Multifunctional Perfluorocarbon Nanoemulsions for $^{19}$F-based Magnetic Resonance and Near-Infrared Optical Imaging of Dendritic Cells

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Preparation of IRDye800-coated PFOB nanoemulsions. To fabricate perfluoroocetyl bromide (PFOB, Aldrich, St. Louis, MO) nanoemulsions, PFOB liquids were emulsified in an aqueous solution using surfactant mixtures. A surfactant mixture comprising of 64 mol % lecithin (L-$\alpha$-Phosphatidylcholine 95%, chicken egg, Avanti Polar Lipids, Alabaster, AL), 35 mol % cholesterol (Avanti Polar Lipids, Alabaster, AL) and 1 mol % 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG(2000) Amine, Avanti Polar Lipids, Alabaster, AL) were dissolved in chloroform, and the organic solvent was evaporated using a rotary evaporator and a vacuum oven (50°C) for 24 h. After dispersing the surfactant mixture into sterilized distilled water, the solution was sonicated. PFOB liquids (40% v/v), glycerin (2.0% w/v), surfactant mixture and distilled water were mixed for 30 s using a homogenizer. The mixture was processed through an emulsifier (M-110S, Microfluidics, Newton, MA) at 20000 psi for 3 min. The fabricated PFOB nanoemulsions were stored at 4°C. As a near-infrared fluorophore, IRDye800 NHS ester was purchased from LI-COR Biosciences (Lincoln, Nebraska). For conjugation, an aliquot of PFOB nanoemulsion solutions were mixed with IRDye800 NHS ester (50 μL/mg) dissolved in water. The conjugation reaction was carried out for 2 hrs at 20°C. IRDye800-coated PFOB nanoemulsions were separated using FPLC system.
Characterization of IRDye800-coated PFOB nanoemulsions. The mean particle diameters and zeta (ζ)-potentials of the IRDye800-coated PFOB nanoemulsions were determined using a particle size analyzer (ELS-Z, Otsuka Electronics, Japan). The intensity autocorrelations were measured at a scattering angle (θ) of 90° with electrophoretic light scattering at 25 ± 0.1 °C. TEM images were obtained using a 200 kV Field Emission Transmission Electron Microscope (JEM-2100F, JEOL, LTD, Tokyo, Japan). IRDye800-coated PFOB nanoemulsions were also prepared as a thin liquid layer supported on a cryo-grid, and were immediately plunged in liquid ethane to prevent evaporation from the thinly spread sample. The frozen grids were stored in liquid nitrogen and transferred in a GATAN model 630 cryotransfer (Gatan, Inc., Warrendale, PA) in liquid nitrogen at a temperature of approximately -185°C. Direct imaging was carried out using an acceleration voltage of 120 kV and a Multiscan 600W charge-coupled device (CCD) camera (Gatan, Inc, Pleasanton, CA) at a temperature of approximately -170°C.

Fluorescence and 19F MR spectroscopy of nanoemulsions. The emission spectra of IRDye800-coated PFOB nanoemulsions were measured using a fluorescence spectrometer (LS 55, PerkinElmer Instruments, Wellesley, MA). All 19F spectral and imaging experiments of IRDye800-coated PFOB nanoemulsions were performed on a 600 MHz (14 T) Bruker NMR spectrometer (Avance DMX600, Bruker, Rheinstetten, Germany) equipped with a triple gradient system for microscopic imaging. The maximum gradient strength was 200 G/cm, and a 5 mm double-tuned 1H/19F saddle-type RF coil was used. For 19F spectral measurement, IRDye800-coated PFOB nanoemulsions were inserted into standard Wilmad 5 mm NMR tubes, and fluorine spectra (30° flip angle; 2 acquisitions; 5 sec acquisition time) were acquired from the sample.

In Vitro and In Vivo Fluorescence and 19F MR imaging of DCs. DC2.4 cells (murine dendritic cells) were cultured in 10 mm dishes (1×10^7/dish) in Dulbecco’s modified Eagle Medium (DMEM; Gibco, Invitrogen, Carlsbad, CA) supplemented with 10% heatinactivated FBS, 50 IU/ml penicillin and 50 µg/ml streptomycin. Cells were incubated with 0.63 ~ 100 µl/ml IRDye800-coated PFOB nanoemulsions for 24 h. After incubation, labeling efficiency was analyzed by flow cytometry. For fluorescence imaging, DC2.4 were incubated with 50 µl/ml IRDye800-coated PFOB nanoemulsions
for 24 h at 37 °C. After washing with PBS, the labeled cells were fixed with Cytofix/Cytoperm solution and stained with DAPI in PBS. To detect intracellular localization of nanoemulsions, DC2.4 cells were stained with the lysosomal marker, Lysosome-Associated Membrane Glycoprotein-1 (LAMP-1) by incubating with FITC-conjugated rat anti-LAMP-1 monoclonal antibody (1D4B) (BD PharMingen) for 30 min at room temperature. Fluorescence images were obtained on a Deltavision RT deconvolution microscope (Applied Precision Technologies, Issaquah, WA).

For in vivo fluorescence and MR imaging, DC2.4 (5×10^6 cells/ml) labeled with IRDye800-coated PFOB nanoemulsions were resuspended in 100 µl HBSS buffer and were subcutaneously injected into a mouse. All experiments involving mice were performed in accordance with the Korean NIH guidelines for the care and use of laboratory research animals. The mice were anesthetized with 300 µl of a 2.5% avertin solution (2,2,2-tribromoethanol-tert-amyl alcohol, Sigma). Thereafter fluorescence images (5 s exposure) were obtained home-made small animal imaging system using an excitation light source (760 nm) and an emission filter (845WB55). All images were processed with Simple PCI software (Compix Inc., Cranberry Township, PA). ^19^F-MR images of the mouse were performed with 4.7 T Bruker scanner (BioSpec, Rheinstetten, Germany) using a double-tuned ^1^H/^19^F Birdcage coil design (outer/inner: 59/35 mm). For the ^19^F-MR image, the mouse was imaged with a gradient echo sequence (128 × 128 matrix; 60 × 60 mm<sup>2</sup> FOV; 50.0 ms TR; 2 ms TE; 5 mm slice thickness; 20 acquisitions).

**Cell viability assay.** Cell viability was measured by analyzing cleavage of thiazoyl blue tetrazolium bromide (MTT; Sigma) by succinate dehydrogenases of living cells to yield formazan. After incubating DC2.4 cells (1 × 10^4 cells/0.1 ml) with IRDye800-coated PFOB nanoemulsions for various times in flat-bottomed 96-well plates (Corning Costar, Cambridge, MA, USA), MTT (10 µl/well of a 5 mg/ml MTT stock solution in PBS) was added directly to each well and plates were incubated at 37°C for 4 h. To assay MTT reduction colorimetrically, dimethyl sulfoxide (DMSO; Sigma) was added to solubilize formazan and absorbance was measured at 562 nm.
Figure S1. Labeling efficiency of dendritic cells labeled with IRDye800-coated PFOB nanoemulsions.
**Figure S2.** The localization of IRDye800-coated PFOB nanoemulsions within dendritic cells. (a) : DIC (differential interference contrast), (b) : FITC- anti-LAMP-1 stained (green; lysosome), (c) : IRDye800 (red), (d) merged. Scale bars : 10 μm. Most of IRDye800-coated PFOB nanoemulsions were localized in the lysosomes.
Figure S3. Viability of dendritic cells labeled with IRDye800-coated PFOB nanoemulsions