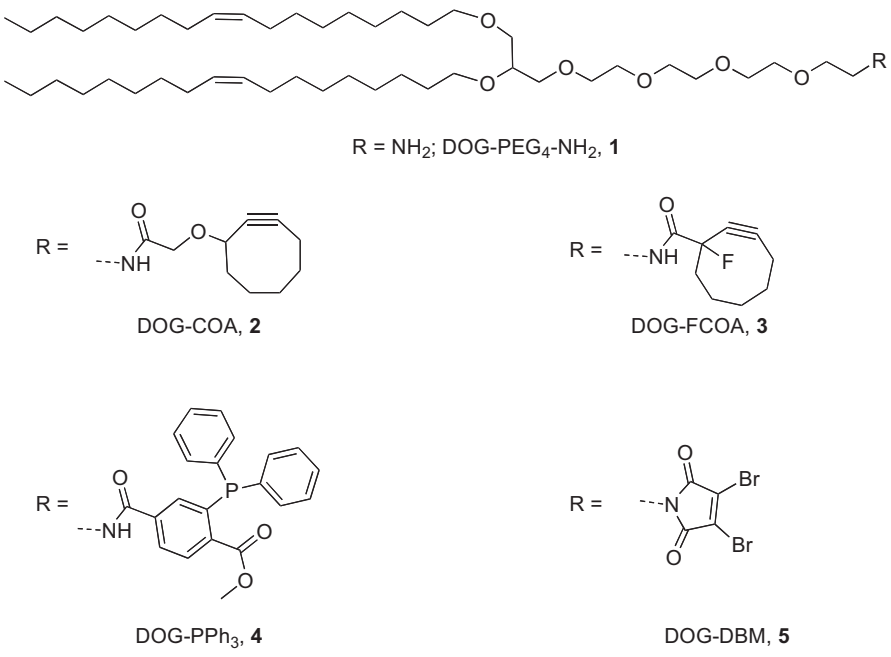


Fig. 1. Four anchors based on a DOG-PEG_4-NH_2 platform

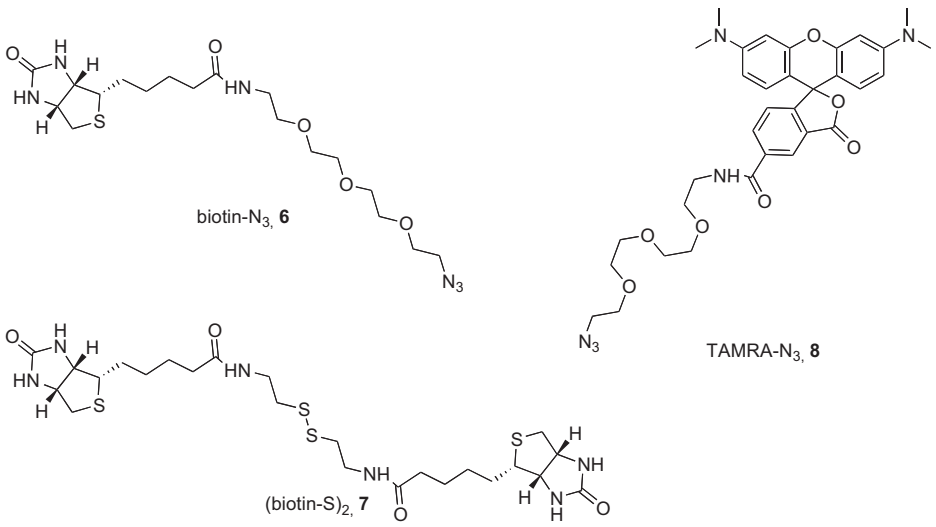


Fig. 2. Molecular tools to study and quantify the bioconjugate chemistry onto liposomes.

2. Material and methods

2.1. Generals

Reagent grade solvents were used without further purification. Egg yolk L-α-phosphatidylcholine (PC), L-α-phosphatidylglycerol (PG) transesterified from egg yolk PC, cholesterol (Chol) (recrystallized in methanol), polymer supported triphenylphosphine and anhydrous CH_2Cl_2 were purchased from Sigma–Aldrich. The PyBOP was purchased from Novabiochem, the DIEA was from Alfa Aesar and both were used without further purification. Column chromatography was carried out on silica gel 60 (Merck, 70–230 mesh). ^{1}H NMR spectra at either 300 MHz, 400 MHz or 500 MHz and ^{13}C NMR spectra at either 75 MHz, 100 MHz or 133 MHz were recorded on Brucker spectrometers either 300, 400 or 500 respectively with residual undeuterated solvent as internal reference. All chemical shift values (δ), coupling constants (J) and the multiplicity (s = singlet, d = doublet, t = triplet, m = multiplet, br = broad) are quoted in ppm and in Hz respectively. High-resolution mass spectra (HRMS) were obtained using an Agilent Q-TOF (time of flight) 6520 and low-resolution mass spectra (LRMS) using an Agilent MSD 1200 SL (ESI/APCI). Analytical RP-HPLC-MS was performed using a C18 column (30 mm × 1 mm; 1.9 μm) using the following parameters: (1) the eluent system A (0.05% TFA in H_2O) and B (0.05% TFA in acetonitrile); (2) the linear gradient t = 0 min with 98% A, t = 5 min with 5% A, t = 6 min with 5% A, t = 7 min with 98% A, and t = 9 min with 98% A; (3) flow rate of 0.3 mL min^{-1}; (4) column temperature 50 °C; (5) ratio of products determined by integration of spectra recorded at 210 or 254 nm; (6) ionization mode ESI. TLC spots were detected by UV irradiation at 254 nm or with $KMnO_4$ stain. IR spectra were performed on Nicolet 380 FT-IR, Thermo Instruments.

2.2. Chemical synthesis

2.2.1. Preparation of dioleylglycero-ethoxy-ethoxy-ethanamide cyclooct-1-yn-3-glycol (**2**)

To a solution of cyclooct-1-yn-3-glycolic acid (20 mg, 0.109 mmol) (Bernardin et al., 2010) in anhydrous CH_2Cl_2 (2 mL), DIEA (68 mg, 0.540 mmol) and PyBOP (68 mg, 0.130 mmol) were added. The resulting mixture was stirred for 10 min for activation. Then previously prepared DOG-PEG$_4$-NH$_2$ (**1**, Supplementary information, Data in Brief) (100 mg, 0.130 mmol) solubilized in 2 mL of anhydrous dichloromethane was added to the above-mentioned activated solution. The resulting mixture was stirred at room temperature overnight and concentrated under vacuum. The crude was purified on silica gel using CH_2Cl_2/MeOH (100/0 to 95.2/4.8) to give 55 mg (55%) of **2** as a yellow oil; Rf = 0.80 (CH_2Cl_2/MeOH: 9.5/0.5); ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.34 (m, 4H), 4.23 (m, 1H), 4.05 (d, 1H, J = 15.3 Hz), 3.86 (d, 1H, J = 15.3 Hz), 3.65–3.40 (m, 23H), 2.72–1.25 (m, 10H and 56H), 0.87 (t, 6H, J = 7.0 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.8, 21.3, 23.3, 26.8, 26.9, 27.9, 29.9, 30.2, 30.3, 30.4, 30.7, 32.6, 33.3, 34.9, 39.2, 42.8, 69.0, 70.9, 71.2, 71.5, 72.0, 72.3, 73.7, 78.5, 91.9, 102.2, 130.5, 130.9, 170.3; HRMS calcd for $C_{57}H_{105}NO_8Na$ 954.7737; found 954.7738.

2.2.2. Preparation of 1-fluoro-N-((Z)-14-((Z)-octadec-9-en-1-yloxy)-3,6,9,12,16-pentaoxatetratriacont-25-en-1-yl) cyclooct-2-ynecarboxamide (**3**)

EDCI (58 mg, 0.30 mmol) and NHS (1 mg, 0.03 mmol) were added to a CH_2Cl_2 solution (1 mL) of 1-fluorocyclooct-2-ynecarboxylic acid (51 mg, 0.30 mmol) (Schultz et al., 2010). The solution was stirred at room temperature (30 min) and a CH_2Cl_2 (1 mL) solution of DOG-PEG$_4$-NH$_2$ (**1**) (120 mg, 0.15 mmol) was added. The solution was stirred at room temperature (16 h). The volatiles were evaporated and the crude was purified by chromatography on silica gel using cyclohexane/EtOAc (70/30 to 0/100) as eluent to obtain 7 mg (25%) of **3** as a yellow oil; IR (cm^{-1}) 3321, 2926, 2849, 2360, 1623, 1568, 1448, 1435, 1309, 1242, 1087, 640; ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.40-5.35 (m, 4H), 3.70-3.43 (m, 25H, 11CH_2O, CH_2N and $(CH_2)_2CHO$), 2.38-2.28 (m, 4H, 2CH_2 cyclooctyne), 2.09-1.98 (m, 10H, 4CH_2 and CH_2 cyclooctyne), 1.62-1.55 (m, 7H, 4H and 3H cyclooctyne), 1.25-1.35 (m, 45H, 44H and H cyclooctyne), 0.82 (t, 6H, J = 7.2 Hz, 2CH_3); ^{13}C NMR (133 MHz, $CDCl_3$): δ 14.1, 14.2, 20.6 (d, J = 3.0 Hz), 21.1, 22.7, 26.1 (d, J = 4.8 Hz), 27.2, 28.9, 29.3, 29.4, 29.5, 29.6, 29.7, 29.7, 29.8, 30.2 (d, J = 9.3 Hz), 31.9, 32.0, 39.3, 46.4 (d, J = 25.0 Hz), 60.4, 69.5, 70.4, 70.5, 70.6, 70.6 70.7, 70.8, 70.9, 71.4, 71.7, 77.9, 109.5 (d, J = 10.9 Hz), 127.8, 128.7, 129.9, 130.0, 171.2; HRMS (ESI) m/z calcd for $C_{53}H_{106}FNO_7SNa^+$ 942.7566; found 942.7557.

2.2.3. Preparation of methyl 2-(diphenylphosphino)-4-(((Z)-14-((Z)-octadec-9-en-1-yloxy)-3,6,9,12,16-pentaoxa-tetratriacont-25-en-1-yl) carbamoyl) benzoate (**4**)

2-(Diphenylphosphino) terephthalic acid 1-methyl 4-pentafluorophenyl diester (10 mg, 0.019 mmol) was added to a DMF (1 mL) solution of DOG-PEG$_4$-NH$_2$ (**1**) (17 mg, 0.022 mmol) and Et_3N (13 μL, 0.095 mmol). The solution was stirred at room

temperature (16 h). The reaction mixture was concentrated under vacuum and the crude was purified by chromatography on silica gel using cyclohexane/EtOAc (100/0 to 50/50) as eluent to obtain 15 mg of DOG-PPh_3 (**4**) as a colorless oil (62%); ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.10 (dd, 1H, J = 8.0, 3.6 Hz, H of C_6H_3), 7.81 (dd, 1H, J = 8.0, 1.5 Hz, H of C_6H_3), 7.48-7.44 (m, 1H, H of C_6H_3), 7.38-7.24 (m, 10H, H of C_6H_5), 6.65-6.59 (br s, 1H, NH), 5.41-5.32 (m, 4H), 3.76 (s, 3H, OCH_3), 3.68-3.41 (m, 25H, $11CH_2O$, CH_2N and $(CH_2)_2CHO$), 2.08-1.94 (m, 8H, $4CH_2$), 1.62-1.51 (m, 4H), 1.38-1.20 (m, 44H), 0.81 (t, 6H, J = 7.0 Hz, $2CH_3$); ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.1 ($2CH_3$), 22.7, 27.2, 29.3, 29.5, 29.5, 29.6, 29.6, 29.7, 29.8, 30.1, 32.6 ($29CH_2$), 39.8 (CH_2CH_2N), 52.2 (OCH_3), 69.6 ($11CH_2O$), 70.3, 70.5, 70.6, 70.7, 70.9, 71.5, 71.7, 77.9 ($CHCH_2O$), 126.7, 126.8, 128.6, 128.7, 129.0, 129.8, 129.9, 132.8, 133.8, 134.0 (10CH), 137.2, 137.3, 137.4, 141.3, 142.6 (4C), 158.7 (C(O)NH), 166.4 (C(O)OMe); HRMS (ESI) m/z calcd for $C_{68}H_{108}NO_9PH^+$ 1114.7834; found 1114.7856.

*2.2.4. Preparation of 3,4-dibromo-1-((Z)-14-((Z)-octadec-9-en-1-yloxy)-3,6,9,12,16-pentaoxatetratriacont-25-en-1-yl)-1H-pyrrole-2,5-dione (**5**)*

Dibromomaleic acid (2.5 mg, 9.11 μmol) and DCC (2 mg, 9.54 μmol) were stirred in CH_2Cl_2 (2.5 mL) at room temperature (1 h) for activation. A CH_2Cl_2 solution of DOG-PEG_4-NH_2 (**1**) (7 mg, 0.01 mmol) was added and the solution was stirred at room temperature (16 h). The volatiles were evaporated and acetic acid (4 mL) was added. Then, the solution was stirred at reflux (16 h). Acetic acid was evaporated under vacuum and the residue was purified by chromatography on silica gel using cyclohexane/EtOAc (100/0 to 50/50) as eluent to obtain 7 mg (60%) of DOG-DBM (**5**) as a colorless oil; ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.35-5.32 (m, 4H), 3.79 (t, 2H, J = 5.2 Hz), 3.67-3.38 (m, 21H), 2.03-1.95 (m, 8H), 1.53-1.49 (m, 4H), 1.42-1.15 (m, 44H), 0.86 (t, 6H, J = 6.7 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.1, 16.8, 22.7, 26.1, 26.2, 27.2, 29.3, 29.5, 29.5, 29.6, 29.7, 29.8, 30.1, 31.9, 32.6, 39.0, 67.5, 70.1, 70.6, 70.6, 70.6, 70.7, 70.8, 70.9, 71.5, 71.7, 77.9, 129.4, 129.8, 129.9; HRMS (ESI) m/z calcd for $C_{51}H_{91}Br_2NO_8H^+$ 1004.5 [^{79}Br, ^{79}Br], 1006.5 [^{79}Br, ^{81}Br], 1008.5 [^{81}Br, ^{81}Br]; found 1004.4 [^{79}Br, ^{79}Br], 1006.4 [^{79}Br, ^{81}Br], 1008.4 [^{81}Br, ^{81}Br].

*2.2.5. Preparation of N-(2-(2-(2-(2-azidoethoxy) ethoxy) ethoxy) ethyl)-5-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl) pentanamide (Biotin-N_3, (**6**)) (Kottani et al., 2006)*

Biotin-OSuc (**14**) (187 mg, 0.55 mmol) was completely dissolved in DMF (5 mL) with gentle warming. After slowly cooling the solution to room temperature without recurring precipitation, the 11-azido-3,6,9-trioxaundecan-1-amine (Borcard et al., 2011) (109 mg, 0.50 mmol) was added. The reaction proceeded at room temperature overnight with stirring and the reaction products were concentrated under vacuum. The remaining solid was triturated in CH_2Cl_2/Et_2O (30/70) solution and the white solid was filtered to obtain *N*-(2-(2-(2-(2-azidoethoxy) ethoxy) ethoxy) ethyl)-5-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl) pentanamide (**6**) (219 mg; quant.); ^{1}H NMR (300 MHz, DMSO-d_6): δ 6.43-6.38 (s, 1H, NH), 6.37-6.34 (s, 1H, NH), 4.33-4.25 (m, 1H), 4.12-4.09 (m, 1H), 3.62-3.34 (m, 12H), 3.12-3.08 (m, 1H), 2.89-2.78 (m, 4H), 1.55-1.19 (m, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 25.6, 28.0, 28.1, 35.9, 39.2, 40.5, 50.7, 55.5, 60.3, 61.8, 70.0, 70.1, 70.4, 70.5, 70.6, 163.9, 173.5; LRMS (ESI) m/z calcd for $C_{18}H_{32}N_6O_5SH^+$ 445.2; found 445.2.

*2.2.6. Preparation of N,N′-(disulfanediylbis(ethane-2,1-diyl))bis(5-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl) pentanamide) (**7**)*

In accordance to a described procedure (Wang et al., 2012), Biotin-OSuc (**14**) (300 mg, 0.87 mmol) was completely dissolved in DMF (3 mL) with gentle warming (40 °C). After slowly cooling the solution to room temperature without recurring precipitation, it was mixed with an aqueous solution (1 mL) containing cystamine dihydrochloride (107 mg, 0.47 mmol) and Et_3N (309 μL, 2.10 mmol). The reaction proceeded at room temperature overnight with stirring. The white solid was filtered and washed with water and EtOH to obtain *N,N′*-(disulfanediylbis(ethane-2,1-diyl)) bis(5-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl) pentanamide) (**7**) as a white solid (250 mg; 87%); ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.08-8.04 (br m, 2H), 6.48 (s, 2H), 6.43 (s, 2H), 4.39-4.34 (m, 2H), 4.22-4.17 (m, 2H), 3.42-3.35 (m, 2H), 3.19-3.14 (m, 2H), 2.88-2.81 (m, 6H), 2.64 (d, J = 12.9 Hz, 2H), 2.13 (t, J = 7.3 Hz, 4H), 1.72-1.53 (m, 8H), 1.40-1.34 (m, 4H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 25.7, 28.5, 28.7, 35.6, 37.8, 38.3, 40.3, 55.9, 59.7, 61.5, 163.2, 172.7.

*2.2.7. N-(2-(2-(2-(2-azidoethoxy) ethoxy) ethoxy) ethyl)-5-(6)-(tetramethylcarboxyrhodamine (TAMRA-N_3,**8**)*

To a solution of 5-(6)-TMCR (43 mg, 0.10 mmol) in anhydrous CH_2Cl_2 (3 mL), DIEA (87 μL, 0.50 mmol) and PyBOP (63 mg, 0.12 mmol) were added. The resulting mixture was stirred for 10 min to get the corresponding activated ester. Then 11-azido-3,6,9-trioxaundecan-1-amine (22 μL, 0.11 mmol) solubilized in anhydrous CH_2Cl_2 (3 mL) were added to the above-mentioned solution. The resulting mixture was stirred at room temperature (3 h) protected from light. Then the solution was concentrated under vacuum and the crude was purified on silica gel using CH_2Cl_2/MeOH (100/0 to 80/20) to give 53 mg (84%) of **8** as a purple solid; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.52-8.48 (br s, 0.4H), 8.19-8.09 (m, 0.8H), 8.03-7.98 (m, 0.4H), 7.70-7.64 (br m, 1.2H), 7.54-7.50 (br m, 1H), 7.21-7.15 (m, 2.2H), 6.79 (t, J = 10.3 Hz, 1H), 6.51-6.45 (m, 3H), 3.72-3.58 (m, 14H), 3.37-3.33 (m, 2H), 3.05-3.07 (m, 12H); ^{13}C NMR (75 MHz, DMSO-d_6), δ (ppm): 29.7, 40.0, 40.1, 40.4, 50.6, 50.7, 69.6, 69.7, 69.8, 69.9, 70.0, 70.1, 70.2, 70.3, 70.41, 70.46, 70.53, 70.56, 70.6, 97.2, 97.4, 111.0, 111.13, 111.14, 111.19, 111.30, 111.34, 111.35, 117.9, 124.4, 125.3, 125.90, 125.96, 126.0, 126.96, 126.98, 127.9, 128.2, 128.3, 128.6, 130.0, 130.3, 132.0, 136.1, 142.2, 154.5, 154.6, 155.1, 154.2, 166.4, 169.3, 169.4; LRMS calcd for $C_{33}H_{39}N_6O_7$ 631.3; found 631.3.

2.3. Liposomes preparation and characterization

2.3.1. SUVs formation

Liposomes were prepared by mixing, in a round-bottom flask, phospholipids (PC, PG), and cholesterol (80/20/50 molar ratio) in chloroform/methanol 9/1 with the appropriate amount of anchors (1, 2.5, 5 and 10 mol% for the initial experiments, then 5 mol% for further experiments with an adjustment of the PC concentration). After solvent evaporation under high vacuum (45 min), the resulting dried lipid film was hydrated by vortex mixing (5 min) after addition of 1 mL of buffer (100 mM NaCl, 10 mM Hepes, pH 7.4) to get a final concentration of 10 μmol of total phospholipids per mL. The resulting suspension was sonicated at 25 °C for 45 min under a continuous flow of argon, using a 3 mm diameter probe sonicator (Vibra Cell, Sonics and Material Inc., Danbury, CT) at 300 W. The small unilamellar vesicle (SUV) suspensions were finally centrifuged for 10 min to remove the titanium dust originating from the probe. Blank liposomes were only composed of PC/PG/Chol (80/20/50 molar ratio) without anchor.

2.3.2. PC assay

The PC content of liposome suspension was quantified using an enzymatic assay with a commercial test kit (Assay™ Phospholipid, Wako Chemicals GmbH, Neuss, Germany). 5 or 10 μL of liposome suspensions were distributed in triplicate in a 96-well microplate before adding 250 μL of phospholipid B reagent solution, prepared according to the manufacturer's instructions. The plates were then incubated at 37 °C for 10 min. A blue color appeared, in presence of

choline, due to the formation of a phenol-4-aminoantipyrine complex. Choline chloride was used for calibration curve. Absorbance was measured at λ = 595 nm using a microplate reader (Bio-Rad model 550, Marnes-la-Coquette, France). It is postulated that a loss in PC is directly correlated to a loss in anchor within the same proportions.

2.3.3. Particle-size measurements

The particle size distribution was measured by dynamic light scattering (DLS) using a Nano ZS (Malvern Instrumentation, Inc., Orsay, France) with an angle of detection of 173 degrees, measured at 25 °C and in intensity. Each sample was diluted with the Hepes buffer until the expected particle concentration was achieved, *i.e.*, 20 μL of a 10 mM liposome suspension and 980 μL of buffer. Each measurement was performed in triplicate.

2.4. Chemistry on liposomes

2.4.1. Absorbance measurement

The absorbance was measured on a Safas Xenius XM spectrometer at λ = 551 nm in a quartz cell. The background absorbance of the blank liposomes was subtracted from the recorder absorbance of the liposome.

2.4.2. Biotin coupling and determination of its functionality

The amount of biotin bound on liposomes was quantified using a commercial kit (Quant*Tag^{TM} Biotin Kit Vector Laboratories, Inc., Burlingame, CA). 5 μL of liposome suspensions were distributed in triplicate in a 96-well microplate before adding 100 μL of reagent solution, prepared according to the manufacturer's instructions. The plates were then incubated at 25 °C for 30 min. A yellow–orange color appeared in presence of biotin. Free biotin was used for calibration. Absorbance was measured at λ = 535 nm using a microplate reader (Bio-Rad model 550, Marnes-la-Coquette, France).

2.4.3. Liposomes ligation: copper-free click

To a suspension of functionalized liposomes in buffer, biotin-N_3 **6** (10 mM in water, 5%) was added. The resulting mixture was gently stirred under argon at 25 °C for 1 h, 6 h, 24 h or 48 h. After the coupling step, the unbound probe was removed by exclusion chromatography on a 1 × 20 cm Sephadex G-100 column equilibrated with the same buffer as that used in the coupling step.

2.4.4. Liposomes ligation: thiol-dibromomaleimide

To a suspension of functionalized liposomes in buffer, (biotin-S)$_2$ **7** (1 eq. based on the anchor quantity, 5 mM in water) and TCEP (1 eq. based on the anchor quantity, 100 mM in water) were added. The resulting mixture was gently stirred under argon at 25 °C for 1 h, 6 h, 24 h or 48 h. After the coupling step, the unbound probe and TCEP derivative were removed by exclusion chromatography on a 1 × 20 cm Sephadex G-100 column equilibrated with the same buffer as that used in the coupling step.

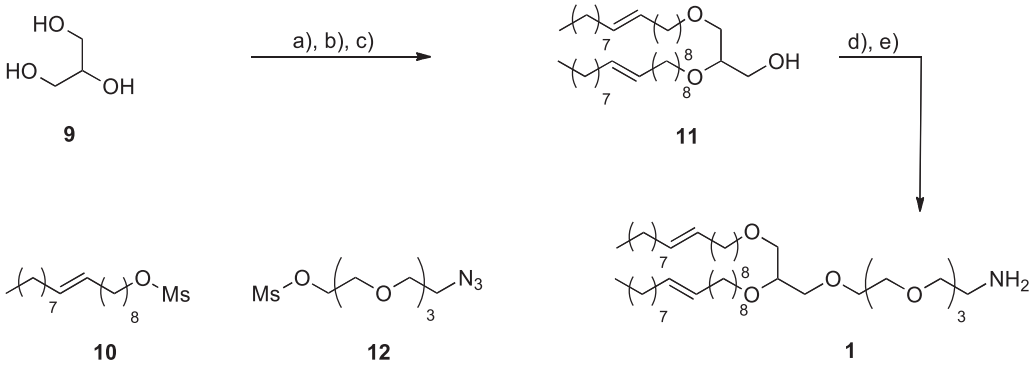
2.4.5. 'One pot' orthogonal double ligation

Liposomes were prepared by mixing in a round-bottom flask, phospholipids (PC, PG) and cholesterol (75/20/50 molar ratio) in chloroform/methanol 9/1 with the DOG-FCOA anchor (2.5% based on phospholipid quantity, 10 mM in chloroform/methanol 9/1) and the DOG-DBM anchor (2.5% based on phospholipid quantity, 10 mM in chloroform/methanol 9/1). After solvent evaporation under high vacuum (45 min), the resulting dried lipid film was hydrated by vortex mixing (5 min) after addition of 1.5 mL of buffer (150 mM NaCl, 10 mM Hepes, pH 7.4), to get a final concentration of 10 μmol of total lipids per mL. The resulting suspension was sonicated at 25 °C for 45 min under a continuous flow of argon, using a 3 mm diameter probe sonicator (Vibra Cell, Sonics and Material Inc., Danbury, CT) at 300 W. The SUV preparations were finally centrifuged for 10 min to remove the titanium dust originating from the probe. TCEP (100 mM in water, 2.5%) and (Biotin-S)$_2$ (5 mM in water, 2.5%) were added successively and the buffer suspension was shaken at room temperature for 1 h to react on anchor **5**. Then, one third (1/3) of the suspension (500 μL) was purified on SEPHADEX-100, lyophilized and re-solubilized (DMSO/H_2O) for biotin quantification and yield determination. One equivalent of TAMRA-N_3 **8** (10 mM in water, 2.5%) was added to the remaining suspension (2/3; 1 mL) and the mixture was shaken for 23 h. After the coupling step, the unbound probe was removed by exclusion chromatography on a 1 × 20 cm Sephadex G-100 column equilibrated with the same buffer as the one used in the coupling step. After that purification, lyophilization and re-solubilization (DMSO/H_2O), the rhodamine was quantified by UV/visible absorption spectrometry at 551 nm (after a standard curve was obtained with a DMSO/H_2O solution of compound **8** – TAMRA-N_3).

3. Results and discussion

3.1. Chemistry

First of all, a key amphiphilic intermediate dioleylglycero-ethoxy-ethoxy-ethoxy-ethanamine (DOG-PEG-NH_2, **1**) was synthesized as a racemic mixture in five steps starting from glycerol, tetraethylene glycol and oleyl alcohol according to a previously described procedure (Espuelas et al., 2003). Considering the low overall yield of the reported procedure (16%), we optimized the oleyl alcohol alkylation and the azide reduction to reach 27% overall yield. For the first step, we found the modified phase-



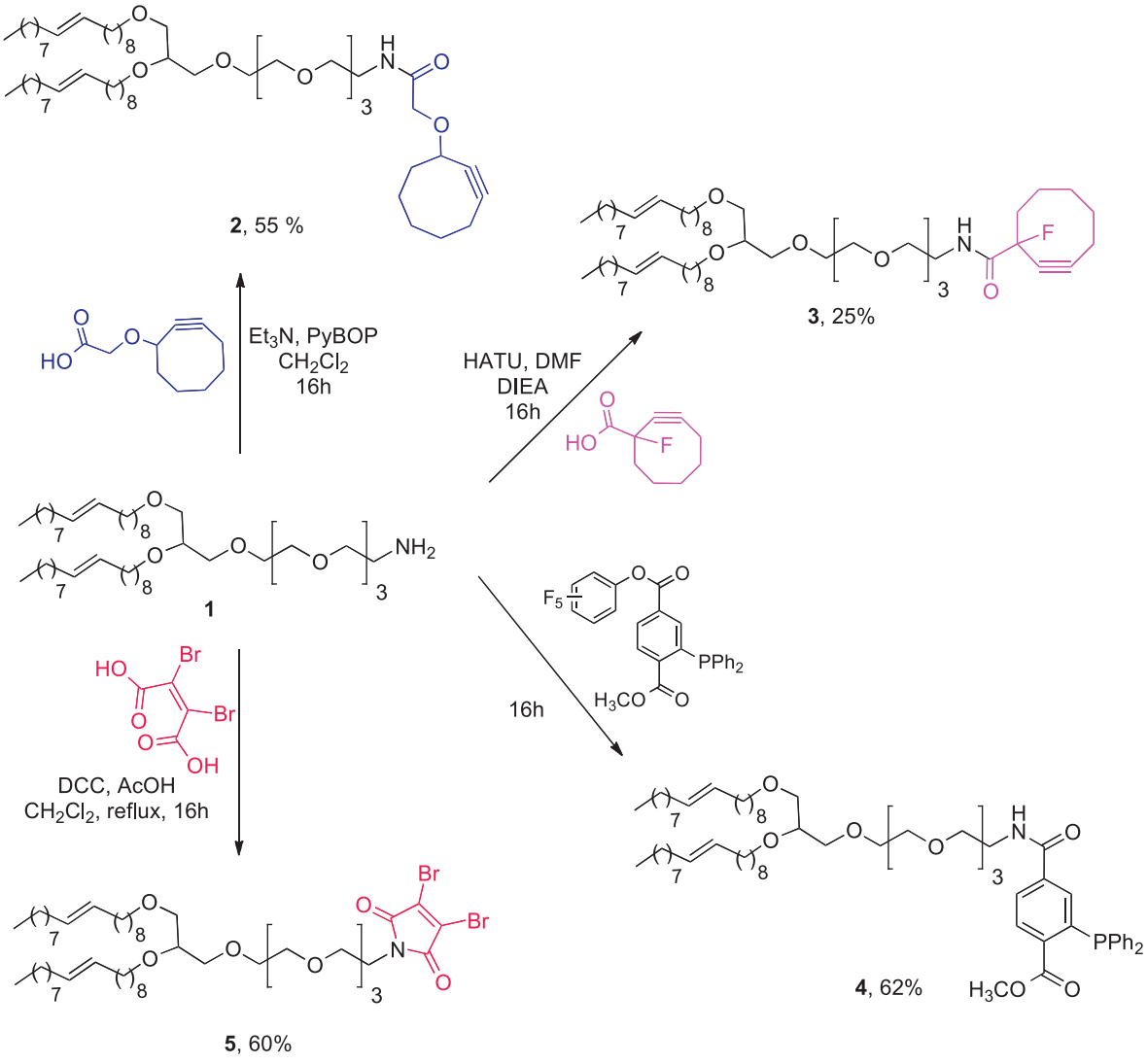
Scheme 1. Synthesis of DOG-PEG$_4$-NH_2: (a) Trityl chloride, DMAP cat., Et_3N, THF, rt (22 h); (b) **10**, TBAS 0.1%, NaOH, H_2O, 65 °C; (c) PTSA, THF/MeOH (1/1), rt (16 h); (d) **12**, NaH (60%), THF, HMPA; (e) Polymer-bound PPh_3, H_2O, THF, 50 °C (16 h).

transfer-catalyzed procedure (TBAS, H_2O, NaOH), already described (Bartsch et al., 1989), very efficient and it allowed us to increase the yield to 80%. In the same way, the azide reduction was optimized by using polymer-supported triphenylphosphine thus helping for the purification stage by a simple filtration of a triphenylphosphine oxide derivative immobilized on the resin (Scheme 1). Thus the chemical route toward **1** was improved and the work-up was simplified.

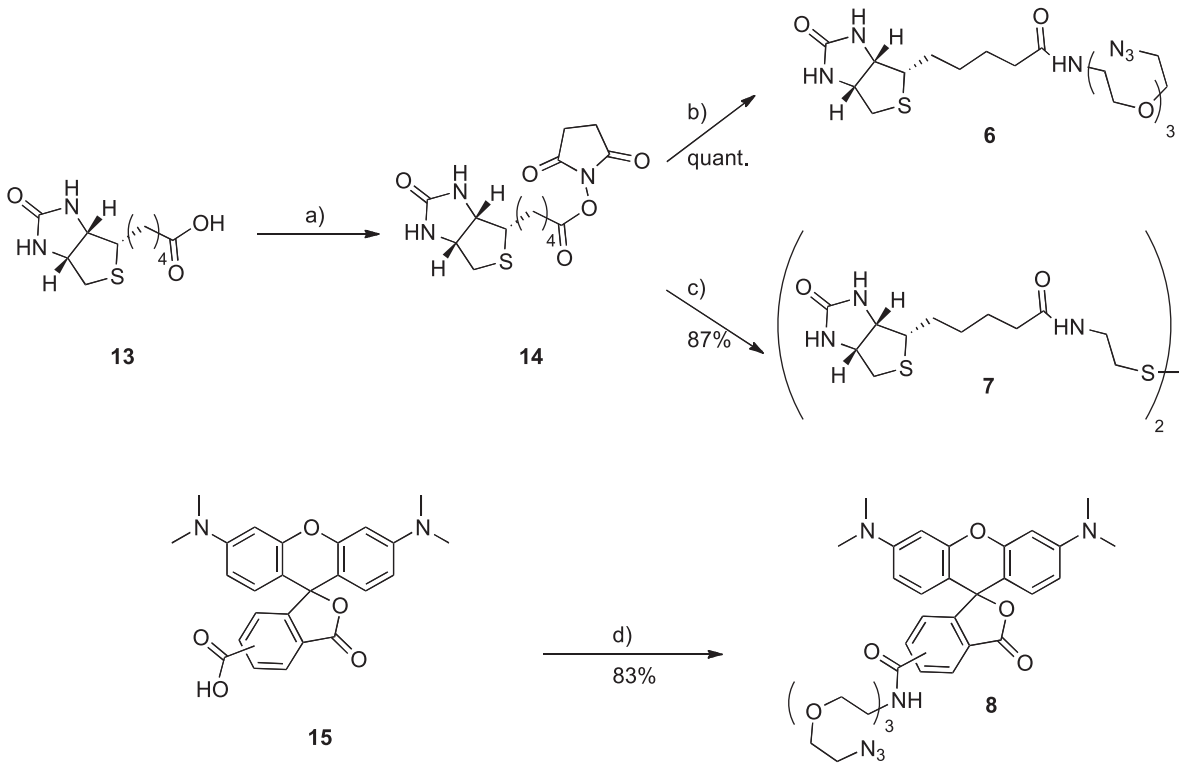
Embarked on the study of new ways of coupling biomolecules of interest to the surface of preformed liposomes, we designed two dioleyl cyclooctyne-based anchors (DOG-COAs, **2** and **3**) and one dioleyl phosphine-based anchor (DOG-PPh_3, **4**) capable of reacting selectively and successfully on azides (Brase and Barnert, 2010; Zhang et al., 2009; Vabbilisettya and Sun, 2014) and one further dioleyl-dibromomaleimide-based anchor (DOG-DBM, **5**) suitable for reacting selectively and efficaciously on thiols (Smith et al., 2010; Schumacher et al., 2011). This last one shows very interesting benefits, as its reversible modification is possible. Advantageously, dibromaleimide/thiol bioorthogonal ligation is a way to double anchor proteins onto lipid vesicles. As a consequence, an extremely efficient disulfide bridging bioconjugation could be obtained. For example, it was showed that conjugation proceeds rapidly and efficiently to give the expected adducts while maintaining the disulfide bridge and thus peptide conformation (Jones et al., 2012). The amphiphilic properties of the target molecule allow it to be incorporated in the phospholipid bilayer of liposomes while exposing a constrained and reactive head equally distributed on the outer and the inner membrane of the vesicle.

A first DOG-COA, dioleylglycero-ethoxy-ethoxy-ethoxy-ethanamide cyclooct-1-yn-3-glycole (**2**) was prepared: the free amine **1** reacted with active hydroxybenzotriazolyl ester of cyclooct-1-yn-3-glycolic acid (Bernardin et al., 2010), obtained with PyBop in the presence of DIEA in CH_2Cl_2, to give the alkyne anchor **2** with a satisfactory overall isolated yield (55%) (Scheme 2). The second cyclooctyne anchor was then prepared: substituted by a fluorine, it was chosen to react more efficiently on azides due to the electron withdrawing effect of the halogen as recently shown (Schultz et al., 2010; Gold et al., 2013). As previously, the amine **1** was coupled with the 1-fluorocyclooct-2-ynecarboxylic acid in DMF, in the presence of EDCI and Et_3N with only 25% yield. Attempts to increase the yield by changing the coupling agent failed. DCC, HBTU, HATU were successively tried without any success.

For the third anchor, the free amine **1** was simply coupled with the commercially available (diphenylphosphino) terephthalic acid 1-methyl 4-pentafluorophenyl diester in DMF to yield the expected compound **4** (62%). Finally, the DOG-DBM anchor **5** was obtained from the reaction with dibromomaleic acid in DMF using DCC as coupling agent (60%) (Wilson et al., 2006). The overall yields are in



Scheme 2. Synthesis of the anchors.



Scheme 3. Synthesis of the probes. (a) NHS, DCC, DMF; (b) H_2N-PEG_3-N_3, DMF; (c) cystamine. HCl, DMF/H_2O (3/1), Et_3N; (d) H_2N-PEG_3-N_3, PyBOP, CH_2C_{l2}, DIEA.

good range for amphiphilic compounds whose purification is reputed challenging: this tedious last operation is usually responsible for the loss of pure expected final compound and frequently screens off very good to excellent conversion yields.

3.1.1. Probes' synthesis

To get the biotin-based probes, biotin succinimidate **14** was synthesized as a key intermediate following the already described procedures (Kottani et al., 2006; Susumu et al., 2007). Then, the two probes could be prepared by coupling **14** with either the 11-azido-3,6,9-trioxaundecan-1-amine (H_2N-PEG_3-N_3) in DMF to get the azido probe **6** or the cystamine hydrochloride to get the thiol-probe **7**. TAMRA-N_3 probe **8** was synthesized starting from the commercially available 5(6)-carboxytetramethylrhodamine (**15**) and 11-azido-3,6,9-trioxaundecan-1-amine (H_2N-PEG_3-N_3) in the presence of DIEA and PyBOP in CH_2Cl_2 (Scheme 3). After purification, the azido fluorescent probe **8** was obtained with 83% yield. Before conducting further studies, we evaluated the chemical reactivity of the selected functional groups using in solution model reactions. In all cases, the expected compounds were obtained with good to excellent yields (50% to quantitative, Supplementary information, Data in Brief).

3.2. Liposomes formulation

The liposomes were prepared by integrating the anchors (1%, 2.5%, 5% and 10% molar ratio) within the phosphatidyl choline (79%, 77.5%, 75% and 70%, respectively), phosphatidyl glycerol (20%) and an additional half of cholesterol to get a final concentration of total lipids as high as 10 mM (53.3/13.3/33.3% molar ratio). After evaporation and lipid film hydratation, the resulting suspension was sonicated using a probe sonicator to get unilamellar vesicles. The average diameter was finally measured by DLS (Table 1). For all the concentrations and anchors, the sizes were in the expected range (<100 nm which is usual for such a liposome preparation procedure) with a polydispersity index inferior to 0.3. These results show that every anchor is well tolerated in the liposomal formulation and the diameters are not significantly different from each other and from blank liposomes. In the following experiments, we have selected 5% anchor as a constant concentration to ensure a reliable monitoring if ever a low conversion yield is obtained in one ligation or another.

3.3. Click-like chemistry on preformed liposomes

3.3.1. Control ligation

Before performing the ligation reactions, the biotin and TAMRA probes **6**, **7** and **8** respectively were evaluated for their unspecific affinities with blank liposomes (containing no anchor). The liposomes were prepared following the above-mentioned general procedure. Then biotin and TAMRA probes were added and the suspensions were shaken at room temperature (48 h). After purification of the liposomes by gel filtration on SEPHADEX-100, the unspecific binding of biotin was quantified using the biotin kit and that of TAMRA probe was quantified by fluorescence. For each probe, less that 1% of unspecific adsorption was observed (data not shown).

Table 1
Size of the liposomes (∅ nm) depending on the type of anchors and their concentration (between parentheses: polydispersity index, PDI).

Anchor type				
	2	**3**	**4**	**5**
Anchors quantity (%)				
0	89 (0.25)			
1	100 (0.29)	61 (0.25)	101 (0.27)	115 (0.28)
2.5	43 (0.17)	87 (0.25)	73 (0.21)	71 (0.24)
5	97 (0.30)	84 (0.24)	81 (0.17)	90 (0.27)
10	63 (0.24)	89 (0.22)	86 (0.17)	106 (0.27)

Table 2
Yields (%) of the different ligations at four reaction times (1 h, 6 h, 24 h and 48 h).

	Molecular tool	Conversion[a]			
		1 h	6 h	24 h	48 h
2	**6**	8[a]	14[a]	16[a]	17[a]
3	**6**	9[a]	19[a]	37[a]	46[a]
4	**6**	22[a]	28[a]	35[a]	38[a]
5	**7**	Quant.[a,b]	Quant.[a,b]	Quant.[a,b]	Quant.[a,b]

[a] Yield calculated with the Quant Tag Biotin Kit and on the outer exposed anchors.
[b] Conversion based on the DOG-DBM quantity on the outer exposed anchors.

3.3.2. Azide/alkyne chemistry on liposomes

As there above-mentioned, liposomes were prepared by integrating 5% (mol/mol) of either DOG-COA **2** or DOG-FCOA **3** to get a 10 mM final concentration. The suspension was sonicated to get unilamellar vesicles so that half of the alkyne reactive head functions were exposed on the outer membrane (assuming an equimolecular repartition of the anchor between outer and inner membranes). The final liposomes were obtained by simple stirring of the functionalized liposomes with biotinyl azide **6** (1 eq. toward total **2** and **3**, *i.e.* 2 eq. toward outer exposed **2** and **3**). The incubation mixture was left up 1, 6, 24 and 48 h. Unbound compound **6** was removed by gel filtration on SEPHADEX-100. It was checked by DLS that the size of the SUVs remained unchanged after chemical ligation and after gel filtration purification steps (∅ 70–100 nm). Thus, the coupling reaction and the purification steps do not alter the size of the liposomes. The combined fractions were lyophilized. Then 100 μL of DMSO followed by 200 μL of water were added and stirred to obtain a homogenous solution. Then, the biotin was quantified using the Quant-Tag Biotin kit (Vector Laboratories, Inc., cat. No. BDK-2000) to calculate the ligation yield (Table 2).

3.3.3. Staudinger chemistry on preformed liposomes

The same strategy was used for the Staudinger ligation. The liposomes were prepared by integrating 5% (mol/mol) of DOG-PPh_3 **4** and biotinyl azide **6**. The results are presented in Table 2 over 1, 6, 24 and 48 h respectively.

3.3.4. Dibromomaleimide/thiol ligation on preformed liposomes

For this fourth ligation, the procedure was slightly modified. The liposome formulation was unchanged, but for the ligation step, 1 eq. of **7** was used, leading, after reduction with 1 eq. of TCEP, to 2 eq. of biotin-SH. The incubation mixture was left up 1, 6, 24 and 48 h. Unbound compound **7** was removed by gel filtration on SEPHADEX-100 and then the biotin was quantified after lyophilization and re-solubilization in DMSO/H_2O (100/200 μL) to calculate the ligation yield (Table 2).

First of all, as expected, cyclooctyne anchor **3**, bearing a fluoride, was more electrophilic and more reactive than cyclooctyne anchor **2** and greater conversions were reached (46% versus 17% after 48 h). For the Staudinger reaction, surprisingly, the ligation occurred with only 38% yield that could not be improved. We hypothesize that the phosphine was oxidized rapidly, leading to an inactive phosphorous atom in the liposome suspension buffer (Wang et al., 2012). Next, the ligation with dibromomaleimide was effected with (biotin-S)$_2$ **7**. Reducing agent TCEP was chosen for its solubility in aqueous media, selectivity and inertness toward liposome constituents. In these conditions, double-biotinated liposomes were obtained quantitatively after only 1 h. Finally, to demonstrate the versatility of the construct, we intended to decorate liposomes with two different species without any buffer

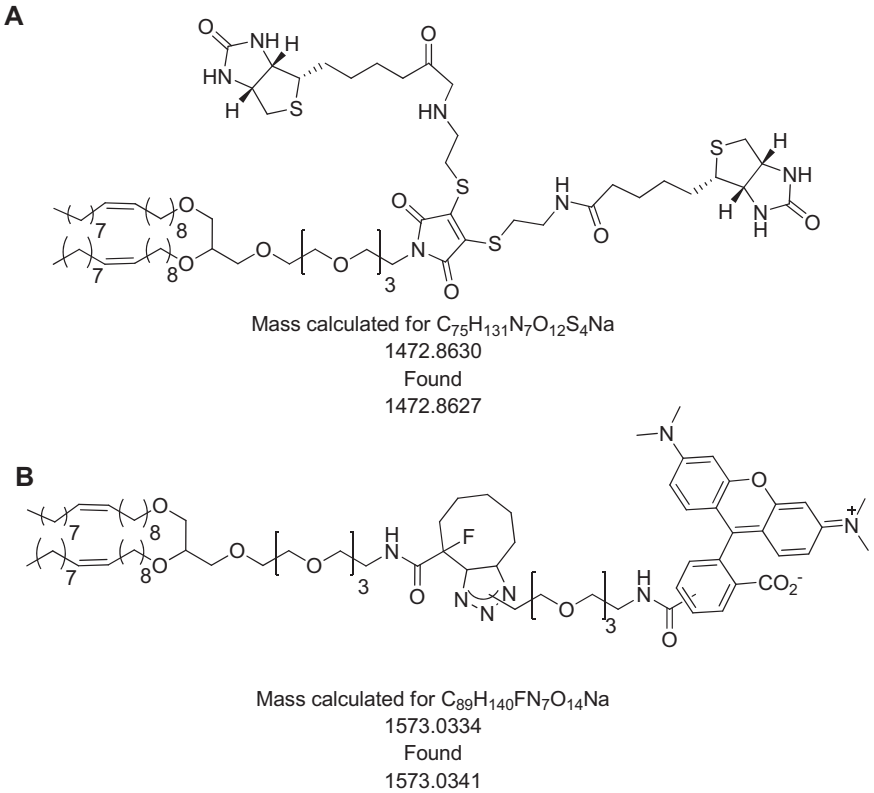


Fig. 3. Bioconjugate compounds found in the DMSO/H_2O solution after destruction of the liposomes.

modification. To this, we selected the two best anchors to set up an orthogonal ligation on liposome.

3.3.5. Double ligation dibromomaleimide/thiol and copper free on the same liposome suspension

For this double ligation, the total anchor's proportion was kept as high as 5%. Thus 2.5% of each anchor were used in the formulation. The couple DOG-FCOA **3** and DOG-DBM **5** was chosen as the best combination for a double ligation without changing buffer. After liposome formation, 1 eq. of TCEP and 1 eq. of **7** were added and left in contact for 1 h to react on **5**. Then, one third (1/3) of the solution was purified on SEPHADEX-100, lyophilized and re-solubilized ($DMSO/H_2O$) for biotin quantification and yield determination.

One equivalent of TAMRA-N_3 **8** was added to the remaining suspension (2/3) and the mixture was shaken for 24 h. After purification on SEPHADEX-100, lyophilization and re-solubilization ($DMSO/H_2O$), the rhodamine was quantified using TAMRA-N_3 **8** solution as standard curve. To strengthen our double ligation, we analyzed by mass spectrometry the resulting liposomes and found evidences (m/z) of both maleimide/biotin conjugate and cyclooctyne/TAMRA conjugate (Fig. 3). Finally, to prove that fluorocyclooctyne function was totally inert toward the biotin **7**, we prepared a liposome suspension bearing only anchor **3**. Then, in place of biotin-N_3 **6**, we added 1 eq. of biotin disulfide **7** and 1 eq. of TCEP. After 24 h, the same work-up was performed. After solubilization in $DMSO/H_2O$ (1/2), the biotin was quantified using the biotin kit and we found that less than 6% of biotin had been non-specifically anchored.

The results of the double ligation show that the first conjugation was obtained with quantitative yield following the above-described quantification procedure. Secondly, the rhodamine quantification demonstrates that the double ligation was effective too. The coupled compound was obtained with yields comparable to those observed with the biotin tool (Table 2). These results demonstrate that a double ligation is possible without changing the buffer and bring up a very simple method to difunctionalize liposomes in a 'one pot' sequential matter. Finally, we proved the total stability of the cyclooctyne toward thiol derivatives. Such additional methodology reinforces the versatility of the liposome tool, especially when some biomolecules to be anchored to these vesicles react very differently and therefore when the use of a statistical mixture is impossible.

4. Conclusion

We have successfully designed and prepared four innovative anchors and integrated them into liposome bilayers. All ligations performed on these functionalized anchors were effective. We also demonstrated that for all four anchors, the conjugated species are at the surface and still accessible to biomolecules (possibly: antibodies, proteins, enzymes...). The best result was unprecedentedly observed with the dibromomaleimide. This ligation is potentially interesting for peptides bearing disulfide bridges and allows the incorporation of two thiol derivatives for one anchor leading to a high atom economy. Another important advantage would be the possible preservation of the cyclic conformation of peptides or the possibility to get loops. Furthermore, we demonstrated that the cyclooctyne derivatives are inert toward thiols. This last observation allowed us to perform a one pot double ligation onto liposomes by using two anchors of the type cyclooctyne and dibromomaleimide without any cross-coupling reaction. This technics could be of great interest for the incorporation of epitopes or immunoadjuvants on lipid particles as ingredients for synthetic vaccines. More widely, those methodologies can be applied to peptide, protein and carbohydrate ligation onto liposomes. These results implement the amount of bioorthogonal chemical bonds available for liposome chemical modification.

Conflict of interest

The authors report no competing financial interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chemphyslip.2015.03.004.

References

Airoldi, C., Mourtas, S., Cardona, F., Zona, C., Sironi, E., D'Orazio, G., Markoutsa, E., Nicotra, F., Antimisiaris, S.G., La Ferla, B., 2014. Nanoliposomes presenting on surface a cis-glycofused benzopyran compound display binding affinity and aggregation inhibition ability towards amyloid β1-42 peptide. Eur. J. Med. Chem. 85, 43–50.

Bartsch, R.A., Cason, C.V., Czech, B.P., 1989. Phase-transfer-catalyzed synthesis of oligoethylene glycols and derivatives. J. Org. Chem. 54, 857–860.

Beal, D.M., Jones, L.H., 2012. Molecular scaffolds using multiple orthogonal conjugations: applications in chemical biology and drug discovery. Angew. Chem. Int. Ed. 51, 6320–6326.

Bernardin, A., Cazet, A., Guyon, L., Delannoy, P., Vinet, F., Bonnaffe', D., Texier, I., 2010. Copper-free click chemistry for highly luminescent quantum dot conjugates: application to in vivo metabolic imaging. Bioconjugate Chem. 21, 583–588.

Boeckler, C., Frisch, B., Schuber, F., 1998. Design and synthesis of thiol-reactive lipopeptides. Bioorg. Med. Chem. Lett. 8, 2055–2058.

Borcard, F.O., Godinat, A., Staedler, D., Comas Blanco, H., Dumont, A.-L., Chapuis-Bernasconi, C., Scaletta, C., Applegate, L.A., Krauss Juillerat, F., Gonzenbach, U.T., Gerber-Lemaire, S., Juillerat-Jeanneret, L., 2011. Covalent cell surface functionalization of human fetal osteoblasts for tissue engineering. Bioconjugate Chem. 22, 1422–1432.

Bostic, H.E., Smith, M.D., Poloukhtine, A.A., Popik, V.V., Best, M.D., 2012. Membrane labeling and immobilization via copper-free click chemistry. Chem. Commun. 48, 1431–1433.

Bourel-Bonnet, L., Pécheur, E.-I., Grandjean, C., Blanpain, A., Baust, T., Melnyk, O., Hoflack, B., Gras-Masse, H., 2005. Anchorage of synthetic peptides onto liposomes via hydrazone and alpha-oxo hydrazone bonds. Preliminary functional investigations. Bioconjugate Chem. 16, 450–457.

Boyce, M., Bertozzi, C.R., 2011. Bringing chemistry to life. Nat. Methods 8, 638–642.

Organic Azides: Syntheses and Applications. In: Brase, S., Barnert, K. (Eds.), Wiley, Chichester, UK.

Cavalli, S., Tipton, R., Overhand, A., Kros, A., 2006. The chemical modifications of liposomes surfaces via a copper-mediated [3 + 2] azide–alkyne cycloaddition monitored by a colorimetric assay. Chem. Commun. 3193–3195.

Chenevier, P., Grandjean, C., Loing, E., Malingue, F., Angyalosi, G., Gras-Masse, H., Roux, D., Melnyk, O., Bourel-Bonnet, L., 2002. Grafting of synthetic mannose receptor-ligands onto onion vectors for human dendritic cells targeting. Chem. Commun. 2446–2447.

Colombo, M., Sommaruga, S., Mazzucchelli, S., Polito, L., Verderio, P., Galeffi, P., Corsi, F., Tortora, P., Prosperi, D., 2012. Site-specific conjugation of ScFvs antibodies to nanoparticles by bioorthogonal strain-promoted alkyne–nitrone cycloaddition. Angew. Chem. Int. Ed. 51, 496–499.

Elliott, J.T., Prestwich, G.D., 2000. Maleimide-functionalized lipids that anchor polypeptides to lipid bilayers and membranes. Bioconjugate Chem. 11, 832–841.

Emmetiere, F., Irwin, C., Viola-Villegas, N.T., Longo, V., Cheal, S.M., Zanzonico, P., Pillarsetty, N., Weber, W.A., Lewis, J.S., Reiner, T., 2013. ^{18}F-labeled-bioorthogonal liposomes for in vivo targeting. Bioconjugate Chem. 24, 1784–1789.

Epand, R.M., 1997. Biophysical studies of lipopeptide-membrane interactions. Pept. Sci. 43, 15–24.

Espuelas, S., Haller, P., Schuber, F., Frisch, B., 2003. Synthesis of an amphiphilic tetraantennary mannosyl conjugate and incorporation into liposome carriers. Bioorg. Med. Chem. Lett. 13, 2557–2560.
Fleiner, M., Benzinger, P., Fichert, T., Massing, U., 2001. Studies on protein–liposome coupling using novel thiol-reactive coupling lipids: influence of spacer length and polarity. Bioconjugate Chem. 12, 470–475.
Frisch, B., Hassane, F.S., Schuber, F., 2010. Conjugation of Ligands to the Surface of Preformed Liposomes by Click Chemistry, in Liposomes. In: Weissig, V. (Ed.), Humana Press, pp. 267–277.
Gold, B., Dudley, G.B., Alabugin, I.V., 2013. Moderating strain without sacrificing reactivity: design of fast and tunable noncatalyzed alkyne–azide cycloadditions via stereoelectronically controlled transition state stabilization. J. Am. Chem. Soc. 135, 1558–1569.
Harding, J.A., Engbers, C.M., Newman, M.S., Goldstein, N.I., Zalipsky, S., 1997. Immunogenicity and pharmacokinetic attributes of poly(ethylene glycol)-grafted immunoliposomes. Biochim. Biophys. Acta – Biomembr. 1327, 181–192.
Heeremans, J.L.M., Kraaijenga, J.J., Los, P., Kluft, C., Crommelin, D.J.A., 1992. Development of a procedure for coupling the homing device glu-plasminogen to liposomes. Biochim. Biophys. Acta – Gen. Subj. 1117, 258–264.
Hein, C., Liu, X.-M., Wang, D., 2008. Click chemistry, a powerful tool for pharmaceutical sciences. Pharm. Res. 25, 2216–2230.
Hojo, H., Kojima, T., Yamauchi, K., Kinoshita, M., 1996. Synthesis and liposome-formation of a thermostable lipid bearing cell adhesion peptide sequence. Tetrahedron Lett. 37, 7391–7394.
Horak, D., Karpísek, M., Turkova, J., Benes, M., 1999. Hydrazide-functionalized poly (2-hydroxyethyl methacrylate) microspheres for immobilization of horseradish peroxidase. Biotechnol. Prog. 15, 208–215.
Jewett, J.C., Bertozzi, C.R., 2010. Cu-free click cycloaddition reactions in chemical biology. Chem. Soc. Rev. 39, 1272–1279.
Jolck, R.I., Sun, H., Berg, R.H., Andresen, T.L., 2011. Catalyst-free conjugation and in situ quantification of nanoparticle ligand surface density using fluorogenic Cu-free click chemistry. Chem. Eur. J. 17, 3326–3331.
Jolimaitre, P., Poirier, C., Richard, A., Blanpain, A., Delord, B., Roux, D., Bourel-Bonnet, L., 2007. Synthesis of versatile chemical tools toward a structure/properties relationships study onto targeting colloids. Eur. J. Med. Chem. 42, 114–124.
Jones, M.W., Strickland, R.A., Schumacher, F.F., Caddick, S., Baker, J.R., Gibson, M.I., Haddleton, D.M., 2012. Polymeric dibromomaleimides as extremely efficient disulfide bridging bioconjugation and pegylation agent. J. Am. Chem. Soc. 134, 1847–1852.
Kamps, J.A.A.M., Swart, P.J., Morselt, H.T.W.M., Pauwels, R., De Bethune, M.-P., De Clercq, E., Meijer, D.K.F., Scherphof, G.L., 1996. Preparation and characterization of conjugates of (modified) human serum albumin and liposomes: drug carriers with an intrinsic anti-HIV activity. Biochim. Biophys. Acta – Biomembr. 1278, 183–190.
Kolb, H.C., Finn, M.G., Sharpless, K.B., 2001. Click chemistry: diverse chemical function from a few good reactions. Angew. Chem. Int. Ed. 40, 2004–2021.
Koo, H., Lee, S., Na, J.H., Kim, S.H., Hahn, S.K., Choi, K., Kwon, I.C., Jeong, S.Y., Kim, K., 2012. Bioorthogonal copper-free click chemistry in vivo for tumor-targeted delivery of nanoparticles. Angew. Chem. Int. Ed. 51, 11836–11840.
Kottani, R., Valiulin, R.A., Kutateladze, A.G., 2006. Direct screening of solution phase combinatorial libraries encoded with externally sensitized photolabile tags. Proc. Natl. Acad. Sci. U. S. A. 103, 13917–13921.
Kung, V.T., Redemann, C.T., 1986. Synthesis of carboxyacyl derivatives of phosphatidylethanolamine and use as an efficient method for conjugation of protein to liposomes. Biochim. Biophys. Acta – Biomembr. 862, 435–439.
Martin, F.J., Papahadjopoulos, D., 1982. Irreversible coupling of immunoglobulin fragments to preformed vesicles. An improved method for liposome targeting. J. Biol. Chem. 257, 286–288.
Meldal, M., Torne, C.W., 2008. Cu-catalyzed azide-alkyne cycloaddition. Chem. Rev. 108, 2952–3015.
Mourtas, S., Canovi, M., Zona, C., Aurilia, D., Niarakis, A., La Ferla, B., Salmona, M., Nicotra, F., Gobbi, M., Antimisiaris, S.G., 2011. Curcumin-decorated nanoliposomes with very high affinity for amyloid-β1-42 peptide. Biomaterials 32, 1635–1645.
Nakano, Y., Mori, M., Nishinohara, S., Takita, Y., Naito, S., Kato, H., Taneichi, M., Komuro, K., Uchida, T., 2001. Surface-linked liposomal antigen induces IgE-selective unresponsiveness regardless of the lipid components of liposomes. Bioconjugate Chem. 12, 391–395.
Rostovtsev, V.V., Green, L.G., Fokin, V.V., Sharpless, K.B., 2002. A stepwise Huisgen cycloaddition process: copper(I)-catalyzed regioselective ligation of azides and terminal alkynes. Angew. Chem. Int. Ed. 41, 2596–2599.
Roth, A., Espuelas, S., Thumann, C., Frisch, B., Schuber, F., 2004. Synthesis of thiol-reactive lipopeptide adjuvants. Incorporation into liposomes and study of their mitogenic effect on mouse splenocytes. Bioconjugate Chem. 15, 541–553 Erratum in: (2005) Bioconjugate Chem. 16, 1049.
Said Hassane, F., Frisch, B., Schuber, F., 2006. Targeted liposomes: convenient coupling of ligands to preformed vesicles using click chemistry. Bioconjugate Chem. 17, 849–854.
Sankaram, M.B., 1994. Membrane interaction of small *N*-myristoylated peptides: implications for membrane anchoring and protein–protein association. Biophys. J. 67, 105–112.
Schelté, P., Boeckler, C., Frisch, B., Schuber, F., 2000. Differential reactivity of maleimide and bromoacetyl functions with thiols: application to the preparation of liposomal diepitope constructs. Bioconjugate Chem. 11, 118–123.
Schultz, M.K., Parameswarappa, S.G., Pigge, F.C., 2010. Synthesis of a DOTA-biotin conjugate for radionuclide chelation via Cu-free click chemistry. Org. Lett. 12, 2398–2401.
Schumacher, F.F., Nobles, M., Ryan, C.P., Smith, M.E.B., Tinker, A., Caddick, S., Baker, J. R., 2011. In situ maleimide bridging of disulfides and a new approach to protein PEGylation. Bioconjugate Chem. 22, 132–136.
Shahinian, S., Silvius, J.R., 1995. Doubly-lipid-modified protein sequence motifs exhibit long-lived anchorage to lipid bilayer membranes. Biochemistry 34, 3813–3822.
Shelbourne, M., Brown, T., El-Sagheer, A.H., Brown, T., 2012. Fast and efficient DNA crosslinking and multiple orthogonal labelling by copper-free click chemistry. Chem. Commun. 48, 11184–11186.
Silvius, J.R., l'Heureux, F., 1994. Fluorometric evaluation of the affinities of isoprenylated peptides for lipid bilayers. Biochemistry 33, 3014–3022.
Sletten, E.M., Bertozzi, C.R., 2009. Bioorthogonal chemistry: fishing for selectivity in a sea of functionality. Angew. Chem. Int. Ed. 48, 6974–6998.
Smith, M.E.B., Schumacher, F.F., Ryan, C.P., Tedaldi, L.M., Papaioannou, D., Waksman, G., Caddick, S., Baker, J.R., 2010. Protein modification, bioconjugation, and disulfide bridging using bromomaleimides. J. Am. Chem. Soc. 132, 1960–1965.
Speers, A.E., Adam, G.C., Cravatt, B.F., 2003. Activity-based protein profiling in vivo using a copper(I)-catalyzed azide–alkyne [3 + 2] cycloaddition. J. Am. Chem. Soc. 125, 4686–4687.
Susumu, K., Uyeda, H.T., Medintz, I.L., Pons, T., Delehanty, J.B., Mattoussi, H., 2007. Enhancing the stability and biological functionalities of quantum dots via compact multifunctional ligands. J. Am. Chem. Soc. 129, 13987–13996.
Tarallo, R., Accardo, A., Falanga, A., Guarnieri, D., Vitiello, G., Netti, P., D'Errico, G., Morelli, G., Galdiero, S., 2011. Clickable functionalization of liposomes with the gH625 peptide from herpes simplex virus type I for intracellular drug delivery. Chem. Eur. J. 17, 12659–12668.
Torne, C.W., Christensen, C., Meldal, M., 2002. Peptidotriazoles on solid phase: [1,2,3]-triazoles by regiospecific copper(I)-catalyzed 1,3-dipolar cycloadditions of terminal alkynes to azides. J. Org. Chem. 67, 3057–3064.
Vabbilisettya, P., Sun, X.L., 2014. Liposome surface functionalization based on different anchoring lipids via Staudinger ligation. Org. Biomol. Chem. 12, 1237–1244.
Wang, T.-P., Ko, N.C., Su, Y.-C., Wang, E.-C., Severance, S., Hwang, C.-C., Shih, Y.T., Wu, M.H., Chen, Y.-H., 2012. Advanced aqueous-phase phosphoramidation reactions for effectively synthesizing peptide-oligonucleotide conjugates trafficked into a human cell line. Bioconjugate Chem. 23, 2417–2433.
Wilson, R.M., Thalji, R.K., Bergman, R.G., Ellman, J.A., 2006. Enantioselective synthesis of a PKC inhibitor via catalytic CH bond activation. Org. Lett. 8, 1745–1747.
Wu, H., Xu, B., Basuli, F., Li, C., Lane, K., Sulima, A., Griffiths, G., 2012. Preparation of an [^{18}F]liposome using a novel 'click' chemistry approach. J. Nucl. Med. 53 (suppl. 1), 1537.
Zalipsky, S., 1993. Synthesis of an end-group functionalized polyethylene glycol-lipid conjugate for preparation of polymer-grafted liposomes. Bioconjugate Chem. 4, 296–299.
Zalipsky, S., Puntambekar, B., Boulikas, P., Engbers, C.M., Woodle, M.C., 1995. Peptide attachment to extremities of liposomal surface grafted PEG chains: preparation of the long-circulating form of laminin pentapeptide, YIGSR. Bioconjugate Chem. 6, 705–708.
Zhang, H., Ma, Y., Sun, X.-L., 2009. Chemically-selective surface glyco-functionalization of liposomes through Staudinger ligation. Chem. Commun. 3032–3034.