

Imaging Raft Cholesterol with Cholesteryl Probes

Dr. Haitao Li

Imaging Probe Development Center, National Heart, Lung, and Blood Institute, National Institutes of Health, 9800 Medical Center Drive, Rockville, MD 20850, ipdc@nhlbi.nih.gov

Background

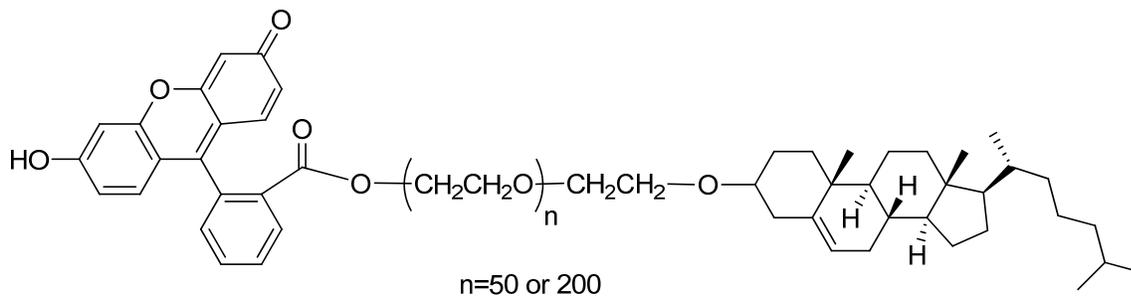
Lipid rafts are cholesterol-rich membrane microdomains that serve as dynamic platforms for signal transduction events in the cell.¹ Depending on cell type, several signaling cascades^{2; 3; 4} have been reported to be assembled and activated in cholesterol-rich lipid rafts, which due to their physicochemical properties are enriched with a unique set of signaling proteins, including receptors, kinases, and G-proteins.⁵ Perhaps the greatest controversy in the field of raft biology is how best to study them, with many studies exploiting their inherent properties by isolating rafts from cells by detergent lysis/sucrose density centrifugation, later shown to impose artefactual cholesterol and protein composition changes.⁶ In addition, diffusion of cholesterol between subcellular compartments can obscure efforts to quantitate its content in raft isolates, while isolation methods generally pool rafts from plasma membranes and intracellular membranes, thereby preventing distinction. More recent efforts have focused on how best to image rafts in intact cells, often by using antibody techniques and confocal microscopy with fluorescence. However these approaches are limited by antibody availability and provide no information about raft cholesterol content. Recently two new raft imaging probes, BC Theta toxin and fPEG-chol,⁷ have been developed that avoid many pitfalls. These agents allow quantitative visualization of raft cholesterol in live cells, with fPEG-chol (Scheme 1) being a selective and quantitative probe for raft cholesterol.

Chemistry

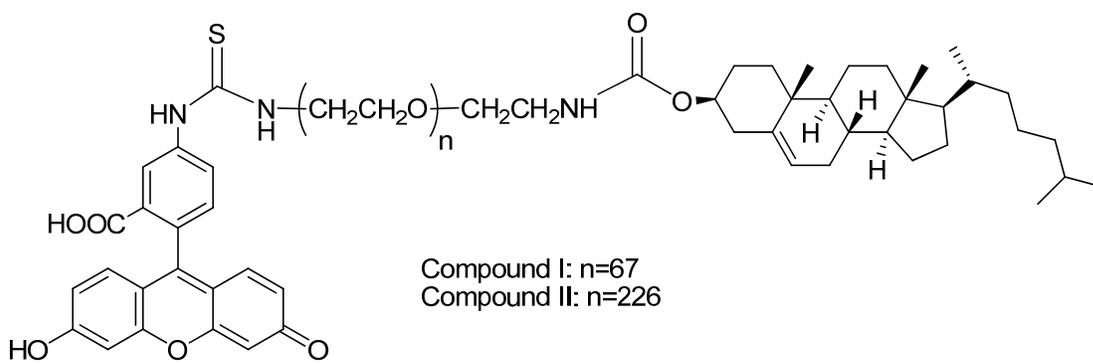
After reviewing the literature on the synthesis of fPEG-Chol, we decided instead to make related analogs, since starting material for fPEG-Chol itself was not readily available. Due to their great structural similarity, differing just in the linkage group, the new probes should have similar properties to fPEG-Chol itself. Syntheses of the cholesteryl probes are shown in Scheme 2.

Monoacylation of PEG diamine with cholesteryl chloroformate followed by reaction with fluorescein isothiocyanate isomer I gave the proposed products.

Scheme 1. Cholesterol probes

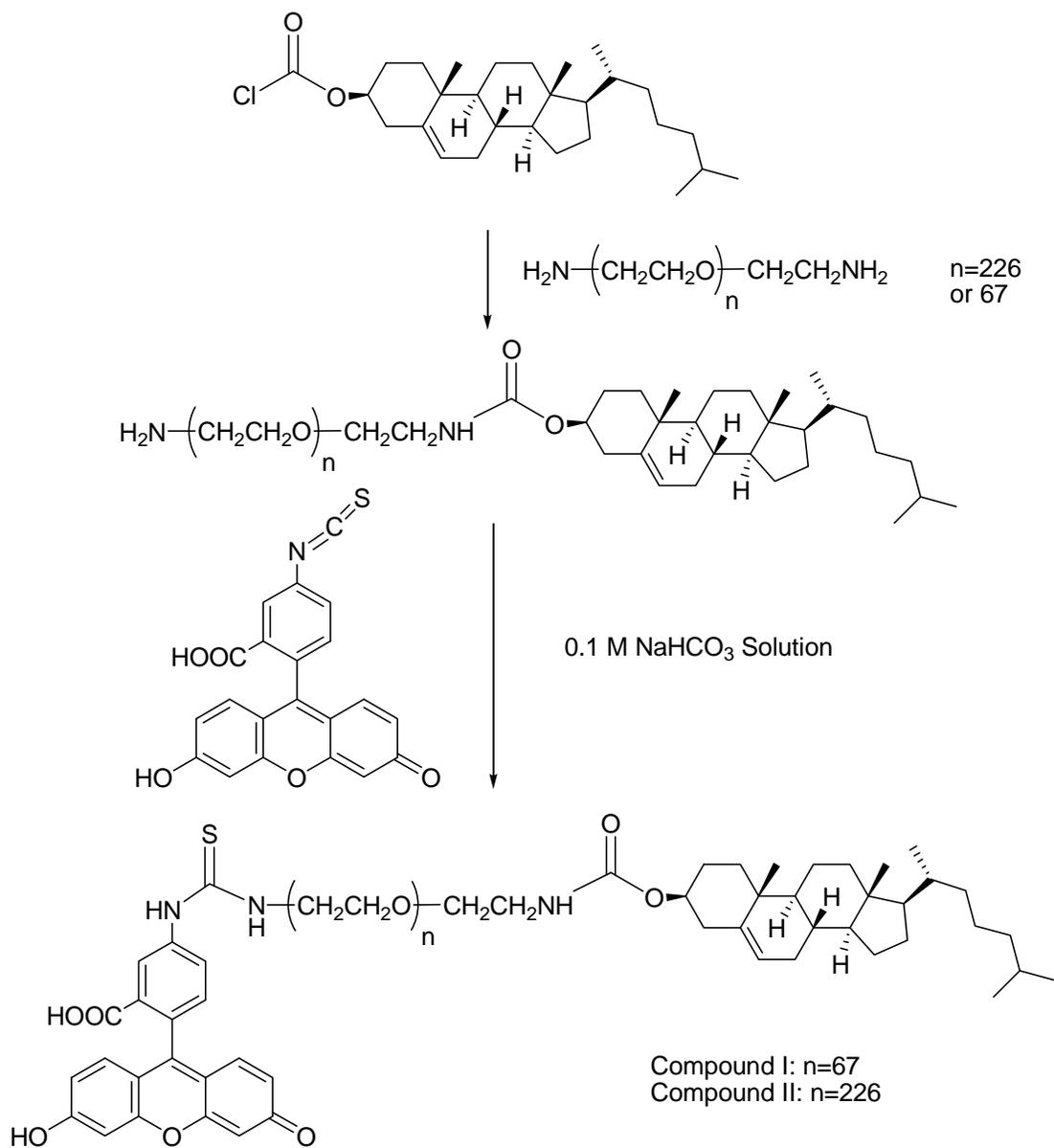


Literature reported cholesterol probes: fPEG-Chol



Our proposed cholesteryl probes

Scheme 2. Syntheses of cholesteryl probes



Experimental

General. Thin-layer chromatography (TLC) analyses were carried out on Analtech (Newark, DE) silica gel GHLF 0.25 mm plates using UV and iodine detection. HPLC analysis was done on a Beckman Coulter System Gold equipped with a Waters Atlantis C18 column (4.5 X 150 mm, 5 μm). MALDI-MS was performed on a Shimadzu Biotech Axima. $^1\text{H-NMR}$ spectra were recorded on a 400 MHz Varian spectrometer. Chemical shifts were recorded in parts per million δ and referenced to tetramethylsilane (TMS). Flash chromatography was carried out on a

Teledyne Isco CombiFlash® Companion® instrument with UV detection at 254 nm. All starting materials and reagents were commercially available from Sigma-Aldrich and were used without further purification.

Synthesis of Chol-PEG₁₀₀₀₀-NH₂. To PEG₁₀₀₀₀-bis(amine) (1 g, 0.1mmol) in dichloromethane (DCM) (75 mL) was added triethylamine (TEA) (41 μ L, 0.3 mmol). Cholesteryl chloroformate (54 mg, 0.12 mmol) dissolved in DCM (5 mL) was added dropwise. After stirring at room temperature overnight, TLC analysis (DCM:methanol=85:15) indicated formation of dicholesteryl (R_f = 0.66) and monocholesteryl (R_f = 0.56) products. The reaction mixture was diluted with DCM (425 mL), washed with 5% potassium hydrogen sulfate, brine and 5% sodium bicarbonate, dried over sodium sulfate and evaporated to dryness. Column chromatography using a gradient of methanol (0-20%) in DCM over 20 minutes afforded monocholesteryl PEG (Chol-PEG₁₀₀₀₀-NH₂) as a white solid (574 mg, 55%). MALDI-MS of PEG₁₀₀₀₀-bis(amine) showed a series of 44-Da spaced peaks centered around 10233. MALDI-MS of monocholesteryl PEG (Chol-PEG₁₀₀₀₀-NH₂) using synaptic acid as matrix showed a series of 44-Da spaced peaks centered around 10674. ¹H NMR (CDCl₃, 400 MHz) δ 0.67-2.34 (cholesteryl protons), 3.35-3.82 (br, m, PEG protons), 5.19 (s, cholesteryl proton), 5.37 (s, cholesteryl proton). Since the proton peaks due to the PEG component is very large, peak integration of was not accurate.

Synthesis of compound I (fPEG₁₀₀₀₀-Chol analog). To Chol-PEG₁₀₀₀₀-NH₂ (133 mg, 0.012 mmol) in 0.1 M sodium bicarbonate (NaHCO₃, 13 mL) was added fluorescein isothiocyanate isomer I (25 mg, 0.06 mmol) and the mixture was stirred overnight protected from light. TLC analysis revealed a new product spot and starting material, so additional fluorescein isothiocyanate isomer I (25 mg, 0.06 mmol) was added and the reaction mixture was stirred a further 72 hours, after which all Chol-PEG₁₀₀₀₀-NH₂ was consumed by HPLC analysis. The reaction mixture was loaded into a Millipore Centriprep filter with a YM-3 membrane (MW cut-off 3000) and centrifuged at 2700 rpm for 30 minutes. After eight rounds of concentration, dilution and centrifugation, HPLC analysis of the retentate showed only one peak, which was lyophilized, giving an orange solid (136 mg). This was loaded onto a PD-10 column and eluted off with water. The desired MW fractions from the PD-10 column were pooled and lyophilized to give the final product as an orange solid (108 mg, 78%). HPLC analysis was done with a

gradient of 50 mM ammonium acetate in water (Solvent A) and acetonitrile (Solvent B) as eluents (5% to 80% solvent B in 20 minutes), and showed the retention time of the desired product at 11.23 minutes. MALDI-MS analysis of the product using dithranol and silver trifluoroacetate as matrix⁸ showed a series of 44-Dalton spaced peaks centered around 11310. ¹H NMR showed a large, broad PEG proton peak while cholesteryl and fluorescein peaks were much smaller. ¹H NMR (*d*⁶-DMSO, δ 0.62-2.37(m, cholesteryl protons), 3.09-3.69 (br, m, PEG protons), 5.32 (m, cholesteryl proton), 6.59-6.70(m, fluorescein peaks), 7.00(m, fluorescein protons), 7.54-7.56(m, fluorescein protons), 8.00(s, fluorescein protons). Since the proton peaks due to PEG were so large, integration was not accurate.

Synthesis of Chol-PEG₃₀₀₀-NH₂. To PEG₃₀₀₀- bis(amine) (1 g, 0.3mmol) in DCM (150 mL) was added TEA (139 μ L, 0.9 mmol). Cholesteryl chloroformate (124 mg, 0.27 mmol) was dissolved in DCM (10 mL) and added to the reaction mixture dropwise. After stirring at room temperature overnight, TLC (DCM:MeOH=85:15) analysis indicated the formation of dicholesteryl (R_f = 0.43) and monocholesteryl (R_f = 0.28) products. The reaction mixture was evaporated to dryness and column chromatography using a gradient of MeOH (0-15%) in DCM over 40 minutes gave monocholesteryl PEG (Chol-PEG₃₀₀₀-NH₂) as a white solid (190 mg, 17%). MALDI-MS of PEG₃₀₀₀- bis(amine) using 2,5-dihydroxybenzoic acid (DHB) as matrix showed a series of 44-Dalton spaced peaks centered around 2968. MALDI-MS of monocholesteryl PEG (Chol-PEG₃₀₀₀-NH₂) using DHB as matrix showed a series of 44-Dalton spaced peaks centered around 3447. ¹H NMR (CDCl₃) δ 0.86-2.34 (cholesteryl protons), 3.35-3.88 (br, m, PEG protons), 5.19 (s, 1H), 5.37 (s, 1H). Since the peaks due to PEG protons are so large, integration was not accurate.

Synthesis of compound II (fPEG₃₀₀₀-Chol analog). To Chol-PEG₃₀₀₀-NH₂ (130 mg, 0.0365 mmol) in 0.1 M NaHCO₃ (15 mL) was added fluorescein isothiocyanate isomer I (71 mg, 0.18 mmol) and the reaction was stirred at 4°C overnight protected from light. TLC analysis revealed a new product spot with some starting material remaining. Additional fluorescein isothiocyanate isomer I (71 mg, 0.18 mmol) was added and the reaction mixture was stirred overnight again at 4°C, whereupon TLC analysis revealed that starting material still existed. A third batch of fluorescein isothiocyanate isomer I (71 mg, 0.18 mmol) was added and the reaction mixture was

stirred a further 72 hours at 4°C. The reaction mixture was centrifuged to remove excess solid, diluted to 100 mL with water and passed three times through a Stir Cell (Millipore, MW cut off 1000) with dilution from 10 mL to 100 mL for each pass. HPLC analysis of the retentate showed one peak and this was lyophilized to an orange solid (140 mg, 97%) which was loaded onto a Sephadex G10 column and eluted with water. The desired fractions were pooled and lyophilized to afford the final product. HPLC analysis using 0.05% TFA in water (Solvent A) and 0.05% TFA in ACN (Solvent B) as eluent (5% to 80% solvent B in 20 min), showed the retention time of the desired product at 16.5 minutes. MALDI-MS analysis of the product using DHB as matrix showed a series of 44 Dalton-spaced peaks centered around 4014. ¹H NMR (*d*⁶-DMSO) δ 0.72-2.39(m, cholesteryl protons), 3.14-3.75 (br, m, PEG protons), 5.39 (m, cholesteryl proton), 5.98-6.41(m, fluorescein peaks), 6.77-6.76(m, fluorescein protons), 6.99-7.08(m, fluorescein protons), 7.74-8.169 (m, fluorescein protons).

References

1. Golub T, Wacha S, Caroni P. Spatial and temporal control of signaling through lipid rafts. *Curr Opin Neurobiol.* 2004 Oct;14(5): 542-50. PMID 15464886
2. Jury EC, Flores-Borja F, Kabouridis PS. Lipid rafts in T cell signalling and disease. *Semin Cell Dev Biol.* 2007 Oct;18(5): 608-15. PMID 17890113
3. Kabouridis PS. Lipid rafts in T cell receptor signalling. *Mol Membr Biol.* 2006 Jan-Feb;23(1): 49-57. PMID 16611580
4. Nishio M, Tajima O, Furukawa K, Urano T. Over-expression of GM1 enhances cell proliferation with epidermal growth factor without affecting the receptor localization in the microdomain in PC12 cells. *Int J Oncol.* 2005 Jan;26(1): 191-9. PMID 15586240
5. Foster LJ, De Hoog CL, Mann M. Unbiased quantitative proteomics of lipid rafts reveals high specificity for signaling factors. *Proc Natl Acad Sci U S A.* 2003 May 13;100(10): 5813-8. PMID 12724530
6. Shogomori H, Brown DA. Use of detergents to study membrane rafts: the good, the bad, and the ugly. *Biol Chem.* 2003 Sep;384(9): 1259-63. PMID 14515986
7. Koseki M, Hirano K, Masuda D, Ikegami C, Tanaka M, Ota A, Sandoval JC, Nakagawa-Toyama Y, Sato SB, Kobayashi T, Shimada Y, Ohno-Iwashita Y, Matsuura F, Shimomura I, Yamashita S. Increased lipid rafts and accelerated lipopolysaccharide-induced tumor necrosis factor-alpha secretion in Abca1-deficient macrophages. *J Lipid Res.* 2007 Feb;48(2): 299-306. PMID 17079792

8. Chaudhary AK, Critchley G, Diaf A, Beckman EJ, Russell AJ. Characterization of Synthetic Polymers Using Matrix-Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry. *Macromolecules*. 1996 29(6): 2213-2221. PMID