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Perfluorocarbon nanoemulsions with fluorescent, colloidal and magnetic properties

Jelena M. Janjic^{a,*,1}, Pin Shao^{b,1}, Shaojuan Zhang^{b,c}, Xun Yang^d, Sravan K. Patel^a, and Mingfeng Bai^{b,e,f,**}

^aGraduate School of Pharmaceutical Sciences, Mylan School of Pharmacy, Duquesne University, Pittsburgh, PA 15282, USA

^bMolecular Imaging Laboratory, Department of Radiology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15219, USA

^cDepartment of Diagnostic Radiology, The First Hospital of Medical School, Xi'an Jiaotong University, Xi'an, Shaanxi 710061, China

^dBayer School of Natural and Environmental Sciences, Duquesne University, Pittsburgh, PA 15282, USA

^eDepartment of Bioengineering, University of Pittsburgh, Pittsburgh, PA 15261, USA

^fUniversity of Pittsburgh Cancer Institute, Pittsburgh, PA 15232, USA

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

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*Corresponding author. 600 Forbes Avenue, 415 Mellon Hall, Duquesne University, Pittsburgh, PA 15282, USA. Tel.: +1 412 396 6369. **Corresponding author. 100 Technology Dr, Suite 452G, Pittsburgh, PA 15219, USA. Tel.: +1 412 624 2565. janjicj@duq.edu (J.M. Janjic), baim@upmc.edu (M. Bai).

¹Authors contributed equally.

Author contributions

Due to multidisciplinary nature of the presented work, J.M.J. is the first and corresponding author, S.P. is co-first author and M.B. is co-corresponding author. Individual author contributions are as follows: J.M.J. wrote the manuscript, designed key experiments, interpreted results, designed and prepared the nanoemulsions, and optimized NIR/¹⁹F MRI combinations in the bimodal nanosystem. S.P. wrote portions of the manuscript, designed and synthesized the NIR dyes, performed photostability experiments and analytical evaluations of the dyes and the bimodal nanosystem. M.B. designed the NIR reporters and key photostability experiments, wrote the manuscript. S.K.P. designed the fluorocarbon colloidal system, performed ¹⁹F NMR analyses and interpreted results. S.Z. performed cell culture and cell imaging experiments, interpreted results. X.Y. performed colloidal stability experiments, analyzed and interpreted data. All authors contributed to the final manuscript and preparation of key figures.

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.

Keywords

Fluorocarbon; Fluorescence; Macrophage; Nanoparticle; Cell viability; Confocal microscopy

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.

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'-Diocetadecyl-3,3,3',3'-Tetramethylindo-tricarbocyanine 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 quaterylene based structure that is fluorescent in lipid rich environments, while it has minimal fluorescence in aqueous solutions. In our recent report, we demonstrated that quaterylene based dendrimeric NIR dye showed superior photo and chemical stability [27]. Moreover, the fluorescence performance of these quaterylene 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 perfluoro-15-crown-5 ether (PCE) nanoemulsions incorporated with quaterylene 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 ^1H and ^{13}C NMR spectra were recorded on the Bruker DRX 300 MHz and Bruker 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. ¹H NMR (400 MHz, CDCl₃, 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; CH(CH₃)₂), 1.12 (d, J = 6.6 Hz, 12H; CH(CH₃)₂). ¹³C NMR (CDCl₃): 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 PdCl₂ (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. ¹H NMR (400 MHz, CDCl₃, 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; CH(CH₃)₂), 1.43 (s, 12H; OC(CH₃)₂ C(CH₃)₂O), 1.15 (d, J = 6.8 Hz, 12H; CH(CH₃)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 H₂O (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(PPh₃)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. ¹H NMR (400 MHz, CDCl₃, 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; CH(CH₃)₂), 1.17 (d, J = 4 Hz, 12H; CH(CH₃)₂), 1.15 (d, J = 4 Hz, 12H; CH(CH₃)₂). ¹³C NMR (CDCl₃): 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. ¹H NMR (400 MHz, CDCl₃, 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; CH(CH₃)₂), 1.16 (d, *J* = 6.8 Hz, 24H; CH(CH₃)₂). ¹³C NMR (CDCl₃): 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 C₈₈H₆₃N₆O₈ [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 kept close to the window and exposed to ambient light. Their emission spectra were recorded over time with excitation wavelength at 700 nm, and all the parameters were kept the same during the measurement.

2.3. Nanoemulsion preparation

Nanoemulsions were prepared following earlier reported procedures with some modifications [17,18]. Briefly, the pre-emulsification was done using planetary mixing on a Planetary Centrifugal Mixer “THINKY MIXER” ARE-310 at 2000 rpm speed for 90 s at room temperature under ambient pressure. The lipophilic NIR dyes (QR-4Py, DiR or CellVue® Burgundy) were dissolved at room temperature at desired concentration in Miglyol, and the labeled oil was combined with PFC and surfactant mixture using planetary high speed mixing forming coarse pre-emulsion. The pre-emulsion was immediately processed on a Microfluidizer M110S at 15,000 psi liquid pressure as reported previously [17,18]. Final nanoemulsions were allowed to equilibrate at room temperature for approximately 1 h before further handling. Bulk emulsions were stored at 4 °C until further testing.

2.4. Nanoemulsion characterization by DLS

All nanoemulsion samples were measured at 1 mL volume at 1:20 v/v dilution in deionized water. Nanoemulsion droplet size and polydispersity index (PDI) were determined by dynamic light scattering (DLS) using a Malvern Zetasizer Nano (Malvern Instruments, Worcestershire, United Kingdom) instrument. A HeNe gas laser (λ = 633 nm) with a detector at 173° was used to carry out measurements at 25°C.

2.5. Filtration stability test

We determined the size distribution of the nanoemulsion droplets before and after filtration. Samples (5–10 mL volume) of undiluted nanoemulsions were filtered using 0.22 μm syringe filters (Millex®-GS, Merck Millipore Ltd, MF-Millipore™ Membrane). DLS measurements were taken after the nanoemulsion samples were diluted in water (1:20 v/v) and allowed to equilibrate at room temperature for at least 30 min prior to each measurement. All DLS measurements were done at room temperature.

2.6. Stability test in model biological media

We determined the size distribution of the nanoemulsion droplets in different environments aimed to represent nanoemulsion environment during cell culture tests. Measurements were taken after the nanoemulsions were diluted (1:20 v/v) in water, serum free Dulbecco's modified Eagle medium (DMEM, Mediatech, Inc., Manassas, VA), 10% fetal bovine serum (FBS) DMEM, 20% FBS DMEM and incubated over time (up to 72 h) at 37 °C. Samples were allowed to equilibrate at room temperature for at least 30 min prior to each DLS measurement. All DLS measurements were done at room temperature. Nanoemulsion stability was assessed by plotting droplet diameter (Z average) and polydispersity versus time (hours or days).

2.7. Centrifugation stability test

We determined the size distribution of the nanoemulsion droplets before and after centrifugation. The nanoemulsions were diluted (1:20 v/v) in water, serum free DMEM, 10% FBS DMEM, or 20% FBS DMEM. Diluted nanoemulsions were centrifuged using Centrifuge 5804R (VWR, Eppendorf AG, 22331 Hamburg) at 1000 rpm for 10 min. The samples were then transferred to DLS cuvettes and measurements were taken after equilibrating at room temperature for at least 30 min prior to each measurement. All measurements were done at room temperature.

2.8. Zeta potential

Zeta potential of nanoemulsions was measured on 1 mL diluted nanoemulsions at 1:20 v/v with deionized (DI) water in zeta cells (folded capillary cells) on a Zetasizer Nano instrument. The data was averaged for three independent measurements. For each measurement, 30 runs were performed.

2.9. ^{19}F NMR and NIR fluorescence of nanoemulsion

Nanoemulsion PM3 (Table 1) loaded with QR-4Py dye was serially diluted with DI water and 0.2 mL of these dilutions were placed in NMR tubes. 0.2% v/v aqueous trifluoroacetic acid (TFA) was used as a reference at equal volume (0.2 mL) for all the dilutions. The highest dilution contained 0.2 mL nanoemulsion in 0.2 mL TFA reference solution. ^{19}F NMR spectra were recorded on a Bruker 500 MHz instrument. Quantification of PCE was performed based on the number of fluorine atoms under TFA ^{19}F NMR spectrum peak at -76.0 ppm. NIR fluorescence imaging was performed on serially diluted NMR samples using a Li-COR Odyssey imaging system with 2.5 mm offset and intensity of 2. Fluorescence emitted above 800 nm was collected for all the samples during the same run.

The fluorescence signal quantification was performed on the same samples prepared for NMR analysis. To minimize operator variability, the NMR tubes used for ^{19}F NMR analysis containing nanoemulsions were imaged on LiCOR Odyssey imager. Integrated values using LiCOR image processing software were used as quantitative measure of fluorescence. Representative NIR images of nanoemulsion dilutions are shown in Fig. S6 (Supporting Information).

2.10. Fluorescent imaging in cells

We used a mouse macrophage cell line RAW 264.7 for *in vitro* study. RAW 264.7 cells were cultured in DMEM containing 10% fetal bovine serum, 4 mM glutamine, 4500 mg/L glucose, 110 mg/L sodium pyruvate, 10 mM HEPES, 100 units/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin. RAW 264.7 cells were treated with 1.44 mg/mL of PM2 or PM1 at 37 °C for 24 h. After being washed for 3 times with serum free medium, cells were fixed with 4% paraformaldehyde/PBS for 20 min at room temperature. Cell nucleus was stained with 1 $\mu\text{g}/\text{mL}$ Hoechst for 15 min at room temperature. Cells were mounted and imaged under a Zeiss Axio Observer fluorescence microscope with ApoTome 2 imaging system. PM2 or PM1 fluorescent images were captured using a NIR camera with an ICG filter set (Excitation/emission: 750–800 nm/820–875 nm). Nuclear images were obtained with a DAPI filter set (Excitation/emission: 335–383 nm/420–470 nm). Differential interference contrast (DIC) images were obtained through Trans light DIC.

2.11. Cell viability assay

RAW 264.7 cells were treated with 0.00–4.96 mg/mL concentration of PM1 or PM2 for 24 h in a water jacketed incubator (37 °C, 5% CO_2). Cell viability was determined using CellTiter-Glo[®] Luminescent Cell Viability Assay kit (Promega) as per the manufacturer's instructions. CellTiter Glo Luminescent Cell Viability Assay depends on measuring the luminescence of viable cells in culture based on quantitation of the ATP present.

3. Results and discussion

3.1. Design of PCE/Miglyol/water nanoemulsion

The goal for the nanoemulsion formulation design was two-fold: 1) stabilize PCE into small size nanodroplets, which remain highly stable during storage and use; 2) provide a platform to carry lipophilic quaterrylene NIR dye with high photo and chemical stability.

PCE has been extensively studied as an ^{19}F MRI reagent for cell tracking [28,29]. PCE and many other small molecular weight PFC nanoemulsions are known to degrade by a process called Ostwald ripening which results in gradual droplet size increase over time and is driven by chemical potential difference between nanoemulsion droplets [30,31]. It was shown that this process is directly influenced by PFC molecular size, with longer PFC chains leading to slower degradation [32]. Because nanoemulsion integrity directly affects *in vivo* imaging quality and reproducibility, it should remain in a narrow droplet size and PDI range upon storage and use.

The earlier reported PCE formulations utilized 20% (v/v) PCE, 2% (w/v) safflower oil, 1.5% (w/v) of a surfactant co-mixture and 1.7% (w/v) glycerin in water [33]. This nanoemulsion had a small size (224 nm) but high PDI of 0.350, suggesting a heterogeneous droplet size distribution. Methods to stabilize PFC nanoemulsions have been extensively studied and a combination of surfactants has been found to be effective in suppressing degradation and droplet size growth [18,25,26,29,32,34,35]. However, there is no report to date of combining PCE with hydrocarbons into a nanoemulsion where the PCE and hydrocarbon are mixed at 1:1 v/v ratio as two-oil internal phase. Also, no reports exist where PCE was used for drug and/or dye delivery where these are introduced into the internal phase of nanoemulsion. Rather, in earlier reports of PCE nanoemulsions aimed at drug delivery, the surfactant layer incorporated the drug, dye or a targeting agent [24,29,36–38]. The rationale behind PCE/hydrocarbon 1:1 v/v ratio is to increase delivery capacity of the PCE nanoemulsion, providing enough hydrocarbon oil to solubilize a water-insoluble dye (or drug) while the PCE content is kept at sufficiently high level for future *in vivo* ^{19}F MRI. We found that PCE in a hydrocarbon oil-containing nanoemulsion is better stabilized than in a nanoemulsion where PCE is stabilized with surfactants as two phase system (PCE/water). Therefore, the nanoemulsions reported in this study were synthesized from PCE and Miglyol[®] 812N (Miglyol), which were kept at constant volume to volume ratio (1:1) in each formulation. The nanoemulsion droplets were stabilized by a combination of two non-ionic surfactants, Pluronic[®] P105 (P105) and Cremophor[®] EL (CrEL), incorporated at 2:3 w/w ratio and shown previously to stabilize PFPE nanoemulsions [17,18]. The resultant PCE nanoemulsions show small droplet size of only 130 nm with low polydispersity <0.15. Nanoemulsion formulations utilized in this study are summarized in Table 1.

Nanoemulsion processing is a critical factor in achieving a stable formulation. The process reported here combined planetary (rotation/revolution) mixing under atmospheric pressure [39] at room temperature in the pre-emulsification steps and high shear liquid processing (microfluidization) at 15,000 psi liquid pressure for size reduction [18,35]. Prior to emulsification, the surfactants are combined in water to form mixed micelles with average size of 14 nm following earlier reported procedure [18]. In the pre-emulsification step, dye-containing Miglyol, PCE and mixed micelle solution were combined by planetary mixing on a Thinky Mixer at 2000 rpm. The resultant coarse emulsion is further processed by microfluidization as reported earlier [18]. The combination of processing steps resulted in a colloiddally stable monodisperse nanoemulsion, Fig. 1. The novelty of this nanoemulsion design lies in combining PCE with Miglyol at 1:1 volume ratio. Although PCE stabilized by lipids has been reported [33], our nanoemulsion system in this paper has combined PCE and hydrocarbon oil as the internal phase. Scheme 1 shows the proposed nanoemulsion droplet structure and composition, where PCE is at the core of the droplet surrounded by a large volume fraction of Miglyol as the dye carrier. We have previously demonstrated that Miglyol can serve as a lipophilic drug carrier in a theranostic nanoemulsion [18]. However, the addition of Miglyol led to dramatic decrease in PCE nanoemulsion droplet size (~130 nm), compared to previous reports, where droplet size ranged from 180 to 250 nm [29,32,34,40–42]. Further, adding Miglyol at high volume fraction to the nanoemulsion system appears to have contributed to significant nanodroplet stabilization, Fig. 2.

3.2. Nanoemulsion colloidal properties and stability

To confirm the stabilizing role of Miglyol on PCE nanoemulsions, two emulsions were prepared side by side; emulsion PM4 with equal volumes of PCE and Miglyol stabilized by P105/CrEL mixed surfactant system (Table 1) and emulsion PM6 where the internal phase was PCE alone. We found that upon storage at 4 °C for 105 days, emulsion PM4 remained with unchanged droplet size and PDI, whereas emulsion PM6 (without Miglyol) nanodroplets increased in size from 140 nm to 165 nm (Fig. 1A–B). Extended monitoring for 182 days of the PCE/Miglyol nanoemulsion samples stored at both 4 and 25 °C showed no change in size and polydispersity, Fig. 1E.

This finding is important for two reasons. First, nanoemulsions with stable size distribution are preferred for MRI due to increased consistency in cell labeling, either *ex vivo* prior to cell introduction into the living animal [20] or *in vivo* by injection [19]. Droplet size growth indicates nanodroplet destabilization that can be further enhanced by nanodroplet environment (e.g. salts, protein, elevated temperature, etc.) during nanoemulsion use for cell tracking *in vivo*. Second, the high lipophilic volume fraction of Miglyol allows for accommodating significant concentration of insoluble dyes and/or drugs [17,18]. When the PCE/Miglyol was loaded with high concentration of lipophilic NIR dyes (DiR or QR-4Py), the droplet size (Fig. 1C) and zeta potential (Fig. 1D) remained unchanged. This finding indicates that PCE/Miglyol nanoemulsion can be used as carriers for chemically and optically stable lipophilic dyes which are not applicable in biomedical imaging alone due to the poor water solubility. We posit that this nanoemulsion could become a platform for future bimodal imaging agent design and theranostic development.

The shelf-life of nanoemulsions was also systematically monitored using DLS. The low droplet size for PCE/Miglyol nanoemulsions loaded with either DiR (PM1) or QR-4Py dye (PM2) remained unchanged upon storage at 4 °C, Fig. 2F. To the best of our knowledge, stability of PCE nanoemulsion reported here is unprecedented. Earlier reported non-ionic surfactants stabilized PCE nanoemulsions showed significant increase in droplet size over time during the first month of storage [43], which is consistent with the size increase shown here for nanoemulsion PM6 with PCE stabilized by surfactants, but lacking Miglyol (Fig. 1A). This further demonstrates that PCE nanoemulsions can be stabilized by a combination of processing, surfactants and high hydrocarbon oil volume fraction in the final product. To evaluate the potential of future biomedical imaging applications, PCE nanoemulsions were exposed to biologically representative *in vitro* conditions. The nanoemulsions were diluted in varied concentration of fetal bovine serum (FBS) containing cell culture media at 37 °C and incubated for up to 72 h. The nanoemulsion dilutions match that of cell labeling studies, where nanoemulsions were diluted in cell culture media and cells were co-cultured for hours to days prior to *in vivo* imaging studies [20,35]. We found that under these conditions, the nanoemulsion droplet size and polydispersity did not change significantly (Fig. 2A and B). In addition, we exposed the nanoemulsions to stress by mixing them with cell culture media and subjecting the mixture to centrifugation at room temperature for 10 min at 1000 rpm. We did not observe significant change in nanoemulsion droplet size (Z average) and polydispersity, as shown in Fig. 2C and D. Filtration through 0.2 µm is employed to obtain sterile nanoemulsion prior to *in vivo* injections for imaging. Therefore, we tested

nanoemulsion stability before and after filtration. No change in droplet size and polydispersity (Fig. 2E) was observed. These findings suggest that the nanoemulsion can serve as a very stable imaging probe and can sustain model stressors (e.g. elevated temperature, prolonged storage and filtration) comparable to those expected during future applications for *ex vivo* cell labeling for *in vivo* imaging.

3.3. Synthesis and photophysical properties of the optically stable NIR dye

Although NIR dye loaded PFC nanoemulsions have been reported [17,23], usually polymethine cyanine dyes are used, which have significant chemical and photostability issues [22]. In our previous study, we developed quaterylene based NIR dyes for biomedical imaging with superior photo and chemical stability [27]. To improve the stability of dual mode, NIR fluorescence and ^{19}F MRI reagent, we synthesized a new quaterylene dye that can be loaded into a lipophilic nanocarrier at high concentration and provide significant long-term signal stability necessary for future *in vivo* longitudinal imaging studies.

The structure and synthetic route to the optically stable lipophilic quaterylene dye QR-4Py is illustrated in Scheme 2. The starting material **1** was prepared by bromination of perylene monoimide following the reported method [44]. Next, the nucleophilic substitution of the two bromide atoms in the *bay*-regions of **1** with 3-hydroxypyridine afforded **2** with a moderate yield (48%), which is likely due to the competition reaction of all three reactive bromide sites. The original attempt of synthesizing biperylene **4** directly from **2** via Yamamoto coupling reaction failed. We then synthesized the intermediate boronic ester **3** via coupling from **2** and bis(pinacolato)diboron with $\text{PdCl}_2(\text{dppf})$ as the catalyst, followed by Suzuki coupling between **2** and **3** to provide **4** with a high yield of 74%. The next cyclodehydrogenation of **4** using K_2CO_3 in ethanolamine at 160°C was completed in 1 h and the target dye QR-4Py was obtained as a green powder in moderate yield (57%).

QR-4Py is highly soluble in chloroform and dichloromethane giving deeply green colored solutions. The absorption and emission spectra of QR-4Py in chloroform are displayed in Fig. 3A. QR-4Py showed intense absorption centered at 776 nm, as well as a shoulder peak at 705 nm. Its molar extinction coefficient is as high as $1.98 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$, which is comparable to those of traditional cyanine dyes [45]. Upon excitation, QR-4Py exhibited the maximum NIR fluorescence at 810 nm in chloroform.

3.4. Formulation of stable bimodal nanoemulsion

Recent studies have emphasized the advantages of using nanoemulsions for optical imaging as carriers for lipophilic fluorescent dyes [46]. The lipid nanodroplets represent attractive alternatives to inorganic and metal-based nanoparticles. It was suggested that nanoemulsions offer advantages such as biocompatibility and ability to increase brightness of non-cytotoxic organic dyes. Here we take this a step further by incorporating highly stable NIR lipophilic quaterylene dye (QR-4Py) into PCE nanoemulsions to provide bimodal MRI and NIR fluorescence imaging agents. PCE/hydrocarbon/water nanoemulsions are attractive as NIR dye carriers as they provide high capacity for dye loading and ^{19}F MRI capabilities. QR-4Py

dye was introduced into the PCE nanoemulsions by dissolving the dye in Miglyol oil first followed by mixing this oil with PCE during processing.

Prone to π - π stacking due to the highly rigid and planar aromatic structure, QR-4Py tends to aggregate and shows extremely poor solubility in aqueous solutions. Fig. 3B demonstrates its absorption and emission properties in different media. QR-4Py shows broad NIR absorption with maximum at 720 nm in DMEM, a widely used cell culture media. The relatively weak and blue shifted absorption compared to in emulsion or Miglyol is due to the poor solubility and aggregation of QR-4Py in the cell culture media. Moreover, no fluorescence signal was detected in DMEM, indicating an “OFF” state of fluorescence. When the solvent was changed to Miglyol, the triglyceride used to synthesize our tri-phasic nanoemulsions, the spectroscopic properties of QR-4Py changed significantly. QR-4Py is well dissolved in Miglyol as a green solution. It shows similar absorption profile in Miglyol to that in chloroform, with the maximum absorption at 766 nm. Importantly, when QR-4Py was dissolved in Miglyol, it became fluorescent (“ON” state) with an intense fluorescence peak at 797 nm. When the QR-4Py Miglyol was formulated into nanoemulsion, the absorption remained the same with slightly enhanced shoulder absorption at around 700 nm (Fig. 3B). The increased absorption in the high-energy region could be ascribed to the scattering of the nanoemulsion. The QR-4Py loaded nanoemulsions (PM2) exhibit desired NIR fluorescence with the maximum at 802 nm. Because of the scattering of the nanoemulsion, its fluorescence intensity is weaker than that in Miglyol under the same measurement conditions (see experimental section) provided that their NIR absorption is the same. Because of the high fluorescence of QR-4Py inside the nanoemulsions and quenched fluorescence signal in aqueous environment, QR-4Py is only fluorescent inside PM2 upon injection into the living system. This allows for accurate imaging of the nanoemulsions. Overall the data clearly demonstrates the need for Miglyol in the nanoemulsion to achieve NIR fluorescence of the QR-4Py dye. Fig. 3 shows the effect of formulation on fluorescent dye performance. The dye shows the high fluorescence in Miglyol containing nanoemulsion PM4 while PM6 nanoemulsion without Miglyol shows no measurable fluorescence signal on an NIR imager (Fig. 3C). Furthermore, there is a concentration dependent signal increase in nanoemulsions carrying QR-4Py dye at increasing concentrations, whereas the micelle solution shows no significant fluorescence (Fig. 3D), indicating that surfactants alone are unable to solubilize the dye or provide sufficiently lipophilic environment for its fluorescence. There is also a linear correlation between nanoemulsion concentrations and signal observed. Overall Fig. 3 demonstrates that formulation is critical for the high fluorescence signal of the QR-4Py dye, as this dye is fluorescent only in a lipid rich environment and shows no fluorescence in either aqueous solution or a simple surfactant solution.

3.5. Photostability of PCE nanoemulsions

Traditional NIR cyanine dyes suffer from poor photostability, which limits their applications when long time signal requisition is required or intense radiance is applied. We recently developed quaterrylenediimide dyes that showed dramatically improved photostability over indocyanine green (ICG), which is the only FDA approved NIR fluorescent and has been used as a gold standard [27]. Here we compared the photostability of the nanoemulsions

incorporated with QR-4Py (PM2) to the same nanoemulsions with lipophilic dialkylcarbocyanine dye DiR (PM1). In our earlier studies we successfully incorporated DiR into PFC nanoemulsions and showed this dye had no effects on nanoemulsion colloidal properties [17,18]. Therefore, DiR was used for the comparison. Fig. 4A compares the fluorescence intensity change of nanoemulsions PM1 and PM2 over time under the same measurement conditions. PM2, loaded with QR-4Py, showed negligible NIR fluorescence signal change when exposed to ambient light for 2 days (Fig. 4B); while the fluorescence intensity of PM1 carrying DiR decreased dramatically and reached baseline level within the same period of time (Fig. 4C). The outstanding photostability of PM2 over PM1 makes it a great NIR fluorescent imaging agent for long-term imaging study. A comparison was also made between nanoemulsions loaded with QR-4Py (PM4) and a commercially available lipophilic membrane tracer Cellvue® Burgundy (PM5) for the fluorescence loss during storage. As shown in Fig. 4D, PM4 remained with high fluorescence signal as compared to complete fluorescence loss of PM5 after 24 h, reiterating the high stability of QR-4Py in these nanoemulsions.

3.6. In vitro evaluation of the QR-4Py labeled PCE nanoemulsion

To confirm that the PCE/Miglyol nanoemulsion can be used for future *in vivo* ^{19}F MRI we tested it by ^{19}F NMR (Fig. S5A, Supporting Information). Based on ^{19}F NMR with TFA as the reference, the fluorocarbon content was calculated to be 113 mg/mL nanoemulsion. We have previously reported that the correlation of NIR signal to ^{19}F NMR is necessary for improved performance of the nanoemulsion as a bimodal imaging agent [7,18]. Fig. S5B (supporting information) shows linear correlation between the two types of imaging signals in series of nanoemulsion dilutions. Due to the lack of concentration dependent changes in correlation between MRI and NIR fluorescence signals, ^{19}F NMR and NIR fluorescence can be used in a complimentary fashion.

The colloiddally and optically stable bimodal nanoemulsions were designed for both *ex vivo* and *in vivo* cell labeling. The small droplet size, colloidal stability and exceptional NIR signal stability make this reagent ideal for future *in vivo* imaging studies where the NIR signal is monitored in the same animal longitudinally. The QR-4Py shows dramatically higher optical stability than two other commercially available dyes (DiR and CellVue® Burgundy dye) after being incorporated into PCE nanoemulsions. To study the intra-cellular uptake of the DiR loaded PCE nanoemulsion PM1 and QR-4Py loaded PCE nanoemulsion PM2, we imaged mouse macrophage cells (RAW 264.7) using fluorescence microscopy. The cells were incubated with 1.44 mg/mL of PM2 or PM1 at 37 °C for 24 h before fixing. Fluorescence images were then collected and compared. As shown in Fig. 5, both PM2 and PM1 showed strong fluorescence signal primarily localized in the cytoplasm, which is consistent with earlier reports [18]. It was also evident that DiR and QR-4Py show comparable fluorescence signal from nanoemulsion droplets in the cells.

Finally, we tested the cell toxicity of nanoemulsions PM2 and PM1. We used CellTiter-Glo® Luminescent Cell Viability Assay kit to evaluate cell toxicity, which is a commonly used method to determine the number of viable cells in culture based on luminescent quantitation of the ATP present, an indicator of metabolically active cells. As shown in Fig.

6, PM2 and PM1 exhibited comparable low cytotoxicity in RAW 264.7 cells to the untreated control.

4. Conclusion

We developed bimodal MRI and optical nanoemulsions with combined colloidal and optical stability where both imaging modalities retain their optimum performance over the life-time of the reagent. Systematic studies directly evaluating colloidal and optical stability of nanosystems design for bimodal imaging (e.g. optical and MRI combined) are lacking. This is especially true for nanoemulsions. Quality of *in vivo* imaging results is directly affected by the quality of imaging agent and we argue that colloidal stability and optical performance must be considered jointly if we are to use the nanoemulsion for multimodal imaging. These PCE/hydrocarbon/water nanoemulsions are exceptionally stable and show no change in nanodroplet size for more than 180 days. Such long-term colloidal stability is unprecedented for PCE nanoemulsions. In addition, a highly photo and chemically stable quaterylene dye was introduced into oil/water nanoemulsions and combined with ^{19}F MRI tracer into a dual mode imaging agent. The synergistic approach of combining highly stable NIR dye and nanoemulsions is expected to bring the nanoemulsion based bimodal imaging to a new level.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Thomas R, Park IK, Jeong YY. Magnetic iron oxide nanoparticles for multimodal imaging and therapy of cancer. *Int J Mol Sci.* 2013; 14(8):15910–30. [PubMed: 23912234]
2. Housni A, Boujraf S. Multimodal magnetic resonance imaging in the diagnosis and therapeutical follow-up of brain tumors. *Neurosciences.* 2013; 18(1):3–10. [PubMed: 23291791]
3. Lee DE, Koo H, Sun IC, Ryu JH, Kim K, Kwon IC. Multifunctional nanoparticles for multimodal imaging and theragnosis. *Chem Soc Rev.* 2012; 41(7):2656–72. [PubMed: 22189429]
4. Fattahi H, Laurent S, Liu F, Arsalani N, Vander Elst L, Muller RN. Magneto-liposomes as multimodal contrast agents for molecular imaging and cancer nanotheragnostics. *Nanomedicine.* 2011; 6(3):529–44. [PubMed: 21542690]
5. Swierczewska M, Lee S, Chen X. Inorganic nanoparticles for multimodal molecular imaging. *Mol Imaging.* 2011; 10(1):3–16. [PubMed: 21303611]
6. Pichler BJ, Judenhofer MS, Pfannenberger C. Multimodal imaging approaches: PET/CT and PET/MRI. *Handb Exp Pharmacol.* 2008; 185(Pt 1):109–32. [PubMed: 18626801]
7. Balducci A, Wen Y, Zhang Y, Helfer BM, Hitchens TK, Meng WS, et al. A novel probe for the non-invasive detection of tumor-associated inflammation. *Oncoimmunology.* 2013; 2(2):e23034. [PubMed: 23526711]

8. Wang XY, Ju S, Li C, Peng XG, Chen AF, Mao H, et al. Non-invasive imaging of endothelial progenitor cells in tumor neovascularization using a novel dual-modality paramagnetic/near-infrared fluorescence probe. *PloS One*. 2012; 7(11):e50575. [PubMed: 23226317]
9. Chen J, Guo Z, Wang HB, Gong M, Kong XK, Xia P, et al. Multifunctional Fe₃O₄@C@Ag hybrid nanoparticles as dual modal imaging probes and near-infrared light-responsive drug delivery platform. *Biomaterials*. 2013; 34(2):571–81. [PubMed: 23092859]
10. Ma LL, Tam JO, Willsey BW, Rigdon D, Ramesh R, Sokolov K, et al. Selective targeting of antibody conjugated multifunctional nanoclusters (nanoroses) to epidermal growth factor receptors in cancer cells. *Langmuir*. 2011; 27(12):7681–90. [PubMed: 21591638]
11. Lee CM, Jang D, Kim J, Cheong SJ, Kim EM, Jeong MH, et al. Oleyl-chitosan nanoparticles based on a dual probe for optical/MR imaging in vivo. *Bioconjug Chem*. 2011; 22(2):186–92. [PubMed: 21243999]
12. Kumar R, Ohulchanskyy TY, Turowski SG, Thompson ME, Seshadri M, Prasad PN. Combined magnetic resonance and optical imaging of head and neck tumor xenografts using Gadolinium-labelled phosphorescent polymeric nanomicelles. *Head Neck Oncol*. 2010; 2:35. [PubMed: 21110873]
13. Melancon MP, Lu W, Huang Q, Thapa P, Zhou D, Ng C, et al. Targeted imaging of tumor-associated M2 macrophages using a macromolecular contrast agent PG-Gd-NIR813. *Biomaterials*. 2010; 31(25):6567–73. [PubMed: 20537382]
14. Melancon MP, Wang Y, Wen X, Bankson JA, Stephens LC, Jasser S, et al. Development of a macromolecular dual-modality MR-optical imaging for sentinel lymph node mapping. *Invest Radiol*. 2007; 42(8):569–78. [PubMed: 17620940]
15. Shan L, Wang S, Sridhar R, Bhujwalla ZM, Wang PC. Dual probe with fluorescent and magnetic properties for imaging solid tumor xenografts. *Mol Imaging*. 2007; 6(2):85–95. [PubMed: 17445503]
16. Uzgiris EE, Sood A, Bove K, Grimmond B, Lee D, Lomnes S. A multimodal contrast agent for preoperative MR imaging and intraoperative tumor margin delineation. *Technol Cancer Res Treat*. 2006; 5(4):301–9. [PubMed: 16866560]
17. Patel SK, Patrick MJ, Pollock JA, Janjic JM. Two-color fluorescent (NIR and visible) triphasic perfluorocarbon nanoemulsions. *J Biomed Opt*. 2013; 18(10):101312. [PubMed: 23912666]
18. Patel SK, Zhang Y, Pollock JA, Janjic JM. Cyclooxygenase-2 inhibiting per-fluoropoly (ethylene glycol) ether theranostic nanoemulsions-in vitro study. *PloS One*. 2013; 8(2):e55802. [PubMed: 23409048]
19. Hitchens TK, Ye Q, Eytan DF, Janjic JM, Ahrens ET, Ho C. 19F MRI detection of acute allograft rejection with in vivo perfluorocarbon labeling of immune cells. *Magn Reson Med*. 2011; 65(4):1144–53. [PubMed: 21305593]
20. Srinivas M, Turner MS, Janjic JM, Morel PA, Laidlaw DH, Ahrens ET. In vivo cytometry of antigen-specific t cells using 19F MRI. *Magn Reson Med*. 2009; 62(3):747–53. [PubMed: 19585593]
21. Lowe KC. Second-generation perfluorocarbon emulsion blood substitutes. *Artif Cell Blood Substit Immobil Biotechnol*. 2000; 28(1):25–38.
22. Mishra A, Behera RK, Behera PK, Mishra BK, Behera GB. Cyanines during the 1990s: a Review. *Chem Rev*. 2000; 100(6):1973–2012. [PubMed: 11749281]
23. Akers WJ, Kim C, Berezin M, Guo K, Fuhrhop R, Lanza GM, et al. Noninvasive photoacoustic and fluorescence sentinel lymph node identification using dye-loaded perfluorocarbon nanoparticles. *ACS Nano*. 2011; 5(1):173–82. [PubMed: 21171567]
24. Akers WJ, Zhang Z, Berezin M, Ye Y, Agee A, Guo K, et al. Targeting of alpha(nu)beta(3)-integrins expressed on tumor tissue and neovasculature using fluorescent small molecules and nanoparticles. *Nanomedicine*. 2010; 5(5):715–26. [PubMed: 20662643]
25. Lim YT, Noh YW, Kwon JN, Chung BH. Multifunctional perfluorocarbon nanoemulsions for (19)F-based magnetic resonance and near-infrared optical imaging of dendritic cells. *Chem Commun*. 2009; 45:6952–4.
26. O'Hanlon CE, Amede KG, O'Hear MR, Janjic JM. NIR-labeled perfluoropolyether nanoemulsions for drug delivery and imaging. *J Fluor Chem*. 2012; 137:27–33. [PubMed: 22675234]

27. Shao P, Bai M. Photostable, hydrophilic and functional near infrared quaterrylene diimide-cored dendrimers for biomedical imaging. *Chem Commun.* 2012; 48(76):9498–500.
28. Lanza GM, Winter PM, Caruthers SD, Hughes MS, Hu G, Schmieder AH, et al. Theragnostics for tumor and plaque angiogenesis with perfluorocarbon nanoemulsions. *Angiogenesis.* 2010; 13(2): 189–202. [PubMed: 20411320]
29. Lee SJ, Schlesinger PH, Wickline SA, Lanza GM, Baker NA. Interaction of melittin peptides with perfluorocarbon nanoemulsion particles. *J Phys Chem B.* 2011; 115(51):15271–9. [PubMed: 22050303]
30. Nazarzadeh E, Anthonypillai T, Sajjadi S. On the growth mechanisms of nanoemulsions. *J Colloid Interface Sci.* 2013; 397:154–62. [PubMed: 23452515]
31. Wooster TJ, Golding M, Sanguansri P. Impact of oil type on nanoemulsion formation and Ostwald ripening stability. *Langmuir.* 2008; 24(22):12758–65. [PubMed: 18850732]
32. Janjic JM, Ahrens ET. Fluorine-containing nanoemulsions for MRI cell tracking. *Wiley Interdiscip Rev Nanomed Nanobiotechnol.* 2009; 1(5):492–501. [PubMed: 19920872]
33. Partlow KC, Chen J, Brant JA, Neubauer AM, Meyerrose TE, Creer MH, et al. ¹⁹F magnetic resonance imaging for stem/progenitor cell tracking with multiple unique perfluorocarbon nanobeacons. *FASEB J.* 2007; 21(8):1647–54. [PubMed: 17284484]
34. Rapoport N. Phase-shift, stimuli-responsive perfluorocarbon nanodroplets for drug delivery to cancer. *Wiley Interdiscip Rev Nanomed Nanobiotechnol.* 2012; 4(5):492–510. [PubMed: 22730185]
35. Janjic JM, Srinivas M, Kadayakkara DK, Ahrens ET. Self-delivering nanoemulsions for dual fluorine-19 MRI and fluorescence detection. *J Am Chem Soc.* 2008; 130(9):2832–41. [PubMed: 18266363]
36. Pan H, Soman NR, Schlesinger PH, Lanza GM, Wickline SA. Cytolytic peptide nanoparticles ('NanoBees') for cancer therapy. *Wiley Interdiscip Rev Nanomed Nanobiotechnol.* 2011; 3(3): 318–27. [PubMed: 21225660]
37. Caruthers SD, Cyrus T, Winter PM, Wickline SA, Lanza GM. Anti-angiogenic perfluorocarbon nanoparticles for diagnosis and treatment of atherosclerosis. *Wiley Interdiscip Rev Nanomed Nanobiotechnol.* 2009; 1(3):311–23. [PubMed: 20049799]
38. Soman NR, Lanza GM, Heuser JM, Schlesinger PH, Wickline SA. Synthesis and characterization of stable fluorocarbon nanostructures as drug delivery vehicles for cytolytic peptides. *Nano Lett.* 2008; 8(4):1131–6. [PubMed: 18302330]
39. Takatsuka T, Endo T, Jianguo Y, Yuminoki K, Hashimoto N. Nanosizing of poorly water soluble compounds using rotation/revolution mixer. *Chem Pharm Bull.* 2009; 57(10):1061–7. [PubMed: 19801859]
40. Rapoport N, Nam KH, Gupta R, Gao Z, Mohan P, Payne A, et al. Ultrasound-mediated tumor imaging and nanotherapy using drug loaded, block copolymer stabilized perfluorocarbon nanoemulsions. *J Control Release.* 2011; 153(1):4–15. [PubMed: 21277919]
41. Kaneda MM, Caruthers S, Lanza GM, Wickline SA. Perfluorocarbon nanoemulsions for quantitative molecular imaging and targeted therapeutics. *Ann Biomed Eng.* 2009; 37(10):1922–33. [PubMed: 19184435]
42. Lee SJ, Olsen B, Schlesinger PH, Baker NA. Characterization of perfluorooctylbromide-based nanoemulsion particles using atomistic molecular dynamics simulations. *J Phys Chem B.* 2010; 114(31):10086–96. [PubMed: 20684632]
43. Janjic, JM.; Ahrens, ET. Compositions and methods for producing emulsions for nuclear magnetic resonance techniques and other applications. US Patent 8,227,610. Issued July 24, 2012
44. Geerts Y, Quante H, Platz H, Mahrt R, Hopmeier M, Böhm A, Müllen K. Quaterrylenebis(dicarboximide)s: near infrared absorbing and emitting dyes. *J Mater Chem.* 1998; 8:2357–69.
45. Johnson, I.; Spence, MT., editors. *Molecular Probes® handbook: a guide to fluorescent probes and labeling technologies.* 11. Life Technologies; 2010. p. 66-73.
46. Klymchenko AS, Roger E, Anton N, Anton H, Shulov I, Vermot J, Melya Y, Vandamme TF. Highly lipophilic fluorescent dyes in nanoemulsions: towards bright non-leaking nano-droplets. *RCS Adv.* 2012; 2:11876–86.

Appendix A. Supplementary data

Supplementary data related to this article can be found online at <http://dx.doi.org/10.1016/j.biomaterials.2014.03.006>.

Fig. 1.

Size and zeta potential measurements of nanoemulsions using dynamic light scattering. A) Miglyol addition to PCE nanoemulsion formulation decreased average droplet size. Size distribution is shown for two nanoemulsions with (PM4) and without Miglyol (PM6) (Table 1). B) PM4 shows no significant change in size distribution after 105 days of storage. C) Presence of high loading of QR-4Py dye in PM2 shows no significant effects on size distribution. D) The nanoemulsion zeta potential is not significantly affected by the chemical structure of the incorporated NIR dye. The DiR and QR-4Py loaded nanoemulsions show identical profile. E) Extended monitoring of PCE/Miglyol nanoemulsion stability at two storage temperatures (4 and 25 °C) for 182 days.

Fig. 2.

Nanoemulsion stability testing by dynamic light scattering. Serum stability for nanoemulsion PM1 (A) and PM2 (B) at 37 °C where W = deionized water, D = serum free media, 10FD = 10% fetal bovine serum (FBS) in media; 20FD = 20% FBS in media. Centrifugation stability for nanoemulsion PM1 (C) and PM2 (D). Filtration stability for nanoemulsion PM1 and PM2 (E); Storage stability for nanoemulsion PM1 and PM2 (F), at 4 °C; In all plots standard deviation represents half width of polydispersity (PDIw/2) indicating the size distribution for each nanoemulsion under testing conditions.

Fig. 3.

The absorption and emission spectra of QR-4Py in (A) chloroform, (B) emulsion, Miglyol and cell culture media. C) NIR fluorescence images at 800 nm of decreasing concentrations of nanoemulsions with QR-4Py dye. D) Concentration dependent fluorescence signal in QR-4Py nanoemulsions (PM2, PM3 and PM4) and surfactant (micelle) solution alone.

Fig. 4.

A) The fluorescence intensity change over time when nanoemulsions were exposed to ambient light. B) The fluorescence spectra change of PM2 over time when exposed to ambient light. C) The fluorescence spectra change of PM1 over time when exposed to ambient light. D) NIR fluorescence images of triplicate samples of nanoemulsions before and after being stored at 37 °C for 24 h. Nanoemulsion PM4 loaded with QR-4Py dye was imaged at 800 nm, while PM5 loaded with CellVue® Burgundy dye was imaged at 700 nm.

Fig. 5.

Cell fluorescent imaging with DiR and QR-4Py loaded PCE nanoemulsions. RAW 264.7 cells were cultured for 24 h at 37 °C before imaging. Cells were incubated for 24 h with 1.44 mg/mL of DiR (PM1) or QR-4Py labeled PCE nanoemulsion (PM2). Fluorescent images were obtained using Zeiss Axio Observer fluorescent microscopy with ApoTome 2 imaging system. QR-4Py nanoemulsion cell staining is shown in A–D: (A) DAPI (blue), (B) QR-4Py (red), (C) QR-4Py (red) + DAPI (blue) merged, and (D) Differential Interference Contrast (DIC). DiR nanoemulsion cell staining is shown in E–H: (E) DAPI (blue), (F) DiR (red), (G) DiR (red) + DAPI (blue) merged and (H) DIC. Scale: 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 6. Cytotoxicity of Bimodal Nanoemulsions. To evaluate cytotoxicity of DiR (PM1) and QR-4Py (PM2) loaded nanoemulsions, RAW 264.7 cells were incubated with increasing nanoemulsion concentrations for 24 h. The nanoemulsion concentrations are presented as the total concentration of the PCE in mM. Cell viability was measured using CellTiter-Glo[®] Luminescent Cell Viability assay. Each data point represents the mean $\pm$ SD based on triplicated samples.

Scheme 1.

Structure and chemical composition of the nanoemulsion designed for bimodal (NIR and ^{19}F MR) molecular imaging.

Scheme 2.
The synthetic route of QR-4Py.

Table 1

Nanoemulsion compositions.

Nanoemulsion	PCE (mL)	Miglyol (mL)	Micelle solution (mL) ^a	Water (mL)	Dye (μM) ^b
PM1	1	1	11.5	11.5	DIR (10)
PM2	1	1	11.5	11.5	QR-4Py (10)
PM3	1	1	11.5	11.5	QR-4Py (5)
PM4	1	1	11.5	11.5	QR-4Py (1)
PM5	1	1	11.5	11.5	CellVue® Burgundy (0.24)
PM6	2	–	11.5	11.5	QR-4Py (1)

^a Micelle solution (5% w/v in deionized-water (DI water)) prepared with P105 and CrEL (2:3 w/w).

^b Dye concentration (μM) as added during processing is shown in parenthesis.