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Development of fluorous lipid-based nanobubbles for efficiently containing perfluoropropane

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ABSTRACT

Nano-/microbubbles are expected not only to function as ultrasound contrast agents but also as ultrasound-triggered enhancers in gene and drug delivery. Notably, nanobubbles have the ability to pass through tumor vasculature and achieve passive tumor targeting. Thus, nanobubbles would be an attractive tool for use as ultrasound-mediated cancer theranostics. However, the amounts of gas carried by nanobubbles are generally lower than those carried by microbubbles because nanobubbles have inherently smaller volumes. In order to reduce the injection volume and to increase echogenicity, it is important to develop nanobubbles with higher gas content. In this study, we prepared 5 kinds of fluoro-lipids and used these reagents as surfactants to generate "Bubble liposomes", that is, liposomes that encapsulate nanobubbles such that the lipids serve as stabilizers between the fluorous gas and water phases. Bubble liposome containing 1-stearoyl-2-(18,18-difluoro)stearoyl-sn-glycero-3-phosphocholine carried 2-fold higher amounts of C_3F_8 compared to unmodified Bubble liposome. The modified Bubble liposome also exhibited increased echogenicity by ultrasonography. These results demonstrated that the inclusion of fluoro-lipid is a promising tool for generating nanobubbles with increased efficiency of fluorous gas carrier.

1. Introduction

Commercially used ultrasound (US) contrast agents (e.g., SonoVue Definity, and Sonazoid) consist of microbubbles with diameters of less than 10 μm, similar in size to that of red blood cells (Quaia, 2007; Claudon et al., 2013; Zheng et al., 2013). The microbubbles are composed primarily of a poorly water soluble gas (C_3F_8, C_4F_{10}, or SF_6) encapsulated within a shell composed of lipid, protein, or polymer. In recent decades, these microbubbles have been used primarily for imaging in clinical practice (Barr et al., 2014). Recently, microbubbles also have been used as enhancers for US-mediated drug and gene delivery, with their associated sonoporation (Sanches et al., 2014; Panje et al., 2013; Zhao et al., 2013).

Lipid-based microbubbles have some advantages for US-mediated imaging and drug/gene delivery. In the context of imaging, lipid-based microbubbles have high echogenicity; the lipid molecules are held together by weak physical forces, without chain entanglement, making the shell compliant to area expansion and compression during US insonification (Sirsi and Borden, 2009). Thus, lipid-based microbubbles have favorable US characteristics, such as resonance with minimal damping and the ability to reseal around the gas core following rupture by US insonification. Also,

Abbreviations: BLs, bubble liposomes; US, ultrasound; DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; DSPE-PEG (2k)-OMe, distearoylphosphatidylethanolamine methoxypolyethyleneglycol; 2-F_1DPPC, 1-palmitoyl-2-(16-fluoro)palmitoyl-sn-glycero-3-phosphocholine; 2-F_1DSPC, 1-stearoyl-2-(18-fluoro)stearoyl-sn-glycero-3-phosphocholine; 1,2-F_2DSPC, 1,2-di(18-fluoro)stearoyl-sn-glycero-3-phosphocholine; 2,2-F_2DSPC, 1-stearoyl-2-(18,18-difluoro)stearoyl-sn-glycero-3-phosphocholine; 2,2,2-F_3-DSPC, 1-stearoyl-2-(18,18,18-trifluoro)stearoyl-sn-glycero-3-phosphocholine; GC, gas chromatography; DLS, dynamic light scattering.

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the surface charge of such microbubbles can be easily controlled by choice of lipid composition. This property permits loading of nucleic acids (Panje et al., 2012; Endo-Takahashi et al., 2012; Sun et al., 2014); nucleic acids complexed with cationic polymers (Wang et al., 2014; Florinas et al., 2013; Borden et al., 2007; Yin et al., 2014); certain drugs (Yan et al., 2011; Tartis et al., 2006; Tinkov et al., 2010); or drug-encapsulating nanoparticles (Lentacker et al., 2010; Geers et al., 2011; Deng et al., 2014). Additionally, the bubbles are readily modified with targeting molecules by attaching these to a lipid molecule that serves as a membrane anchor (Wei et al., 2014; Schumann et al., 2002; Yan et al., 2014). Due to these properties, lipid-based microbubbles are receiving increasing attention as potential theranostic agents.

Most applications of microbubbles in cancer are intravascular events because the short circulation time and relatively large size of microbubbles preclude effective extravasation into tumor tissue, preventing efficient targeting to tumors (Zheng et al., 2013). Recently, Yin et al. (2012) reported that nanobubbles (mean ± SD diameters of 436.8 ± 5.7 nm) have the ability to pass through the tumor vasculature and achieve passive tumor targeting. Thus, nanobubbles would be an attractive approach for US-mediated cancer theranostics. In our own work, we previously developed Bubble liposomes (BLs), which are lipid-coated perfluoropropane nanobubbles encapsulated in liposomes. We reported that BLs worked as US-mediated theranostic agents (Suzuki et al., 2008, 2010; Oda et al., 2012; Negishi et al., 2013). However, BLs contain lower concentrations of perfluoropropane than other microbubbles because of their smaller volumes due to size reduction. For example, Definity has 200 μL/mg lipid of C_3F_8; in contrast, BLs have about 15 μL/mg lipid of C_3F_8. Therefore, in order to reduce the injection volume and to improve echogenicity, it was important to develop BLs with higher gas content.

Generally, highly fluorinated compounds have low miscibility in both hydrophilic and hydrophobic solvents, but high miscibility in fluorous solvents such as perfluorinated alkanes. Based on these points, we hypothesized that nanobubbles containing fluoro-lipids could effectively encapsulate perfluorocarbons due to a favorable interaction between the lipids and the fluorous gas. In the present study, we attempted the development of BLs capable of containing high levels of C_3F_8. Specifically, we prepared 5 kinds of fluoro-lipids and examined the C_3F_8 encapsulation and echogenicity of BLs containing each of these components.

2. Materials and methods

2.1. Structure elucidation

Materials were obtained from commercial suppliers and used without further purification, unless otherwise noted. Melting points were determined using a micro melting point apparatus (Yanaco New Science Inc., Kyoto, Japan) and are presented as uncorrected values. NMR spectra were recorded at 23 °C on a JNM-ECS 400 spectrometer (JEOL RESONANCE Inc., Tokyo, Japan) at 400 MHz (for ^{1}H NMR) or 100 MHz (for ^{13}C NMR). Chemical shifts are given in parts per million (ppm) downfield from tetramethylsilane as an internal standard; coupling constants (*J*) are reported in hertz (Hz). Splitting patterns are abbreviated as follows: singlet (s), doublet (d), triplet (t), quartet (q), quintet (quin), multiplet (m), or broad (br). IR spectra were recorded on a FT/IR-4200 FT-IR spectrometer (JASCO Co., Tokyo, Japan). High-resolution mass spectra (HRMS) were recorded on a LCMS-IT-TOF mass spectrometer (in ESI mode) (Shimadzu Co., Kyoto, Japan). Optical rotations were determined with a P-2200 digital polarimeter (JASCO Co.). Analytical thin-layer chromatography was performed on silica gel 60 F-254 plates (Merck KGaA Group, Darmstadt, Germany). Column chromatography was performed using silica gel (45–60 μm, Fuji Silysia Chemical Ltd., Aichi, Japan). High-pressure liquid chromatography (HPLC) was performed with a Prominence system (Shimadzu Co.).

2.2. Preparation of BLs

2-F_1DPPC was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). 2-F_1DSPC, 1,2-F_2DSPC, 2,2-F_2DSPC, and 2,2,2-F_3-DSPC were synthesized in house. Liposomes consisting of 1,2-distearoyl-sn-glycero-phosphatidylcholine (DSPC), (NOF Co., Tokyo, Japan), 1,2-distearoyl-sn-glycero-3-phosphatidyl-ethanolamine-methoxypolyethyleneglycol (DSPE-PEG(2k)-OMe) (NOF Co.), and fluoro-lipids 44: 6: 50 (mol ratio), were prepared by reverse-phase evaporation. BLs were prepared from the liposomes and perfluoropropane (Takachiho Chemical Industrial Co., Ltd., Tokyo, Japan) as previously reported (Suzuki et al., 2008, 2007). Briefly, 5-mL sterilized vials containing 2 mL of the liposome suspension (lipid concentration: 1 mg/mL) were filled with perfluoropropane, capped, and then supercharged with another 7.5 mL of perfluoropropane. The vials were placed in a bath-type sonicator (42 kHz, 100 W; BRANSONIC 2510J-DTH, Branson Ultrasonics Co., Danbury, CT, USA) for 5 min to form the BLs. Under these conditions, the liposomes are re-constituted by sonication under the condition of supercharge with perfluoropropane in the 5-mL vial. At the same time, perfluoropropane is entrapped within micelles composed of lipids as bubbles, thus forming nanobubbles. The lipid nanobubbles are encapsulated within the reconstituted liposomes, the sizes of which are increased from 150 to 200 nm to ~1000 nm.

2.3. Measurement of sizes of BLs

The BLs were removed to disposable cuvette and the sizes were measured by dynamic light scattering (ELS-Z, Otsuka Electronics Co., Ltd., Osaka, Japan). These data were analyzed with CONTIN method.

2.4. Quantification of C_3F_8 in BLs

Five-hundred microliter of BLs were put into three vials (designed for use in the headspace auto sampler of a gas chromatograph (GC)) and the vials were capped. The samples were sonicated with a bath-type sonicator for 1 min to disrupt the bubbles. The samples then were analyzed using an automatic headspace sampler (PerkinElmer Inc., Wellesley, MA, USA) coupled to the GC (GC-2014; Shimadzu Co.) connected to a flame ionization detector. The amount of C_3F_8 contained in the BLs was determined by comparing the mean area (calculated across the three samples) to a standard curve.

2.5. Echogenicity of BLs as US contrast agent

Degased phosphate-buffered saline (PBS; 500 mL) was transferred to a beaker and maintained with stirring in a thermostatic bath set to 37 °C. BLs (100 μL) were added to the PBS, and the US contrasts and contrast mean power were monitored by ultrasonograpy (VEVO 2100; FUJIFILM VisualSonics Inc., Toronto, ON, Canada) using a non-linear contrast mode for 0–30 min with the following parameters: frequency = 18 MHz (MS-250 linear array transducer), transmit power = 4%, contrast gain = 35 dB. Time-intensity plots were generated for 116 mm^2 ROIs avoiding multiple-reflection echo (Denbeigh et al., 2014; Ingrid Leguerney et al., 2015). Survival ratio was calculated as the contrast intensity at each time point/maximum contrast intensity × 100%.