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To cite this article: Melissa L Hill et al 2013 Phys. Med. Biol. 58 5215

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Towards a nanoscale mammographic contrast agent: development of a modular pre-clinical dual optical/x-ray agent

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Received 24 April 2013, in final form 13 June 2013
Published 12 July 2013
Online at stacks.iop.org/PMB/58/5215

Abstract

Contrast-enhanced digital mammography (CEDM) can provide improved breast cancer detection and characterization compared to conventional mammography by imaging the effects of tumour angiogenesis. Current small-molecule contrast agents used for CEDM are limited by a short plasma half-life and rapid extravasation into tissue interstitial space. To address these limitations, nanoscale agents that can remain intravascular except at sites of tumour angiogenesis can be used. For CEDM, this agent must be both biocompatible and strongly attenuate mammographic energy x-rays. Nanoscale perfluorooctylbromide (PFOB) droplets have good x-ray attenuation and have been used in patients for other applications. However, the macroscopic scale of x-ray imaging (50–100 μm) is inadequate for direct verification that PFOB droplets localize at sites of breast tumour angiogenesis. For efficient pre-clinical optimization for CEDM, we integrated an optical marker into PFOB droplets for microscopic assessment (≪50 μm). To develop PFOB droplets as a new nanoscale mammographic contrast agent, PFOB droplets were labelled with fluorescent quantum dots (QDs). The droplets had mean diameters of 160 nm, fluoresced at 635 nm and attenuated x-ray spectra at 30.5 keV mean energy with a relative attenuation of 5.6 ± 0.3 Hounsfield units (HU) mg⁻¹ mL⁻¹ QD-PFOB. With the agent loaded into tissue phantoms, good correlation between x-ray attenuation and optical fluorescence was found ($R^2 = 0.96$), confirming co-localization of the QDs with PFOB for quantitative assessment using x-ray or optical methods. Furthermore, the QDs can be removed from the PFOB agent...
without affecting its x-ray attenuation or structural properties for expedited translation of optimized PFOB droplet formulations into patients.

(Some figures may appear in colour only in the online journal)

1. Introduction

X-ray mammography provides good sensitivity and specificity of breast cancer detection for most women (Barlow et al 2002, Pisano et al 2005). However, conventional mammography has a low sensitivity for cancers in radiographically dense breasts (Carney et al 2003) due to a combination of overlapping tissue signals that may obscure a lesion, and limited differences in x-ray attenuations between the healthy fibroglandular tissues and cancers (Johns and Yaffe 1987). One approach to increase the x-ray attenuation of a lesion relative to the surrounding tissue is by the use of an exogenous contrast agent. Compared to normal vasculature, blood vessels in solid tumours are often poorly formed and are leaky, with gaps forming in the vessel wall up to several hundred nanometres in diameter (Hashizume et al 2000). Clinical pilot studies of contrast-enhanced digital mammography (CEDM) have shown that this characteristic leakiness can be exploited to facilitate visualization and characterization of breast tumours using an intravenously administered contrast agent in combination with digital mammography (Jong et al 2003, Dromain et al 2006, 2011, Lewin et al 2003, Diekmann et al 2005, Thibault et al 2012).

Although the performance of CEDM using clinically available iodinated contrast agents is promising (Dromain et al 2012), these agents are not ideal for breast cancer detection and characterization (Diekmann et al 2007, Lawaczeck et al 2003, Suryanarayanan et al 2007), especially for imaging the leaky vasculature characteristic of tumour angiogenesis. These iodinated agents are water-soluble and are small enough (≈1 nm) to extravasate from normal vasculature into the extravascular–extracellular space. This results in the molecular agents being quickly cleared from the vascular space, with a plasma half-life on the order of minutes (Bourin et al 1997), such that enhanced image contrast in the tumour must be assessed within minutes after injection (Froeling et al 2013). This presents a limitation of a short working time for effective lesion detection, which could be a concern in exams with multiple views (Jochelson et al 2013), and for imaging large breasts, where tiling of the views may be necessary. Also, the non-specificity of the agent due to its small size results in a large dose of contrast media (≈1.5 mL kg\(^{-1}\)) being required in CEDM to elucidate the subtle differences between tissues with normal and tumour vasculature, because the media will be diluted in the whole of the body’s vascular and extracellular–extravascular spaces. This high dose of contrast agent is of concern because the toxicity poses an appreciable risk to patients, especially those with impaired renal function (Morcos and Thomsen 2001).

A biocompatible contrast agent of intermediate size (≈100 to 200 nm in diameter), which can selectively extravasate from leaky vasculature and provide good x-ray attenuation over the length of time required for examination, would be better suited for use in combination with CEDM than existing agents. Nanoscale PFOB (C\(_8\)F\(_{17}\)Br, PFOB) droplets are promising candidates for CEDM because they are well-tolerated, biologically compatible with a long history in patients (Reiss 2001, Castro and Briceno 2010, Kraft and Reiss 2007, Bruneton et al 1989, Behan et al 1993), and should have high x-ray absorption at CEDM energies due to the bromine atom in the molecule. As demonstrated in figure 1, it is predicted that, due to its superior x-ray attenuation in the mammographic imaging energy range, a lower concentration of bromine will be required to produce image contrast equivalent to that of iodine. As PFOB
QD-PFOB dual optical and x-ray contrast agent

Figure 1. Concentrations of iodine (dashed line) and bromine (solid line) in a 1 cm lesion required to produce 5% image contrast in CEDM between the lesion and surrounding breast tissue, as a function of x-ray energy (Hubbell and Seltzer 2004) (no x-ray scatter, no normal tissue contrast uptake, and an ideal detector assumed). The shaded regions indicate an approximate range of x-ray beam mean energies used in mammography conventional diagnostic x-ray imaging. Br is more attenuating than I in the highlighted mammographic energy region, requiring a lower concentration of Br for detectability, and the reverse is true at conventional diagnostic x-ray energies.

is hydrophobic, it is not miscible with water and must be emulsified for intravenous injection. Conveniently, the emulsified droplets formed tend to be between 100 and 200 nm in diameter; small enough to permeate through blood vessels with leaky vasculature, but too large to permeate normal vasculature (Danquah et al 2011).

We propose that the characteristics of PFOB make this material particularly well-suited for imaging breast tumours with CEDM. However, a comprehensive evaluation of any novel x-ray contrast agent for human use requires extensive pre-clinical assessment to determine the mechanism of agent localization, and to optimize the accumulation of the PFOB agent in breast tumours (Matsuura and Rowlands 2008). This is difficult because the signals observed in x-ray images at a macroscopic scale (> 100 μm) do not adequately reflect the microscopic interactions of the agent that determine its localization in relation to the tumour properties (i.e., the cancer cells, the gaps in the blood vessels, and the extravascular space). Ideally, for efficient pre-clinical assessment, the agent could be visualized under a microscope to confirm its spatial distribution at high-resolution.

In this work we develop nanoscale PFOB droplets that have dual x-ray and optical signals for evaluation in a pre-clinical environment. We propose to engineer these dual-modality droplets with appropriate characteristics of x-ray attenuation and size for their ultimate use in CEDM for sensitive and specific breast cancer detection. To allow efficient clinical translation, a modular design is employed so that the optical component could be removed without affecting the physical or x-ray properties of an optimized agent formulation.

To generate optical signal from the PFOB droplets, we use solid QD nanoparticles that are highly fluorescent and non-photobleaching, and that can be fluorinated for miscibilization into PFOB. Since the fluorinated QDs are not soluble in water or lipids, the QDs do not leak out of the PFOB droplets, and because the method of incorporation of the QDs into PFOB does not involve changing the PFOB molecule in any way, the QDs are added to the PFOB droplets in a modular fashion. Also, because the fluorination procedure is not specific to a nanoparticle type, different QDs can be similarly fluorinated for integration into PFOB, such that any type of QD can be interchanged to create a variety of application-dependent optical labels.
Figure 2. A block diagram illustrating the conditions (solid) and the methods of characterization (dashed) of three contrast agent materials. The PFOB compound proposed for clinical use in contrast-enhanced mammography, the dual-modality QD-PFOB material for pre-clinical development, and the PFC control compound were assessed: (a) as a raw material; (b) after formation into a droplet agent; and (c) after droplet agent loading into tissue phantoms.

We characterize the novel QD-labelled PFOB material before and after formation of the droplet agent. We first show that PFOB alone strongly attenuates an x-ray beam in an energy range appropriate for CEDM, and that the inclusion of QDs within the PFOB material renders strong optical fluorescence from the PFOB while not significantly affecting the x-ray attenuation. We then form a first-generation QD-PFOB droplet agent suspended in saline, compatible with intravenous injection, and determine that the x-ray and optical properties are retained after agent formation, and that the inclusion of QDs does not affect the size or surface properties of the unlabelled PFOB droplets. Next, using tissue phantoms, we assess the optical fluorescence and x-ray attenuation of the agent under more realistic, simulated in vivo imaging conditions. We confirm that the QD fluorescence is detectable, even at low concentrations, and that the fluorescence of the dual-modality agent strongly correlates with the x-ray signal. Correlation between optical signal and the x-ray attenuation of the tissue phantoms confirms the co-localization of the QDs and PFOB droplets, and demonstrates that the optical signal can be a surrogate for x-ray attenuation.

2. Materials and methods

The block diagram in figure 2 outlines three materials that were investigated in this study: (1) the PFOB compound proposed for clinical use in mammography; (2) the novel dual-modality combination of QD and PFOB for pre-clinical development; and (3) a perfluorocarbon (PFC) control compound, perfluorotributylamine, that does not contain the x-ray attenuating Br atom or any QDs to confer exogenous optical signal. These materials were assessed for their optical properties and x-ray attenuation under three conditions: (a) as a raw material; (b) after formation into the droplet agent; and (c) after loading of the droplet agent into tissue phantoms.

In the following sub-sections we describe the preparation and physical characterization of the droplet agents, the preparation of the droplet agent-loaded imaging phantoms, and the methods applied for the characterization of the optical signal and x-ray attenuation of these materials.
QD-PFOB dual optical and x-ray contrast agent

2.1. Droplet agent synthesis and characterization

CdSe/ZnS core/shell QDs (~5 nm in diameter) (Gorelikov and Matsuura 2008) were coated with silica (Yi et al 2005) and fluorinated for miscibilization into PFOB (C₈F₁₇Br, bp 142 °C, Synquest Laboratories, Alachua, FL, USA) (Gorelikov et al 2011, Matsuura et al 2009). All chemicals for silica-coated QD synthesis and PFOB miscibilization were purchased from Sigma-Aldrich and used as-received. For all experiments, the concentration of QDs in PFOB was ~20 μM (Yu et al 2003). A single batch of QDs was prepared for the entire experimental work to avoid potential discrepancies resulting from different QD preparations.

The PFOB, QD-PFOB, and PFC agents were made by forming each raw material into nanoscale droplet suspensions using a biocompatible fluorosurfactant (Zonyl FSP, Sigma-Aldrich, Oakville, ON, Canada). The QD-PFOB agent is shown schematically in Figure 3. Droplets were prepared at 3% volumetric concentration by emulsification of PFOB, QD-PFOB and PFC (CF₃(CF₂)₃N, bp 178 °C, Synquest Laboratories, Alachua, FL, USA) solutions with fluorosurfactant in deionized water, and sonication of the mixtures (Gorelikov et al 2011, Matsuura et al 2009). The emulsions were then purified by centrifugation (Gorelikov et al 2011), resuspended in PBS (Wisent Inc.; St-Jean-Baptiste, QC, Canada), and filtered using a 0.8 μm membrane syringe filter.

Droplets were physically characterized before and after the droplet agent purification procedure. Physical characteristics were quantified by mean hydrodynamic size, size distribution (i.e., polydispersity index (PDI) (Nobbmann et al 2007)), and droplet surface charge (i.e., zeta potential). These quantities were measured on four independent preparations of droplets using a dynamic light scattering instrument (Malvern Zeta-Sizer 3000 HS; Malvern Instruments, Worcestershire, UK).

2.2. Preparation of imaging phantoms for in vitro droplet agent assessment

Cell-based in vitro imaging phantoms were prepared to provide autofluorescence and x-ray attenuations representative of normal tissue, and against which the additional signal enhancement conferred by the PFOB and QD-PFOB droplet agents could be measured.
Imaging phantoms were prepared by incubating 1.4 mL of the droplet agent in 4 mL of fresh medium with phagocytic murine alveolar macrophage cells (RAW264.7, ATCC, Manassas, VA, USA) seeded on 10 cm dishes (∼3 × 10^7 cells/dish) in vitro for 2 h at 37 °C with 5% CO₂ (Gorelikov et al. 2011, Martin et al. 2012). Loading of the QD-PFOB agent in cells was controlled by mixing PFC droplets with QD-PFOB droplets in desired loading ratios, at QD-PFOB droplet fractions of 0, 0.10, 0.25, 0.75, and 1.00. Control cells that contained no droplets, and cells containing only PFOB droplets were also prepared. After aspirating and washing the incubated cells with sterile PBS several times to remove excess droplets, 0.6 mL of PBS solution was added per plate and cells were detached by scraping. An aliquot of cells was taken for spectrofluorometry analysis. Cell concentration was measured after optical imaging to normalize the fluorescence by the number of cells in the aliquot using a Beckman Coulter Counter Multisizer III (Beckman Coulter Inc., Brea, CA, USA). For x-ray attenuation assessment, the cell suspensions were centrifuged into pellets and fixed (2% glutaraldehyde in 0.1 M sodium cacodylate buffer (Gorelikov et al. 2011). Although fixation is known to affect the x-ray attenuation of tissues (Benders et al. 2010, Chen et al. 2010), the parameter of interest for the phantom evaluation was the change in x-ray attenuation due to the addition of QD-PFOB agent relative to that of control pellets that were also fixed. Given that soft tissues have a low contrast compared to water, but have a good contrast against lipids, the fixed cell pellets were immersed in vegetable oil to facilitate the accurate selection of regions of interest (ROIs) such that they were located entirely within the pellet images.

For independent verification that QD-PFOB agent was successfully loaded in cells, fluorescence microscopy and transmission electron microscopy (TEM) were used (Gorelikov et al. 2011). For fluorescence microscopy, the cells were seeded in six-well plates with glass slides prior to droplet incubation, followed by staining on ice with Alexa488-Concanavalin A (molecular probes) to label the cell membrane, and DAPI (Invitrogen) to stain nuclei, and fixation in 4% paraformaldehyde (Gorelikov et al. 2011). For TEM verification, 100 nm thick sections of the fixed cell pellets were viewed in an FEI Tecnai 20 TEM, equipped with a Gatan Dual view digital camera (Gorelikov et al. 2011).

Four independent sets of the full panel of cell cultures were prepared, each of which was incubated with fresh formulations of the droplet suspensions that was characterized using spectrofluorometry prior to incubation with the cells. For all four sets of droplet-loaded cells, fluorescence spectrometry, x-ray attenuation, fluorescence microscopy, cell counting, and TEM were performed.

### 2.3. Optical signal characterization

#### 2.3.1. Optical evaluation of raw materials and droplet agents

Fluorescence emission spectra of PFOB, QD-PFOB and PFC solutions were obtained using a Horiba Jobin Yvon FluoroMax-4 Spectrofluorometer (Horiba Scientific, Edison, NJ, USA) at an excitation wavelength of 350 nm. The fluorescence enhancement due to QDs was evaluated by measuring the intensity of the QD 635 nm peak (in counts per second, cps), and subtracting the wavelength-independent background signal off-peak at 585 nm. Due to the brightness of the QDs, the original QD-PFOB material prior to droplet formation was measured at a dilution of 0.04% in pure PFOB to prevent detector saturation. The fluorescence of the QD-PFOB material was assessed in comparison to pure PFOB and pure PFC. After droplet formation, all suspensions were measured at 5% dilution in PBS under the same fluorescence imaging conditions.

#### 2.3.2. Optical evaluation of droplet agents in cells

The optical signal of the PFOB, QD-PFOB and PFC control droplet agents in cells was measured using spectrofluorometry, in
Figure 4. Geometry of the experimental setup for measuring x-ray attenuation. Samples were loaded into an 8.3 cm diameter PMMA cylinder and placed on a rotating stage for x-ray characterization with cone-beam CT. The cylinder was centred 53.3 cm from the source, and 23.3 cm from the detector, with about 8 cm of its height exposed to x-rays.

600 μL cell aliquots placed in quartz cuvettes. The signal was quantified in terms of the relative fluorescence enhancement of the droplet-loaded phantoms over the unloaded control cells. The fluorescence enhancement due to QD-PFOB droplets in cells was evaluated by measuring the intensity of the QD 635 nm peak relative to the 585 nm background signal, after normalizing for number of cells in the suspension. Confirmation of cellular uptake of the QD-PFOB agent was performed by fluorescence microscopy using a Zeiss Axiovert 200 M inverted epifluorescence microscope (Carl Zeiss Canada Ltd, Toronto, ON, Canada) using Axiovision imaging software and standard FITC, cy3, and DAPI filter sets to image the cell membrane, QDs, and nucleus, respectively (Gorelikov et al 2011, Martin et al 2012).

2.4. X-ray attenuation characterization

X-ray characterization was performed using a custom-built tabletop cone-beam CT system based on a digital mammography x-ray tube (22–49 kV) (GE DMR v. 2, GE Healthcare, Chalfont St. Giles, UK) and a flat-panel CsI(Tl)/photodiode detector (GE Senographe 2000D detector) with 100 μm detector elements. The experimental imaging setup is illustrated in figure 4. Up to 11 samples were loaded for imaging in 8 mm diameter wells, located at a 28.5 mm radius from the centre of an 83 mm diameter polymethyl methacrylate (PMMA) cylinder. The source-to-detector distance was set to 75.6 cm, with the PMMA cylinder placed on a rotating stage with the isocentre 53.3 cm from the source. Specimen imaging was performed using a rhodium target and 3.1 mm aluminium filtration, with an x-ray tube potential of 49 kV, and 30 mAs/projection. The x-ray spectrum is shown in figure 5, as estimated using the spectral model of Boone et al (Boone et al 1997, Carton et al 2006). The appropriateness of the model was verified with half-value layer measurements using high purity aluminium foils (HVL = 1.43 mm Al), and the spectrum was estimated to have an effective mean energy of 30.5 keV. The air kerma per projection image at the isocentre was measured with an ionisation chamber (Radcal Corp., metre 2026C, chamber 20 × 6–6 M) to be approximately 1.5 mGy.
Figure 5. Estimated x-ray spectrum used in this study for all x-ray measurements. (Boone et al. 1997, Carton et al. 2006) The imaging technique is 49 kVp, with a Rh target and 3.1 mm of Al filtration, resulting in a mean beam energy of 30.5 keV.

Projection images \( (n = 237) \) of the phantom were acquired at equal intervals over \( 360^\circ \), and reconstruction was performed at a voxel size of \( 0.2 \times 0.2 \times 0.2 \) mm\(^3\) using a commercial filtered backprojection algorithm (COBRA v6.1.8; Exxim Computing Corp., Pleasanton, CA, USA). The image reconstructions were corrected for spatial variations in signal across the image (i.e., scattering and beam hardening) by division with a normalized signal profile obtained by averaging 50 reconstructed image slices from a uniform segment of the PMMA cylinder.

The ROIs for x-ray attenuation evaluation were manually selected as demonstrated in figure 6, encompassing a volume of between 250 and 360 voxels \( (2 \text{ to } 2.88 \text{ mm}^3) \). The reconstructed image signal intensity is proportional to the material linear attenuation coefficient, and can be transformed to the relative x-ray attenuation, \( \mu_{\text{rel}} \), as measured in HU, using air and water references via the following calculation:

\[
\mu_{\text{rel}} = 1000 \frac{\mu_{\text{object}} - \mu_{\text{water}}}{\mu_{\text{water}} - \mu_{\text{air}}},
\]  

(1)

Figure 6. Example of ROI selection within the cell pellets used for analysis for relative x-ray attenuation. (a) Image of a cell pellet incubated with QD-PFOB droplets, with the ROI indicated as a black box; and (b) a volumetric view of ROI (175 voxels) selection in the cell pellet CT image. Axes are scaled as voxel indices.
where the effective linear attenuation coefficients of the object of interest, $\mu'_{\text{object}}$, air, $\mu'_{\text{air}}$, and water, $\mu'_{\text{water}}$, are obtained from the averaged reconstructed image intensities as measured in image regions within samples of each material. Air and water reference standards were included in the PMMA cylinder for each imaging experiment. The sample x-ray attenuation measurements are expressed as mean ± standard deviation in HU. A two-sample Student’s $t$-test was used to compare differences between x-ray measurements of independent samples. Differences with a $p$-value less than 0.05 were considered significant.

2.4.1. X-ray evaluation of raw materials and droplet agents. The x-ray attenuation of the raw PFOB compound as a function of its concentration in PFC was measured at concentrations of 0, 0.5, 1, 3, and 5% by volume. Samples of QD-PFOB (QD concentration of $\sim 20 \mu$M) diluted to 5% in PFC were also measured. The relative x-ray attenuations of the PFOB, QD-PFOB and PFC droplet agents were measured as prepared and after purification.

2.4.2. X-ray evaluation of droplet agents in imaging phantoms. The increase in the relative x-ray attenuation of the droplet-loaded cell pellets compared to unloaded control pellets was calculated as the difference between their relative x-ray attenuations, $\Delta \mu_{\text{rel}} = \mu_{\text{rel, loaded}} - \mu_{\text{rel, control}}$. Finally, using the optical and x-ray measurements from all of the imaging phantoms, the correlation between the fluorescence enhancement per cell and the increase in x-ray attenuation, $\Delta \mu_{\text{rel}}$, per cell pellet was determined.

3. Results and discussion

This study investigates a promising biocompatible candidate material for formulation into a nanoscale droplet CEDM agent to improve breast cancer detection and characterization. We demonstrate that the approach of modular incorporation of non-interacting, fluorescent labels into x-ray contrast media can address a separate need that exists in the research community to be able to expedite clinical translation of this, or other candidate contrast materials. The optical/x-ray droplet agent described can potentially improve the efficiency of the in vitro and pre-clinical development stages of PFOB as an x-ray contrast medium, by allowing complementary, independent and high-resolution optical validation of the agent’s spatial location by incorporating fluorescent QD labels into the nanoscale PFOB droplets.

3.1. Characterization of raw materials

3.1.1. Optical signal of raw materials. The fluorescent optical signal was added to PFOB by fluorinating the surface of the QDs to overcome their native immiscibility in PFOB. Although any type of QD could potentially be used, red-emitting CdSe/ZnS QDs were selected here due to their compatibility with standard filters used in fluorescence microscopy, thereby permitting high-resolution optical microscopy of QD-PFOB droplets. Red-emitting QDs also emit light at wavelengths spectrally shifted from tissue autofluorescence, which reduces optical signal interference for in vivo testing.

Under ambient light, QD-PFOB appears uniformly orange–red, while PFOB and PFC are transparent (figure 7(a), samples 1–3, respectively). Upon illumination by UV light, the strong fluorescence of QD-PFOB can be observed, whereas no fluorescence was observed in PFOB and PFC (figure 7(b), samples 1–3, respectively). High dilutions of the QD-PFOB material (i.e., 0.04% QD-PFOB in PFOB) appear almost colourless under ambient light (figure 7(a), sample 4), while at this same dilution the QD-PFOB material exhibits strong fluorescence
Figure 7. Solutions of (1) QD-PFOB, (2) PFOB, (3) PFC, and (4) 0.04% QD-PFOB diluted in PFOB: (a) under ambient light; and (b) illuminated by UV light of wavelength 365 nm. (c) Fluorescence spectra of diluted QD-PFOB solution (solid line) illuminated using an optical excitation wavelength of 350 nm. PFOB and PFC solutions (overlapping dotted red and dashed blue lines) are shown as comparison.

under UV illumination (figure 7(b), sample 4). Upon excitation by 350 nm light, the QD-PFOB material exhibited a strong photoluminescence (PL) peak at 635 ± 1 nm (FWHM of 35 ± 1 nm) (figure 7(c)) with negligible background fluorescence. A small secondary PL peak was observed at 504 nm (FWHM of 46 ± 1 nm), but since the intensity of the secondary peak was low (i.e., 7.5% of the 635 nm peak), it was not used for optical quantification. Pure PFOB and pure PFC exhibited little fluorescence (figure 7(c)). Because all three materials, QD-PFOB, PFOB, and PFC, exhibit negligible fluorescence at 585 nm, this wavelength was selected as a reference (background) signal against which the fluorescence at the PL peak (635 nm) was compared to determine the relative content of QD-PFOB in each sample.

3.1.2. X-ray attenuation of raw materials. The x-ray attenuation of PFOB material loaded in a PMMA cylinder was characterized using cone-beam CT to permit quantitative measurements of the relative x-ray attenuation of materials (Jones and Mahvash 2012). The PMMA provides a realistic imaging environment, including x-ray attenuation and x-ray scatter conditions (Galper et al 2012), that approximate in vivo conditions for contrast agent detection in a small animal (e.g., rabbit, rat) or in a dense human breast (~50% fibroglandular tissue equivalent) (Huang et al 2011). The x-ray beam used here, at 49 kV tube potential, with a Rh anode and Al filtration, approximates the beam characteristics used clinically for CEDM (Jong et al 2003, Dromain et al 2011).

For x-ray attenuation measurements, it was necessary to dilute the pure materials to prevent photon starvation due to the strong attenuation by Br of the mammographic-energy x-ray beam. Because PFOB is immiscible in standard aqueous and non-polar solvents (Babiak et al 2008), we diluted PFOB in the PFC control (no Br) material. A calibration for the relative
the x-ray attenuation of PFOB per unit concentration in PFC, as plotted in figure 8, demonstrates a relative attenuation of 5621 ± 268 HU g⁻¹ mL⁻¹ PFOB ($R^2 = 0.998$). Compared to PFC, the bromine in PFOB (16% mass fraction of Br) provides strong additional attenuation at 35.1 ± 1.7 HU mg⁻¹ mL⁻¹ Br. This attenuation compares well to that provided by the iodine in conventional diagnostic x-ray applications (Miles et al. 2007).

The linear attenuation coefficient of the QD material is estimated to be 13 times greater than that of PFOB under the x-ray beam conditions in the study (Hubbell and Seltzer 2004, Boone et al. 1997). However, at 20 μM, their concentration is very low and the dissolved QDs only displace a volume fraction of PFOB equivalent to about $7.9 \times 10^{-5}$ of a unit volume (spherical QDs assumed). The uncertainty in x-ray attenuation of the PFOB for the imaging conditions was equivalent to about $±4.5$ mg ml⁻¹ PFOB, or a $2.3 \times 10^{-3}$ volume fraction of PFOB. Therefore, the specimen imaging did not have the contrast resolution to distinguish such small amounts of QD material. Consequently, their addition to the PFOB solution did not raise the relative attenuation significantly above the unlabelled PFOB ($p = 0.40$, 1439 ± 33 HU PFOB versus 1452 ± 26 QD-PFOB at 5% volumetric concentrations, figure 8) This suggests that pre-clinical measurements with QD-PFOB are predictive of those from equal quantities of unlabelled PFOB.

### 3.2. Characterization of droplet agents

#### 3.2.1. Physical characteristics of the droplet agents

A simple two-component formulation illustrated in figure 3 was used to form the QD-PFOB droplet agent, comprised of the QD-PFOB material (forming the fluorescent and x-ray attenuating core), and a biocompatible emulsifier (forming the droplet-stabilizing shell). The choice of the emulsifier determines the droplet physical properties and their interactions with cells. In this work an anionic synthetic fluorosurfactant was used to optimize the loading of QD-PFOB droplets into cells for in vitro evaluation of the QD-PFOB agent in imaging phantoms (Dobrovolskaia et al. 2008, Moghimi et al. 2001).

Results of the physical characterization of four independent preparations each of PFOB, QD-PFOB and PFC droplets are summarized in table 1. All droplets had similar mean

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**Figure 8.** Relative x-ray attenuation of PFOB diluted in PFC (black squares), up to 5% PFOB in PFC, using an x-ray spectrum with 30.5 keV mean energy. A 5% QD-PFOB dilution in PFC (red triangle) and pure PFC (blue circle) are shown for comparison. The dashed line is a linear least-squares fit to the data, and has an $R^2$ value of 0.998. The error bars are of the same order as the size of the symbols (± 18 to 26 HU), so are not shown.
Table 1. Mean size and PDI of PFOB, QD-PFOB, and PFC droplets, as-prepared and after purification by centrifugation and filtering.

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<th>PFOB droplets</th>
<th>QD-PFOB droplets</th>
<th>PFC droplets</th>
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<tr>
<td>Mean size (nm)</td>
<td>As-prepared</td>
<td>Purified</td>
<td>As-prepared</td>
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<td></td>
<td>158.3 ± 2.5</td>
<td>161.1 ± 1.9</td>
<td>159.8 ± 2.7</td>
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<tr>
<td>PDI</td>
<td>0.089</td>
<td>0.066</td>
<td>0.083</td>
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</table>

sizes of about 160 nm; a size suitable for application as an intravascular agent for tumour imaging (Moghimi et al 2001, 2005, Jain and Stylianopoulos 2010, Maeda 2010). Although the purification step did not shift the mean of the droplet size distributions, the distributions became narrower for the PFOB and QD-PFOB droplets post-purification, suggesting that the centrifugation and filtering procedure improved the agents’ monodispersity. Importantly, the addition of the QD to PFOB did not result in different mean droplet sizes or size distributions compared to PFOB alone. Surface charge zeta-potential measurements resulted in values of $-62.5$, $-62.0$, and $-61.0$ mV, for QD-PFOB, PFOB and PFC droplets, respectively. All droplet agents were stable in suspension in saline and aggregation was not observed over the course of the experiments. Consistent and robust physical properties demonstrated by all three droplet types suggested that the PFC droplets were suitable controls to the QD-PFOB and PFOB droplet agents in these experimental investigations.

3.2.2. Optical signal of droplet agents. Optical assessment of the agent demonstrated that the strong fluorescence of the QD-PFOB material was retained after droplet synthesis and purification. As shown in figure 9(a), the primary fluorescence emission at 635 nm was virtually identical for the four independent preparations of purified QD-PFOB droplet suspensions. Consistent with measurements of the raw materials, the PFOB and PFC droplets exhibited only background fluorescence levels at 635 nm. As shown in figure 9(b), the PFOB and PFC droplets had intensities of 1.5% and 2.8% of the QD-PFOB droplet signal, respectively. It is noteworthy that the QD-PFOB droplets retained their strong fluorescence even after purification (figure 9(b)), demonstrating that QDs were successfully integrated into the PFOB droplets and were not suspended freely in the initial, as-prepared population.

The linearity in the optical calibration in figure 10 demonstrates that the fluorescence enhancement from the QD-PFOB agent was directly related to the fraction of QD-PFOB droplets in a QD-PFOB/PFC droplet mixture ($R^2 = 0.997$).

3.2.3. X-ray attenuation of droplet agents. The relative x-ray attenuations of the PFOB, QD-PFOB and PFC droplet agents are shown in figure 11. For each droplet agent, no significant difference in the relative attenuations was observed after purification. Using the relative x-ray attenuation calibration curve obtained from the PFOB material (figure 8), and the relative attenuations of the droplet suspensions in figure 11, we estimated that the PFOB droplets were in suspension at a volumetric concentration of about 3% (3.8 ± 0.8% and 3.7 ± 0.7%, before and after purification, respectively). These results are within error of the target PFOB droplet volume fraction according to the agent preparation method (i.e., 3%). Similarly, for the PFC droplets, the measured relative attenuations indicated droplet volume fractions of 9 ± 6% and 9 ± 6%, before and after purification, respectively. The uncertainty and apparent discrepancy from the desired agent volume fraction of 3% can be attributed to the low attenuation of the PFC droplets at this concentration, which is close to the attenuation sensitivity limit of our measurement technique.
3.3. Characterization of droplet agents in imaging phantoms

The cell-based in vitro phantoms were intended to provide a simple and controlled model to test selected properties that may be predictive of in vivo imaging performance. Since the PFC droplets contributed negligible optical signal and x-ray attenuation compared to the QD-PFOB agent, loading the cells with mixtures of these droplets in different proportions simulated variable conditions of droplet accumulation under both imaging modalities. For example, imaging phantoms prepared with phagocytic cells incubated with 100% QD-PFOB agent are anticipated to represent an upper limit of in vivo droplet accumulation, whereas the smaller fractions of QD-PFOB should represent more realistic levels of droplet regional localization (Ding et al 2013). Also, individual cells (~15 μm in size) loaded with the QD-PFOB droplet agent can be used for high-resolution optical imaging to demonstrate that the
Figure 10. Average QD fluorescence (peak intensity at 635 nm with background signal level at 585 nm subtracted) of QD-PFOB droplets diluted by PFC droplets (squares). Unlabelled PFOB droplet fluorescence is shown for comparison (circle), and shows no fluorescence. Each point represents the average of four different samples. The error bars are of the same order as the symbols, and are not shown.

Figure 11. Mean relative x-ray attenuation of QD-PFOB, PFOB, and PFC droplets suspended in saline as-prepared, and after purification.

agent’s interactions with target and non-target cells can be directly observed in biological media.

Fluorescence microscopy (figures 12(a) and (b)) clearly demonstrates that sub-cellular optical imaging of the QD-PFOB droplets is possible. QD-PFOB droplets (red) were observed to be loaded into cells, within the cell membrane (stained green) and outside the nucleus (stained blue). The dependence of the fluorescence on the concentration of the QD-PFOB droplets within the cells can be qualitatively observed upon suspension of the cells in saline and illumination with UV light (figure 13(a)). Increasing concentrations QD-PFOB droplets in the cells can be clearly seen, and cells loaded with unlabelled PFOB droplets appear identical to those loaded with PFC droplets and the unloaded control cells.

The effect of increased loading of QD-labelled PFOB droplets could also be clearly seen as increased x-ray attenuation in comparison to control cells after the QD-PFOB droplet-loaded cells were pelleted into tissue phantoms (figure 13(b)). Given that the cell preparations
Figure 12. Fluorescence images of fixed RAW264.7 cells (cell membrane stained green and nucleus stained blue): (a) unloaded; and (b) incubated with QD-PFOB droplets (orange) for 2 h at 37 °C.

Figure 13. (a) Optical image of vials containing RAW264.7 cells in suspension (~3 × 10^6 cells), illuminated by 365 nm UV light (from left to right): unloaded cells; QD-PFOB droplets mixed with PFC and loaded in cells with fractions of QD-PFOB droplets in the mixture of 0, 0.10, 0.25, 0.50, 0.75, and 1.00; and cells loaded with PFOB droplets. (b) CT slice images of cell pellets in Eppendorf tubes (at 2× magnification) created from the suspensions in (a). All cells were incubated with droplets for 2 h under physiological conditions (37 °C).
Figure 14. TEM images of fixed RAW264.7 cells after a 2 h incubation with QD-PFOB droplets at 37°C: (a) an entire cell; and (b) higher magnification of a specific area of the cell shown by the red box, selected to show vacuoles containing QDs.

were washed before imaging with either modality, the presence of both x-ray and optical contrast indicates that the cells must have entirely engulfed the QD-PFOB droplets. This was confirmed via TEM (figure 14) of the tissue pellets, which showed that the QD-PFOB droplets were internalized by the cells within vacuoles in the cytoplasm. It should be noted that the sizes of the vacuoles in the TEM images are larger than the original 160 nm droplet size, as measured using dynamic light scattering. The TEM images cannot verify whether the vacuoles contained single or multiple QD-PFOB droplets, as the TEM specimen preparation procedure prevents any liquid from being retained in the sample. The apparent size increase of the droplets in vitro may have been due to temperature-induced droplet instability during TEM specimen preparation or due to droplet growth within the vacuoles after incubation with cells (Gorelikov et al 2011, Martin et al 2012). In either of these cases, the x-ray attenuation from the PFOB droplet and optical signals from the QD labels were co-localized within the cells.

Due to the shallow penetration of UV light in biological tissue, the fluorescence of cells loaded with different fractions of QD-PFOB droplets cannot be measured reliably in millimetre scale pellets. Instead, individual cells were suspended in saline, and the fluorescence enhancement of the cell suspensions was measured and normalized to the number of cells in each sample (figure 15). The optical signal was normalized by the number of cells to account for the fact that four independent sets of cell cultures were used for each experimental condition, which leads to the expected variability in cell number per plate. While the PFC droplet-loaded cells could not be distinguished from unloaded cells, the fluorescent enhancement from the cells loaded with a 0.10 QD-PFOB droplet mixture was clearly measurable. As demonstrated qualitatively in figure 13 and quantitatively in figure 15, the fluorescence enhancement of QD-PFOB agent in single cells directly correlated with the x-ray attenuation of the same cells in pelleted form. This strong correlation between signals in each modality suggests that the fluorescence of the QD-PFOB can be used in a predictive manner, to sensitively evaluate the biological targeting potential of PFOB droplet formulations in vitro, at a cellular level, without the use of CT imaging. Thus, a qualitative evaluation of cellular targeting by microscopy would be straightforward using the QD signal, in that we have demonstrated the optical marker to be co-localized with the x-ray attenuation arising from PFOB. We note that a quantitative assessment of QD-PFOB droplet loading using purely optical methods is more difficult. This would require careful assessment of the batch-dependent optical properties of the QDs, the
environment in which the QD-PFOB droplets are measured and calibration of the QD-PFOB fluorescence to the droplet concentration of the samples using the intended x-ray imaging technique. For example, in figure 10 we demonstrated that the fluorescence enhancement of the QD-PFOB droplet concentration can be used to estimate the agent concentration in solution before its experimental use with cells. However, these data could not be used to directly predict the fluorescence enhancement from the concentration of QD-PFOB droplets loaded into cells because the local distribution of QDs, the absorption of incident light and light scattering from both droplets and cells will determine the absolute measured fluorescence.

Using the linear dependence between the relative x-ray attenuation and PFOB concentration (figure 8), the measured increase in relative x-ray attenuation by droplet agent loading of the cell pellets was calibrated to an equivalent concentration of PFOB, as indicated on the right y-axis of figure 15. This calibration demonstrates that the increased relative x-ray attenuation of the cell pellets maximally loaded with QD-PFOB droplets compared to unloaded cells (~465 ± 166 HU, n = 4, left y-axis, figure 15) corresponds to about a 3% droplet concentration by volume of droplets in the cell pellet (i.e., similar to attenuation of the 3% droplet suspension in figure 11).

We have shown here that the QD marker can distinguish the distribution of PFOB droplets at sub-cellular resolution in vitro. However, the QD label also has potential for in vivo pre-clinical use. We suggest that this bi-modal droplet agent can be an important tool for future in vivo biodistribution studies, not only to understand pharmacokinetics, but also to validate x-ray image signals. For example, the QD label may differentiate the case where an observed x-ray signal within a voxel is due to very high local concentrations of PFOB droplets in a few cells, from the case where an equivalent number of PFOB droplets are spread uniformly at lower concentrations in tissue contained within the voxel. Given the strong correlation between the optical and x-ray signals, this investigation could be done independently of x-ray imaging, using the results to predict whether the tumour-to-normal tissue particle sequestration is adequate for visualization in an x-ray image. For example, a region of higher attenuation at a tumour site could be validated as being due to the presence of QD-PFOB droplets by removing the tumour, sectioning it post-sacrifice, and performing fluorescence imaging of histology sections. The QDs are unaffected by tissue fixation procedures (Gorelikov et al 2011) and as we demonstrated here, they can be easily distinguished from the background

![Figure 15. Correlation of optical signal of cells in suspension (e.g., as shown in figure 12(a)) with relative x-ray attenuation of cell pellets (e.g., as shown in figure 12(b)). Relative attenuation is expressed as HU (left axis) and in terms of effective PFOB concentration (right axis). The line of best fit is shown on the plot as the dashed line ($R^2 = 0.96$).](image-url)
autofluorescence (see figure 12(a) versus (b)) of the surrounding tissue in unstained sections. Furthermore, it may be possible to co-register the optical images of the tissue sections with the x-ray images, which will assist in determining the location of smaller concentrations of the PFOB droplets due to the increased sensitivity and resolution of fluorescence imaging in comparison to x-ray imaging. Finally, although the primary intent of the use of a non-interacting optical label in this work was for easy removal for clinical translation, following the methods illustrated here, it is also possible to exchange types of QDs for those with different emission characteristics, or to integrate biocompatible QDs for direct optical imaging in vivo (Ntziachristos et al 2005).

In the future, in vivo studies of the QD-PFOB agent will be conducted. Significant debate exists in the literature how nanoscale agents can be designed to most effectively accumulate in tumours (Moghimi et al 2001, 2005, Jain and Stylianopoulos 2010, Maeda 2010). Given this uncertainty, and the fact that different PFOB droplet shell formulations are known to strongly influence their biodistribution, the goal will be to evaluate the localization of different droplet formulations, and to tune the droplet shells accordingly for maximum specificity. We anticipate the QD label to be invaluable for these experiments.

4. Conclusions

In this study, we have demonstrated that a novel, dual-modality, QD-labelled PFOB material can serve as a platform for the development and optimization of new, nanoscale PFOB droplet formulations for contrast-enhanced mammographic imaging via high-resolution and complementary optical imaging. The method of adding the bright and easily distinguished QDs to the PFOB did not require any alterations to the PFOB molecules, which makes it possible to remove the QDs after optimization of the PFOB agent without changing the physical properties of the agent. Furthermore, as the QDs did not significantly contribute to the x-ray attenuation of the PFOB material, the QD-PFOB droplets can be used in pre-clinical studies as a surrogate to the unlabelled PFOB materials, with equivalent x-ray attenuation characteristics. QD-PFOB droplets with mean sizes of 160 nm were successfully synthesized, with a demonstrated co-localization of x-ray and optical signals from these droplets in tissue phantoms. This direct relationship between the signals in each modality demonstrates that quantitative and predictive measurements can be made with this material. In the future, we believe that the QD-labelled PFOB droplets as introduced here, can be an important tool in the high-resolution assessment of formulation-dependent in vivo agent biodistribution, as well as the validation and optimization of x-ray techniques for sensitive detection of PFOB in vivo, and may ultimately allow for expedited development and clinical translation of this agent for mammographic imaging applications.

Acknowledgments

Authors would like to thank Dr J Booth for providing the RAW264.7 cells used in this work. This study was supported, in part, by the CIHR Excellence in Radiation Research for the 21st Century (EIRR21) Research Training Program, the Ontario Institute for Cancer Research through funding provided by the Province of Ontario Ministry of Research and Innovation, the Department of Defense Breast Cancer Research Program Concept Award (BC075873), the Ontario Research Fund-Research Excellence Program, and a program project grant entitled ‘Imaging for Cancer’ from the Terry Fox Foundation.
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