In situ synthesis of fluorescent membrane lipids (ceramides) using click chemistry

María Garrido · José Luis Abad · Alicia Alonso ·
Félix M. Goñi · Antonio Delgado · L.-Ruth Montes

Received: 4 January 2012 / Accepted: 3 April 2012 / Published online: 18 April 2012
© Springer-Verlag 2012

Abstract Ceramide analogues containing azide groups either in the polar head or in the hydrocarbon chains are non-fluorescent. When incorporated into phospholipid bilayers, they can react in situ with a non-fluorescent 1,8-naphthalimide using click chemistry giving rise to fluorescent ceramide derivatives emitting at ≈ 440 nm. When incorporated into giant unilamellar vesicles, two-photon excitation at 760 nm allows visualization of the ceramide-containing bilayers. This kind of method may be of general applicability in the study of model and cell membranes.

Keywords Ceramide · GUV · Fluorescence · Click chemistry

Electronic supplementary material The online version of this article (doi:10.1007/s12154-012-0075-0) contains supplementary material, which is available to authorized users.

M. Garrido · J. L. Abad · A. Delgado
Consejo Superior de Investigaciones Científicas (CSIC),
Research Unit on Bioactive Molecules (RUBAM),
Institut de Química Avançada de Catalunya (IQAC-CSIC),
Jordi Girona 18-26,
08034 Barcelona, Spain

A. Alonso · F. M. Goñi · L.-R. Montes (✉)
Unidad de Biofísica (CSIC-UPV/EHU),
and Departamento de Bioquímica, Universidad del País Vasco,
Aptdo. 688,
48080 Bilbao, Spain
e-mail: lidiaruth.montes@ehu.es

A. Delgado
Facultad de Farmacia, Unidad de Química Farmacéutica
(Unidad Asociada al CSIC), Universidad de Barcelona (UB),
Avda. Joan XXIII s/n,
08028 Barcelona, Spain

Abbreviations

DMSO	Dimethylsulfoxide
NMR	Nuclear magnetic resonance
HRMS	High-resolution mass spectrometry
ePC	Egg phosphatidylcholine

Introduction

The use of specifically designed lipid probes for biophysical applications is a well-recognized strategy [1]. In particular, the use of fluorophore tags is a common strategy for the visualization of the membrane architecture and the study of its dynamic properties. However, the incorporation of a generally bulky fluorescent tag as part of a relatively small lipid molecule can exert a dramatic effect on the properties of the resulting probe, especially if trafficking, sorting and/or domain formation are investigated [2]. In order to avoid these drawbacks, the ideal probe should be structurally similar to its natural counterpart. In this context, incorporation of a small functional group amenable to further chemoselective functionalization may allow the synthesis of a fluorescent probe in a natural environment. These requirements can be fulfilled by adaptation of the so-called bioorthogonal chemical reporter strategy, a technique that has become very popular for the labelling of biomolecules [3, 4]. Among the different approaches described so far, the Huisgen copper(I)-catalysed alkyne–azide [3+2] cycloaddition (CuAAC) to afford 1,2,3-triazoles has rapidly evolved as a paradigm of what is known as “click chemistry”. This strategy relies on the use of very selective transformations that are usually free of background reactions [5]. Adaptations of this strategy to the design of “click-on” fluorogenic dyes have been recently reported [6, 7]. The method relies on the use of suitable azides or alkynes with no or low baseline fluorescence, which, after the click

reaction, affords an extended conjugated system with increased fluorescence (Fig. 1a).

According to Baskin and Bertozzi [4], a good click reaction should satisfy several criteria: it should be high yielding, produce minimal by-products and be stereospecific when applicable. Further, it should involve readily available starting materials, take place in benign solvents (ideally water) or under solvent-free conditions, and allow simple isolation of products. In practice, click reactions tend to have large negative free energies and hence involve carbon–heteroatom bond-forming processes. Thus, unlike many conventional synthetic reactions, the power of click chemistry lies in its simplicity and ease of use. In just a few years, click chemistry has proven itself in the arenas of small molecule library synthesis [8], supramolecular materials construction [9] and selective biomolecule conjugation [10].

In this communication we have made use of click chemistry for the synthesis of fluorescent ceramide analogues in a lipid membrane environment. The fluorescent analogues are synthesized from non-fluorescent azide-tagged ceramides (**1** and **2**) and a non-fluorescent naphthalimide (**F**; Fig. 1b) [11]. A related procedure has been applied to the in situ synthesis of fluorescent glycerol-based phospholipids [12]. In our case the molecules have been designed to mimic the behaviour of natural ceramides in model and cell membranes. Ceramides are important metabolic signals [13, 14], and they are known to separate laterally and give rise to gel-like ceramide-enriched domains [15–18]. Because of their structural similarity, compounds **1** and **2** were expected to orient in lipid bilayers in parallel with the phospholipids, and eventually to give rise to domains similarly to natural ceramides. Our in situ synthetic

method would allow the observation of ceramide domains in living cells. Very recently [19] an azide–alkyne click chemistry approach has been used to assemble phospholipid chains in a biomimetic coupling reaction leading to artificial membranes containing a triazole ring. Although conceptually related, in our case, the click chemistry reaction is not essential for membrane formation but only for ceramide visualization in pre-existing bilayers.

Experimental

Synthesis of probes **1** and **2**

The required probes **1** and **2** were prepared by acylation of the corresponding ω -azidoaminodiol **6** or β -azidoaminoalcohol **7**, respectively (Fig. 2), following standard protocols. These precursors were, in turn, prepared by a multistep synthesis from a common allylic alcohol **3** [20] (Fig. 2). Thus, compound **6** required metathesis of **3** with 11-bromo-1-undecene, bromide displacement with sodium azide and simultaneous isopropylidene and *N*-Boc deprotection under acidic conditions. On the other hand, metathesis of **3** with 1-pentadecene, followed by selective *N*, *O*-isopropylidene removal, mesylation of the primary alcohol, azide displacement and final *N*-Boc deprotection afforded precursor **7**. Methathesis reactions afforded cleanly the corresponding *trans* adducts **4** and **5**, and no trace of the corresponding *cis* isomers was detected. All the above intermediates showed spectroscopic and analytical data consistent with the expected structures. A full account of the synthesis of these and related azido-substituted sphingoid probes will be reported elsewhere.

Fig. 1 **a** Example of a “click-on” fluorogenic reaction leading to a fluorescent lipid probe. **b** Lipid probes (**1** and **2**), “click-on” dye adducts **1F** and **2F** and fluorogenic naphthalimide **F** used in this study

Fig. 2 Starting allylic alcohol (**3**) and key intermediates (**4–7**) for the synthesis of probes **1** and **2**

(2S,3R,E)-N-Palmitoyl-2-amino-14-azido-4-tetradecen-1,3-diol (1)

Thirty-five percent yield as a white solid; 1H nuclear magnetic resonance (NMR) (δ , 500 MHz, $CDCl_3$): 6.26 (d, $J=7.0$ Hz, 1 H, NH amide), 5.78 (dt, $J_1=15.5$ Hz, $J_2=7.0$ Hz, 1 H, C5H), 5.53 (dd, $J_1=15.5$ Hz, $J_2=6.5$ Hz, 1 H, C4H), 4.32 (t, $J=4.5$ Hz, 1 H, C3H), 3.95 (dd, $J_1=11.5$ Hz, $J_2=4.0$ Hz, 1 H, C1Ha), 3.91 (dq, apparent $J=3.5$ Hz, 1 H, C2H), 3.70 (dd, $J_1=11.5$ Hz, $J_2=3.0$ Hz, 1 H, C1Hb), 3.25 (t, $J=7.0$ Hz, 2 H, C14H2), 2.75 (b.s., 1 H, OH), 2.23 (t, $J=8.0$ Hz, 2 H, C1'H2), 2.07 (dt, apparent $J=7.5$ Hz, 2 H, C6H2), 1.69–1.55 (m, 4 H, C13H2+C2'H2), 1.41–1.22 (m, 36 H), 0.88 (t, $J=7.0$ Hz, 3 H, CH3); ^{13}C NMR (δ , 101 MHz, $CDCl_3$): 174.0 (C=O), 134.4 (C5), 129.1 (C4), 74.9 (C3), 62.7 (C1), 54.7 (C2), 51.7 (C14), 37.0 (C1'), 32.4 (C6), 32.1, 29.9–29.0 (16 carbons), 26.9, 25.9 (C2'), 22.9, 14.3 (CH3). High-resolution mass spectrometry (HRMS) calculated for $C_{30}H_{58}N_4NaO_3$: 545.4407 $[M+Na]^+$. Found: 545.4415.

(2S,3R,E)-N-Palmitoyl-2-amino-1-azido-4-octadecen-3-ol (2)

Eighty-two percent yield; 1H NMR (δ , 400 MHz, $CDCl_3$): 5.83 (d, $J=8.5$ Hz, 1 H, NH amide), 5.76 (dt, $J_1=15.0$ Hz, $J_2=7.0$ Hz, 1 H, C5H), 5.45 (dd, $J_1=15.5$ Hz, $J_2=6.5$ Hz, 1 H, C4H), 4.18 (t, $J=6.0$ Hz, 1 H, C3H), 4.06 (dq, apparent $J=4.5$ Hz, 1 H, C2H), 3.63 (dd, $J_1=13.0$ Hz, $J_2=5.0$ Hz, 1 H, C1Ha), 3.52 (dd, $J_1=12.5$ Hz, $J_2=4.0$ Hz, 1 H, C1Hb), 2.47 (b.s., 1 H, OH), 2.23–2.17 (m, 2 H, C1'H2), 2.07 (m, 2 H, C6H2), 1.67–1.57 (m, 2 H, C2'H2), 1.40–1.19 (m, 46 H), 0.88 (t, $J=7.0$ Hz, 6 H, $2\times CH_3$); ^{13}C NMR (δ , 101 MHz, $CDCl_3$): 173.6 (C=O), 135.1 (C5), 128.6 (C4), 73.5 (C3), 53.0 (C2), 51.0 (C1), 37.0 (C1'), 32.4 (C6), 32.1, 29.9–29.2 (21 carbons), 25.8 (C2'), 22.8, 14.3 ($2\times CH_3$). HRMS calculated for $C_{34}H_{66}N_4NaO_2$: 585.5083 $[M+Na]^+$. Found: 585.5086.

Liposome preparation

For fluorescence spectroscopy, multilamellar vesicles (MLV) were used. The sample was hydrated in 50 mM HEPES, pH 7.4 at 65 °C, with stirring. In order to ensure homogeneous dispersion, the hydrated samples were extruded between two syringes through a narrow tubing (0.5 mm internal diameter, 10 cm long) 100 times at 65 °C. Final

lipid concentration of egg phosphatidylcholine (ePC):**1** (10 mol%) and ePC:**2** (10 mol%) was 0.3 mM. Phospholipid concentration was measured as lipid phosphorus.

Giant unilamellar vesicles (GUVs) were prepared following the electroformation method described previously [21, 22], using a home-made chamber that allows direct visualization under the microscope. Stock solutions of lipids (0.2 mg/ml total lipid) were prepared in a chloroform to methanol (2:1, v/v) solution. Three microlitres of the appropriate stocks was added onto the surface of platinum electrodes, and solvent traces were removed under vacuum for 2 h. Electroformation was performed in 400 μ l of 50 mM HEPES pH 7.4, preheated to 65 °C, using an electric wave generator (TG330 function generator; Thurlby Thandar Instruments, Huntingdon, UK). The application of the AC field followed three increasing amplitude steps, all performed at 65 °C and at a frequency of 500 Hz: (1) 220 mV for 5 min, (2) 1.9 V for 20 min and (3) 5.3 V for 90 min. The electroformation chamber was allowed to cool down for 30 min before treating the GUVs with 100 μ l of click labelling solution.

Click reaction

In all experiments, liposomes were treated with click labelling solution at a final concentration of 0.1 mM $CuSO_4$, 0.5 mM L-ascorbic acid, 0.1 mM tris(triazolyl)amine ligand [100 mM stock in dimethylsulfoxide (DMSO)] and 0.05 mM naphthalimide ligation probe F (50 mM stock in DMSO; final total concentration of DMSO 0.2 %). Samples were incubated in the dark for 1.5 h at room temperature to allow cycloaddition reaction.

Fluorescence spectroscopy

Fluorescence measurements were performed in a Jobin Yvon Fluoromax-3 spectrofluorometer. CuAAC reaction was monitored as an increase in the fluorescence intensity of emission spectra when exciting the sample at 368 nm. Excitation spectra were obtained when emitted fluorescence was collected at 420 nm.

Confocal fluorescence

Giant vesicles were visualized in an inverted confocal fluorescence microscope with a high-efficiency spectral detector (Leica TCS SP5; Leica Microsystems CMS GmbH, Mannheim, Germany). Two-photon excitation mode was used at 760 nm (MaiTai HP DS laser; Spectra Physics, Mountain View, CA), and the fluorescence signal was collected in the 450–500-nm channel. The experiments were performed using a 62 $\times$ water immersion, NA 1.2 objective. Images were collected and analysed with the LAS AF software (Leica Microsystems).

Results and discussion

We have characterized ceramide analogues that contain an azido functionality, incorporated into MLV. These molecules can be used as fluorogenic membrane probes for in situ “clicked on” assays within a lipidic environment. For this purpose we have synthesized and tested two analogues that contain the azido functionalization in different positions and explored whether this characteristic interferes with the reactivity of the molecules, facilitating or preventing the cycloaddition reaction. In **1**, carbons C15–C18 from the sphingoid tail were substituted with the N3 group, which will remain buried in the hydrophobic interphase of a lipidic membrane. For **2** we added the azide tag to the polar head of the ceramide through the OH group, inducing it to appear positioned towards the aqueous solvent. The CuAAC reaction between naphthalimide **F** and its coupling azide-modified partners **1** and **2** requires the presence of ascorbic acid and a 1:1 M ratio complex of CuSO_4 –tris(triazolyl) amine. In both cases a highly fluorescent signal was observed within 1.5 h incubation at room temperature, meaning that both azides are accessible to the click labelling reaction solution.

Fluorescent properties of ceramide analogues and the products of the ligation reaction **1F** and **2F** were tested by fluorescence spectroscopy. Excitation spectra (Fig. 3, lines EX) showed a maximum at 368 nm for both probes. Thus, the samples were excited at 368 nm, and 375–600 nm emission spectra were collected (Fig. 3, lines EM). Control MLV composed of ePC+10 mol% of either **1** (A) or **2** (B) were incubated for 1.5 h in the absence of click labelling solution or with a solution lacking CuSO_4 (Fig. 3, dashed lines). Control experiments revealed an observable emission maximum at 400 nm. However when equivalent samples

Fig. 3 Fluorescence spectra of MLV composed of ePC and 10 mol% of either **1** (A) or **2** (B) in the presence (solid lines) or absence (dashed lines) of CuSO_4 . Naphthalimide **F** was present in both cases. EX—300–420 nm excitation spectra collected at emission wavelength 420 nm. EM—375–600 nm emission spectra exciting at 368 nm. Observed fluorescence intensities are due to naphthalimide **1** (control without CuSO_4 , dashed lines) and its click adducts **1F** and **2F** (solid lines)

were incubated with the complete labelling solution for 1.5 h (Fig. 3, solid lines), the emission maximum shifted to ≈ 440 nm and the emission intensity increased greatly.

With the purpose of visualizing “in situ” the photoactivation of our newly synthesized probes, we performed microscopy experiments using GUVs of ePC:1 and ePC:2 (10 mol% of clickable probe in both cases). The utilized electroformation chamber allows real-time direct visualization of the reaction. When GUVs were treated with the complete labelling solution, a clear fluorescence intensity was collected in both cases between 450 and 500 nm, which was attributed to the formation of **1F** and **2F** species in the membrane (Fig. 4, +Cu). To diminish photobleaching of the fluorophore, the sample was excited in the two-photon mode via an infrared laser set at 760 nm. This allowed us to greatly overcome the photobleaching phenomenon, thus obtaining highly fluorescent images of the membranes. The excitation wavelength was shifted to 760 nm in order to avoid contribution of non-clicked naphthalimide **1** emitted fluorescence. To confirm that the imaging data were a consequence of click activation, GUV were also incubated under control conditions (absence of click labelling solution or with a solution that lacks CuSO_4), where no fluorescence signal could be observed even after 3 h incubation (Fig. 4, –Cu).

Thus, our results constitute a proof of principle that fluorescent ceramide derivatives may be formed within lipid membranes starting from non-fluorescent reagents, one of them a ceramide analogue differing only by an azide tag from the native molecule. This technique offers a new approach to the studies of ceramide-enriched domain

Fig. 4 Fluorescent images (false colour representation) of GUVs composed of ePC+10 mol% of either **1** or **2** in the presence of click labelling solution with (+Cu) or without (–Cu) CuSO_4

formation based on fluorescent confocal microscopy [16, 23–26] and may provide new insights into currently debated problems, e.g. the proposed displacement of cholesterol from sphingomyelin by ceramide [24, 27, 28]. The procedure described in this paper should also be applicable to the study of the so-called ceramide platforms [29] and other phenomena at the cell level. Even if Cu^{2+} at the concentrations used in this paper may be toxic to cells, localization of ceramide-rich domains in cell membranes could be actually performed on fixed cell preparations.

The Baskin–Bertozzi criteria for a good click reaction were mentioned in the “Introduction” section. As discussed in the above paragraphs, in our case, the reaction fulfils virtually all the criteria mentioned. In addition, it proceeds in water at neutral or near neutral pH at room temperature. Our demonstration of in situ synthesis of ceramide fluorescent analogues may open the way to novel applications of click chemistry in the field of biomembranes.

Acknowledgments This work was supported in part by Spanish Ministerio de Ciencia e Innovación grants BFU2011-28566 (AA), SAF 2011-22444 (JLA/AD) and BFU 2007-62062 (FMG), and by Generalitat de Catalunya grant SGR 2009-1072 (JLA/AD). LRM is grateful to Consejo Superior de Investigaciones Científicas for a JAEdoc fellowship and MG for a JAEpredoc fellowship.

References

- Loura LM, Ramalho JP (2011) Recent developments in molecular dynamics simulations of fluorescent membrane probes. *Molecules* 16(7):5437–5452
- Maier O, Oberle V, Hoekstra D (2002) Fluorescent lipid probes: some properties and applications (a review). *Chem Phys Lipids* 116(1–2):3–18
- Lim RK, Lin Q (2010) Bioorthogonal chemistry: recent progress and future directions. *Chem Commun (Camb)* 46(10):1589–1600
- Baskin JM, Bertozzi CR (2007) Bioorthogonal click chemistry: covalent labeling in living systems. *QSAR Comb Sci* 26:1211–1219
- Sletten EM, Bertozzi CR (2009) Bioorthogonal chemistry: fishing for selectivity in a sea of functionality. *Angew Chem Int Ed Engl* 48(38):6974–6998
- Qi J, Han MS, Chang YC, Tung CH (2011) Developing visible fluorogenic ‘click-on’ dyes for cellular imaging. *Bioconjug Chem* 22(9):1758–1762
- Qi J, Tung CH (2011) Development of benzothiazole ‘click-on’ fluorogenic dyes. *Bioorg Med Chem Lett* 21(1):320–323
- Kolb HC, Sharpless KB (2003) The growing impact of click chemistry on drug discovery. *Drug Discov Today* 8(24):1128–1137
- Lutz JF (2007) 1,3-Dipolar cycloadditions of azides and alkynes: a universal ligation tool in polymer and materials science. *Angew Chem Int Ed Engl* 46(7):1018–1025
- Wu P, Fokin VV (2007) Catalytic azide–alkyne cycloadditions: reactivity and applications. *Aldrichimica Acta* 40(1):7–17
- Sawa M, Hsu TL, Itoh T, Sugiyama M, Hanson SR, Vogt PK, Wong CH (2006) Glycoproteomic probes for fluorescent imaging of fucosylated glycans in vivo. *Proc Natl Acad Sci U S A* 103(33):12371–12376
- Neef AB, Schultz C (2009) Selective fluorescence labeling of lipids in living cells. *Angew Chem* 48(8):1498–1500
- Kolesnick RN, Goni FM, Alonso A (2000) Compartmentalization of ceramide signaling: physical foundations and biological effects. *J Cell Physiol* 184(3):285–300
- Zeidan YH, Hannun YA (2010) The acid sphingomyelinase/ceramide pathway: biomedical significance and mechanisms of regulation. *Curr Mol Med* 10(5):454–466
- Sot J, Ibarguren M, Busto JV, Montes LR, Goni FM, Alonso A (2008) Cholesterol displacement by ceramide in sphingomyelin-containing liquid-ordered domains, and generation of gel regions in giant lipidic vesicles. *FEBS Lett* 582(21–22):3230–3236
- Goni FM, Alonso A (2009) Effects of ceramide and other simple sphingolipids on membrane lateral structure. *Biochim Biophys Acta* 1788(1):169–177
- Carrer DC, Schreier S, Patrino M, Maggio B (2006) Effects of a short-chain ceramide on bilayer domain formation, thickness, and chain mobility: DMPC and asymmetric ceramide mixtures. *Biophys J* 90(7):2394–2403
- Loura LM, de Almeida RF, Silva LC, Prieto M (2009) FRET analysis of domain formation and properties in complex membrane systems. *Biochim Biophys Acta* 1788(1):209–224
- Budin I, Devaraj NK (2012) Membrane assembly driven by a biomimetic coupling reaction. *J Am Chem Soc* 134(2):751–753
- Bedia C, Casas J, Garcia V, Levade T, Fabrias G (2007) Synthesis of a novel ceramide analogue and its use in a high-throughput fluorogenic assay for ceramidases. *Chembiochem* 8(6):642–648
- Montes LR, Alonso A, Goni FM, Bagatolli LA (2007) Giant unilamellar vesicles electroformed from native membranes and organic lipid mixtures under physiological conditions. *Biophys J* 93(10):3548–3554
- Montes LR, Ahyauch H, Ibarguren M, Sot J, Alonso A, Bagatolli LA, Goni FM (2010) Electroformation of giant unilamellar vesicles from native membranes and organic lipid mixtures for the study of lipid domains under physiological ionic-strength conditions. *Methods Mol Biol* 606:105–114
- Sot J, Bagatolli LA, Goni FM, Alonso A (2006) Detergent-resistant, ceramide-enriched domains in sphingomyelin/ceramide bilayers. *Biophys J* 90(3):903–914
- de Almeida RF, Loura LM, Prieto M (2009) Membrane lipid domains and rafts: current applications of fluorescence lifetime spectroscopy and imaging. *Chem Phys Lipids* 157(2):61–77
- Chiantia S, Kahya N, Schwille P (2007) Raft domain reorganization driven by short- and long-chain ceramide: a combined AFM and FCS study. *Langmuir* 23(14):7659–7665
- Ira ZS, Ramirez DM, Vanderlip S, Ogilvie W, Jakubek ZJ, Johnston LJ (2009) Enzymatic generation of ceramide induces membrane restructuring: correlated AFM and fluorescence imaging of supported bilayers. *J Struct Biol* 168(1):78–89
- Megha, London E (2004) Ceramide selectively displaces cholesterol from ordered lipid domains (rafts): implications for lipid raft structure and function. *J Biol Chem* 279(11):9997–10004
- Busto JV, Sot J, Requejo-Isidro J, Goni FM, Alonso A (2010) Cholesterol displaces palmitoylceramide from its tight packing with palmitoylsphingomyelin in the absence of a liquid-disordered phase. *Biophys J* 99(4):1119–1128
- Cremesti AE, Goni FM, Kolesnick R (2002) Role of sphingomyelinase and ceramide in modulating rafts: do biophysical properties determine biologic outcome? *FEBS Lett* 531(1):47–53