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Near-infrared-fluorescence imaging of lymph nodes by using liposomally formulated indocyanine green derivatives

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ABSTRACT

Liposomally formulated indocyanine green (LP-ICG) has drawn much attention as a highly sensitive near-infrared (NIR)-fluorescence probe for tumors or lymph nodes in vivo. We synthesized ICG derivatives tagged with alkyl chains (ICG-Cn), and we examined NIR-fluorescence imaging for lymph nodes in the lower extremities of mice by using liposomally formulated ICG-Cn (LP-ICG-Cn) as well as conventional liposomally formulated ICG (LP-ICG) and ICG. Analysis with a noninvasive preclinical NIR-fluorescence imaging system revealed that LP-ICG-Cn accumulates in only the popliteal lymph node 1 h after injection into the footpad, whereas LP-ICG and ICG accumulate in the popliteal lymph node and other organs like the liver. This result indicates that LP-ICG-Cn is a useful NIR-fluorescence probe for noninvasive in vivo bioimaging, especially for the sentinel lymph node.

1. Introduction

In the biological and medical sciences, noninvasive imaging technologies such as radioactive isotope imaging,[1–4] ultrasonic echo imaging,[5–7] and nuclear magnetic resonance imaging[8–10] are used. Among these, near-infrared (NIR) fluorescence imaging has drawn much attention because of low tissue autofluorescence and deep penetration of light into tissues at wavelengths between 650 and 900 nm.[11–18]

Both NIR-fluorescent dyes[19–28] and colloidal particles[29–40] are commonly used in biomedical analyses, and NIR-fluorescent carbocyanine dyes are of great interest because of their ease of synthesis, biocompatibility, tunable spectral properties, and exceptionally high molar absorptivity in the NIR region; therefore, they have been extensively used in cellular and animal imaging applications.[41–59] Indocyanine green (ICG) is one of such NIR-fluorescent dyes, and it has recently garnered much attention in detecting sentinel lymph nodes (SLNs)[60–62] owing to its abovementioned characteristics, but more importantly, because it was approved by the Food and Drug Administration in the United States.[63] SLNs are, hypothetically, the first nodes to receive drainage from primary tumors, and detection of these during operation could prevent morbidity of surgery, improve lymph node staging, and thus contribute to survival and quality of life after surgery. Thus far, SLN mapping procedures with ICG have been tested in various types of malignancies and found to be equally useful and less cumbersome than conventional techniques using radioactive colloids or dyes.[60–62,64–67] However, one of the issues to be considered while using this technique is the rapid diffusion characteristic of this dye. Wide blurring of the dye around injection sites and rapid migration of the dye beyond the SLN have been reported, and these could hamper the accuracy and simplicity of detecting SLNs with this technique.

Liposomes, which are closed bilayer membranes composed of phospholipids, have traditionally been of interest to medical researchers for use as drug carriers and recently have been used as colloidal probes for bioimaging because their size allows for the targeting of a specific region of tumor.[68–76] As a result, liposomally formulated ICG (LP-ICG) has been recently considered

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the ideal candidate as an NIR-fluorescence imaging probe. For instance, Jeong et al. have reported the modification of liposome via mannosylation of phospholipid and the lymph node imaging by ICG contained in the modified liposome.[74] Proulx et al. have reported on the high stability and high sensitivity of LP-ICG for NIR-fluorescence imaging of lymph nodes in vivo by its administration to lower extremities of mice.[75] However, LP-ICG has still shown to accumulate in not only a first draining lymph node but also a second-tier node in the lower extremities of mice. We hypothesized that ICG can leak out from the liposomal membrane in vivo; thus, we synthesized a novel ICG, which is more hydrophobic than conventional ICG, by substitution of one sulfonate groups of the ICG with an alkyl chain (ICG-Cn; n = 4, 6, 8, 10, and 18). We examined the spectral properties of ICG-Cn in both an organic solution and a liposomal dispersion, and we visualized the lymph nodes in the lower extremities of mice by using liposomally formulated ICG-Cn (LP-ICG-Cn) as well as LP-ICG and ICG under a NIR-fluorescence imaging system.

2. Material and methods

2.1. Synthesis of 18-(2-((1*E*,3*E*,5*E*,7*E*)-7-(1,1-dimethyl-3-butyl-1*H*-benzo[*e*]indol-2(3*H*)-ilydene)hepta-1,3,5-trienyl)-1,1-dimethyl-1*H*-benzo[*e*]indolium-3-yl)octadecane-1-sulforic acid (ICG-C18) and other ICG-Cn (n = 4, 6, 8, and 10)

The process of ICG-C18 synthesis follows the conventional processes for synthesis of ICG and other ICG derivatives reported elsewhere (Scheme 2).[45,56,77] 2,3,3-Trimethyl-4,5-benzo-3*H*-indole (3.1 g, 15 mmol) and 1,4-butane sultone (2.1 g, 15 mmol) were mixed in a round-bottomed flask (25 mL) under a nitrogen atmosphere, and the reaction solution was stirred at 80 °C for 4 h and then cooled at room temperature. By addition of acetone to the reaction mixture, the residue was dissolved, and the product was crystallized. The reaction product, 2,3,3-trimethyl-1-(sulfobutyl)-4,5-benzoindolium inner salt, was separated by filtration and rinsed with acetone. The product was obtained as a gray crystal (1.17 g, 23%).

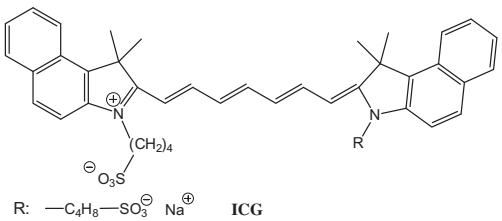
A reaction mixture of the gray crystal (1.04 g, 3.0 mmol) and glutaconaldehyde dianil hydrogen chloride salt (0.94 g, 3.3 mmol) was stirred at 120 °C for 1 h and then cooled at room temperature to crystallize the product, 2-(6-acetanilido-1,3,5-hexatrienyl)-3,3-dimethyl-1-(sulfobutyl)-4,5-benzo[*e*]indolium inner salt. The salt was then separated by filtration and rinsed with acetone. The product was obtained as a dark purple crystal (0.97 g, 58%).

A 2-butanone solution (40 mL) of 2,3,3-trimethyl-4,5-benzo-3*H*-indole (8.4 g, 40 mmol) and 1-iodooctadecane (16.8 g, 44 mmol) was stirred in a round-bottomed flask (100 mL) at 70 °C for 18 h and then cooled at room temperature. After addition of ethyl acetate (40 mL) to crystallize the product, 1-octadecyl-2,3,3-trimethyl-4,5-benzo[*e*]indolium iodide, was separated by filtration and rinsed twice with ethyl acetate. The product was obtained as a gray crystal (4.4 g, 19%).

Finally, a pyridine solution (16 mL) of the indolium inner salt (1.58 g, 3.0 mmol) and the indolium iodide (1.77 g, 3.0 mmol) was stirred at 50 °C for 1 h under a nitrogen atmosphere. After cooling at room temperature and addition of distilled water (40 mL), the crystallized product was separated by filtration and dissolved in ethyl acetate. The residue of the solution was filtered and recrystallized in 40 mL of chloroform/ethyl acetate (1/1, volume ratio), and the product was obtained as a dark green crystal (1.39 g, 53%). The other ICG derivatives: ICG-Cn (n = 4, 6, 8, and 10), with different alkyl chains from ICG-C18, were synthesized using the same procedure and the corresponding indolium salts. Yields of 46% (ICG-C4), 59% (ICG-C6), 57% (ICG-C8), and 44% (ICG-C10) were obtained.

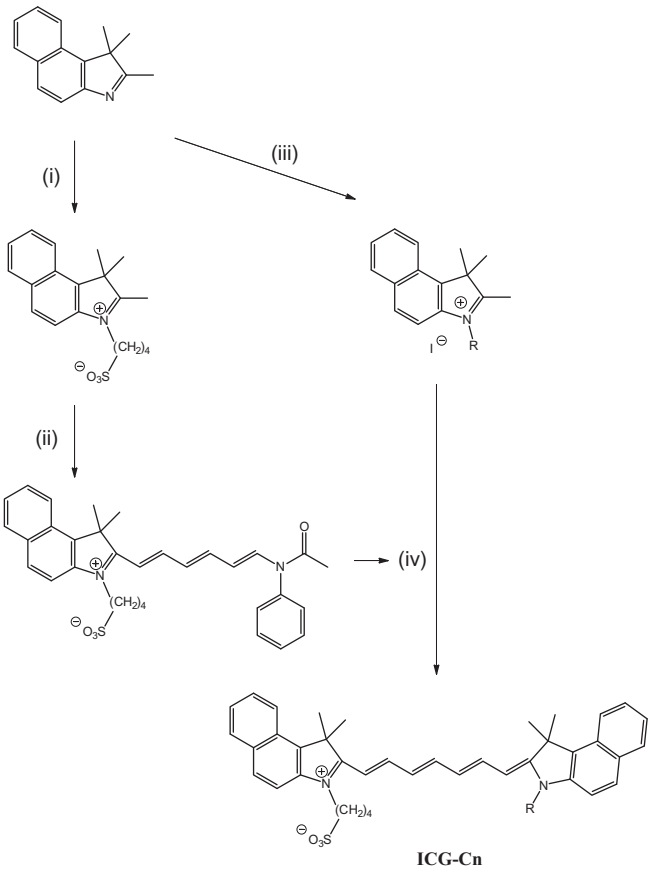
The ^{1}H NMR spectra of ICG-Cn (n = 4, 6, 8, 10, and 18) were run at 300 MHz in DMSO-d_6 by using a Lambda-300 NMR spectrometer (JEOL, Tokyo, Japan). The infrared spectra of these compounds were recorded on an IRPrestige-21 spectrometer (Shimadzu, Kyoto, Japan) in the range of 4000–400 cm^{-1} using potassium bromide pellets. Electron spray ionization mass spectroscopy (ESI-MS) was examined using LCMS-2010EV (Shimadzu, Kyoto, Japan).

ICG-C4: ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.94 (t, 3H, J = 7.0 Hz); 1.43 (m, 2H); 1.79 (m, 18H); 2.54 (m, 2H); 4.20 (m, 4H); 6.36 (d, 1H, J = 13.8 Hz); 6.57 (m, 3H); 7.47 (m, 2H); 7.63 (m, 3H); 7.78 (m, 2H); 7.99 (m, 6H); 8.22 (d, 2H, J = 8.5 Hz). IR (KBr, cm^{-1}): 3051, 2930, 2871, 1630, 1418, 1310, 1138. ESI-MS (methanol/acetonitrile = 1:1): m/z = 673 [M+H$^+$].



R:		
	—C_4H_8—SO_3^- Na^+	**ICG**
	—C_4H_9	**ICG-C4**
	—C_6H_{13}	**ICG-C6**
	—C_8H_{17}	**ICG-C8**
	—$C_{10}H_{21}$	**ICG-C10**
	—$C_{18}H_{37}$	**ICG-C18**

Scheme 1. Structures of ICG and ICG-Cn (n = 4, 6, 8, 10, and 18).



Scheme 2. Synthesis of ICG-Cn: (i) 1,4-butane sultone, (ii) glutacon-aldehyde dianil hydrogen chloride salt, (iii) $C_nH_{2n+1}I$ (n = 4, 6, 8, 10, and 18), 2-butanone, and (iv) pyridine.