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Perfluorocarbon nanoemulsions with fluorescent, colloidal and magnetic properties

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ABSTRACT

Bimodal imaging agents that combine magnetic resonance imaging (MRI) and nearinfrared (NIR) imaging formulated as nanoemulsions became increasingly popular for imaging inflammation *in vivo*. Quality of *in vivo* imaging using nanoemulsions is directly dependent on their integrity and stability. Here we report the design of nanoemulsions for bimodal imaging, where both photostability and colloidal stability are equally addressed. A highly chemically and photo stable quaterrylenediimide dye was introduced into perfluoro-15-crown-5 ether (PCE) nanoemulsions. The nanoemulsions were prepared with PCE and Miglyol 812N mixed at 1:1 v/v ratio as internal phase stabilized by non-ionic surfactants. Data shows exceptional colloidal stability demonstrated as unchanged droplet size (~130 nm) and polydispersity (<0.15) after 182 days follow up at both 4 and 25 °C. Nanoemulsions also sustained the exposure to mechanical and temperature stress, and prolonged exposure to light without changes in droplet size, ^{19}F signal or fluorescence signal. No toxicity was observed *in vitro* in model inflammatory cells upon 24 h exposure while confocal microscopy showed that nanoemulsions droplets accumulated in the cytoplasm. Overall, our data demonstrates that design of bimodal imaging agents requires consideration of stability of each imaging component and that of the nanosystem as a whole to achieve excellent imaging performance.

1. Introduction

Introduction of multimodality into the development of molecular imaging agents has become common practice in preclinical research in recent years [1–6]. The multimodal imaging approaches combine the advantages of each individual imaging modality aiming to improve imaging accuracy and better characterize disease processes. Specifically, bimodal imaging agents that combine magnetic resonance imaging (MRI) and optical imaging have grown in popularity [7–16]. MRI provides high-resolution images with no tissue penetration limitation and moderate instrumentation costs, and is widely used in clinical and preclinical imaging studies. Optical imaging has high sensitivity and resolution, and is suitable for cell imaging and small animal studies. Tissue absorption and autofluorescence is at a minimum in the near infrared (NIR) optical window (650–900 nm). Therefore, NIR fluorescence is typically used for *in vivo* optical imaging applications.

Recently several perfluorocarbon (PFC) nanoemulsions were reported as MR-optical bimodal imaging platforms [7,17,18]. PFCs are biologically inert, background-free MRI reagents that can be quantitatively detected *in vivo* by ^{19}F MRI. [19,20] ^{19}F MRI emerged as a unique non-invasive quantitative molecular imaging technique where ^{19}F nuclei provide specific signal for the introduced PFC molecule. This allows unambiguous *in vivo* monitoring of the injected PFC nanoemulsion and quantitative monitoring of its biodistribution [19,20]. In addition, NIR dyes had been incorporated into PFC nanoemulsions to provide fluorescence signature.

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Although PFC nanoemulsions have been shown to have great potential as bimodal agents, there are several issues needed to be addressed. PFC nanoemulsion degradation is a known problem since the early 90s when they were formulated as blood substitutes [21]. Colloidal stability problems in PFC nanoemulsions can lead to changes in the nanoemulsion biodistribution *in vivo* and consequently erroneous MR and NIR imaging conclusions. Once injected *in vivo*, nanoemulsion is exposed to complex biological environment in the circulatory system, which could further accelerate the nanodroplet degradation.

In addition to the nanoemulsion colloidal stability, the photostability of NIR dye incorporated into the nanosystem has large effect on its fluorescence intensity, imaging time and signal quantification. The photostability of the dye is therefore critical for the overall quality of the bimodal imaging nanosystem. Most NIR dyes used in biomedical imaging belong to the polymethine cyanine dye family, which suffers from poor photo and chemical stability [22]. As such, long-term imaging and signal quantification can prove challenging. We have recently shown that a widely used lipophilic NIR probe (1,1′-Dioctadecyl-3,3,3′,3′-Tetramethylindotricarbocyanine Iodide, DiR) when incorporated into a perfluoropolyether (PFPE) nanoemulsion, quickly loses fluorescence upon storage due to degradation to a lower wavelength emitting byproduct [17]. Further, the methods of incorporating NIR dyes into PFC nanoemulsions for bimodal imaging purpose also must be carefully considered. The overall chemical environment of the NIR dye when incorporated into the PFC nanoemulsions will impact its fluorescence properties and photostability. NIR dyes are commonly introduced into the PFC nanoemulsions by either incorporation into the surfactant layer [23,24] or droplet surface conjugation [25]. In either approach, there is a possibility of dissociation of the NIR dye from the PFC nanodroplet due to chemical degradation mechanisms or colloidal destabilization. Our group recently reported the formulations where PFPE was combined with a NIR reporter for bimodal imaging and drug delivery [17,18,26]. In these nanoemulsions, the lipophilic NIR tracer was introduced into the hydrocarbon oil core of the nanoemulsion droplet, providing adequate environment for the NIR dye fluorescence and minimizing its exposure to aqueous environment, which for cyanine dyes can lead to loss of fluorescence. By this approach, the PFC nanoemulsion can serve as stabilizing carrier for poorly water soluble NIR dyes and increase the dye performance for imaging. As a lipophilic NIR dye we chose to synthesize a highly stable quaterrylene based structure that is fluorescent in lipid rich environments, while it has minimal fluorescence in aqueous solutions. In our recent report, we demonstrated that quaterrylene based dendrimeric NIR dye showed superior photo and chemical stability [27]. Moreover, the fluorescence performance of these quaterrylene dyes enhanced dramatically in lipophilic micelle environment compared to that in aqueous solutions [27]. Therefore, the high affinity for the lipid environment combined with high photostability was expected to add to overall photostability of the bimodal PFC nanosystem.

In this study, we set out to develop perfuoro-15-crown-5 ether (PCE) nanoemulsions incorporated with quaterrylene dye molecules and perform comprehensive *in vitro* evaluation of the nanoemulsion's properties under biologically relevant conditions and upon prolonged storage. The synergistic approach of combining highly stable NIR dye and nanoemulsions is expected to bring the nanoemulsion based bimodal imaging to a new level.

2. Materials and methods

2.1. Synthesis of NIR dyes

The solvents used are of commercial grade. Compound **1** was synthesized by following the reported procedures [27]. Silica gel (standard grade, 60A, Sorbtech) column chromatography was used to purify synthesized compounds. The ^{1}H and ^{13}C NMR spectra were recorded on the Brucker DRX 300 MHz and Brucker Avance III 400 MHz instruments. MALDI-TOF mass spectra were recorded on a PerSeptive Voyager STR MS spectrometer. UV/Vis spectra were recorded on a Cary 100 Bio UV-Vis spectrophotometer, and fluorescence spectra were recorded on a Cary Eclipse fluorescence spectrophotometer.

2.1.1. Compound **2**

1 (2.16 g, 3 mmol), 3-hydroxypyridine (0.63 g, 6.6 mmol), and potassium carbonate (0.91 g, 6.6 mmol) were stirred in N-methyl-2-pyrrolidone (250 mL) at 80 °C under argon atmosphere for 6.5 h. After being cooled to room temperature, the mixture was poured into brine (1 L). The precipitate was collected, washed with water and dried. The crude product was purified over silica gel column chromatography using ethyl acetate/dichloromethane (3/40) as the eluent resulting in **2** (1.07 g, 48%) as a red solid. ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C, TMS): δ = 9.24 (d, J = 7.8 Hz, 1H; ArH), 9.01 (d, J = 8.7 Hz, 1H; ArH), 8.49 (t, J = 3.3 Hz, 2H; ArH), 8.42–8.44 (m, 2H; ArH), 8.37 (d, J = 8.1 Hz, 1H; ArH), 8.31 (s, 1H; ArH), 8.29 (s, 1H; ArH), 7.89 (d, J = 8.4 Hz, 1H; ArH), 7.70 (t, J = 8.1 Hz, 1H; ArH), 7.38–7.47 (m, 3H; ArH), 7.27–7.33 (m, 4H; ArH), 2.68 (sep, J = 6.6 Hz, 2H; C<u>H</u>$(CH_3)_2$), 1.12 (d, J = 6.6 Hz, 12H; CH(C<u>H</u>$_3)_2$). ^{13}C NMR ($CDCl_3$): δ = 162.73, 152.35, 145.59, 140.81, 132.13, 131.60, 131.19, 130.09, 129.62, 128.91, 128.01, 127.66, 127.28, 126.75, 126.27, 125.37, 125.18, 125.06, 124.62, 124.03, 122.33, 29.69, 29.14, 23.99.

2.1.2. Compound **3**

2 (0.75 g, 1 mmol), bis(pinacolato)diborane (0.39 g, 1.5 mmol), and potassium acetate (0.31 g, 3 mmol) were stirred in dioxane (30 mL) under argon atmosphere, and then $PdCl_2$ (dppf) (40 mg, 0.05 mmol) was added. The mixture was heated to 70 °C for 24 h. After being cooled to room temperature, the solvent was removed by rotary evaporation. The crude product was purified over silica gel column chromatography using methanol/dichloromethane (3/100) as the eluent resulting in **3** (0.4 g, 50%) as a red solid. ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C, TMS): δ = 9.19 (dd, J = 1.2, 8 Hz, 1H; ArH), 9.15 (d, J = 7.6 Hz, 1H; ArH), 8.90 (dd, J = 1.2, 8.4 Hz, 1H; ArH), 8.50 (dd, J = 7.8 Hz, 2H; ArH), 8.40 (d, J = 4.4 Hz, 2H; ArH), 8.35 (s, 2H; ArH), 8.17 (d, J = 8 Hz, 1H; ArH), 7.64 (t, J = 8 Hz, 1H; ArH), 7.46 (t, J = 8 Hz, 1H; ArH), 7.35–7.39 (m, 2H; ArH), 7.27–7.32 (m, 4H; ArH), 2.71 (sep, J = 6.8 Hz, 2H; C<u>H</u>$(CH_3)_2$), 1.43 (s, 12H; OC(C<u>H</u>$_3)_2$ C(C<u>H</u>$_3)_2$O), 1.15 (d, J = 6.8 Hz, 12H; CH(C<u>H</u>$_3)_2$).

2.1.3. Compound **4**

2 (376 mg, 0.5 mmol) and **3** (397 mg, 0.5 mmol) were dissolved in freshly distilled toluene (50 mL). A solution of potassium carbonate (210 mg, 1.5 mmol) in H_2O (2 mL) and ethanol (0.2 mL) was purged with argon for 10 min and added to the toluene solution under argon atmosphere. After $Pd(PPh_3)_4$ was added, the resulting mixture was stirred at 80 °C for 24 h. The reaction mixture was then cooled to room temperature and concentrated by rotary evaporation. The crude product was purified by column chromatography (silica gel) using methanol/dichloromethane (3/100 to 1/10) as the eluent resulting in **4** (490 mg, 74%) as a claret solid. ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C, TMS): δ = 9.34 (d, J = 8 Hz, 2H; ArH), 9.24 (dd, J = 1.2, 8 Hz, 2H; ArH), 8.54 (d, J = 2.8 Hz, 2H; ArH), 8.50 (d, J = 2.8 Hz, 2H; ArH), 8.46 (dd, J = 1.2, 4.8 Hz, 2H; ArH), 8.44 (dd, J = 1.2, 4.8 Hz, 2H; ArH), 8.37 (s, 2H; ArH), 8.36 (s, 2H; ArH), 7.65 (d, J = 8 Hz, 2H; ArH), 7.61 (dd, J = 1.2, 8 Hz, 2H; ArH), 7.51–7.52 (m, 1H; ArH), 7.45–7.5 (m, 7H; ArH), 7.31–7.38 (m, 8H; ArH), 2.7–2.75 (m, 4H; C<u>H</u>$(CH_3)_2$), 1.17 (d, J = 4 Hz, 12H; CH(C<u>H</u>$_3)_2$), 1.15 (d, J = 4 Hz, 12H; CH(C<u>H</u>$_3)_2$). ^{13}C NMR ($CDCl_3$): δ = 162.94, 152.66, 152.56, 152.46, 145.77, 145.74, 145.62, 145.54, 140.97, 140.83, 140.61, 132.93, 132.06, 130.48, 129.80, 129.61, 129.38, 129.25, 128.62, 128.39, 128.08, 127.38, 127.37, 127.35, 125.73, 125.61, 125.43, 125.27, 124.84, 124.80, 124.46, 124.21, 122.30, 122.29, 29.29, 24.19, 24.18.

2.1.4. QR-4Py

A mixture of **4** (490 mg, 0.37 mmol) and potassium carbonate (1.27 g, 9.2 mmol) in ethanolamine (5 mL) was heated at 160 °C for 1 h under argon atmosphere. After being cooled to room temperature, the mixture was poured into chloroform (50 mL). The inorganic salt was removed by filtration, and the filtrate was concentrated and purified over silica gel column chromatography using ethyl acetate/dichloromethane (1/2) and methanol/dichloromethane (1/10) as the eluents. The compound was further purified by precipitation in chloroform/ethyl ether. After being dried in vacuum oven overnight, QR-4Py (280 mg, 57%) was obtained as a green solid. ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C, TMS): δ = 9.41 (d, J = 8.8 Hz, 4H; ArH), 8.57 (d, J = 8.8 Hz, 4H; ArH), 8.55 (d, J = 3.2 Hz, 4H; ArH), 8.45 (dd, J = 1.2, 4.4 Hz, 4H; ArH), 8.36 (s, 4H; ArH), 7.45–7.49 (m, 6H; ArH), 7.34–7.36 (m, 4H; ArH), 7.32 (d, J = 8 Hz, 4H; ArH), 2.71 (sep, J = 6.8 Hz, 4H; C<u>H</u>$(CH_3)_2$), 1.16 (d, J = 6.8 Hz, 24H; CH(C<u>H</u>$_3)_2$). ^{13}C NMR ($CDCl_3$): δ = 162.75, 152.65, 152.54, 145.61, 145.55, 140.90, 131.84, 130.43, 129.89, 129.61, 127.69, 127.59, 127.34, 125.39, 124.83, 124.68, 124.50, 124.07, 123.21, 121.95, 29.17, 24.03. MS (MALDI-TOF): m/z = 1331.70, calcd. for $C_{88}H_{63}N_6O_8$ $[M + H]^+$ m/z = 1331.47.

2.2. Photostability of the nanoemulsions

To test the photostability of the QR-4Py nanoemulsions (PM2, Table 1), we compared the photobleaching behavior of PM2 with that of DiR nanoemulsions (PM1, Table 1). Nanoemulsion solutions in pure Millipore water were kept in disposable cuvettes sealed with parafilm. The initial peak fluorescence intensities of the nanoemulsion solutions were adjusted to about 900 units and the cuvettes were