Drug delivery monitoring by photoacoustic tomography with an ICG encapsulated double emulsion

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Abstract: The absorption spectrum of indocyanine green (ICG), a nontoxic dye used for medical diagnostics, depends upon its concentration as well as the nature of its environment, i.e., the solvent medium into which it is dissolved. In blood, ICG binds with plasma proteins, thus causing changes in its photoacoustic spectrum. We successfully encapsulated ICG in an ultrasound-triggerable perfluorocarbon double emulsion that prevents ICG from binding with plasma proteins. Photoacoustic spectral measurements on point target as well as 2-D photoacoustic images of blood vessels revealed that the photoacoustic spectrum changes significantly in blood when the ICG-loaded emulsion undergoes acoustic droplet vaporization (ADV), which is the conversion of liquid droplets into gas bubbles using ultrasound. We propose that these changes in the photoacoustic spectrum of the ICG emulsion in blood, coupled with photoacoustic tomography, could be used to spatially and quantitatively monitor ultrasound initiated drug delivery. In addition, we suggest that the photoacoustic spectral change induced by ultrasound exposure could also be used as contrast in photoacoustic imaging to obtain a background free image.

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OCIS codes: (170.5120) Photoacoustic imaging; (110.7170) Ultrasound; (170.6510) Spectroscopy.

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1. Introduction

Photoacoustic tomography (PAT) is a hybrid tissue imaging modality that possesses the contrast advantage of optical imaging and the resolution advantage and penetration of ultrasonography [1, 2]. PAT is based on the photoacoustic effect [3], in which the absorbed optical energy is converted into acoustic waves. The image contrast in PAT is provided by the absorption of endogenous chromophores, such as oxygenated hemoglobin, deoxygenated hemoglobin, lipid, melanin and water present in the tissues. In addition, exogenous contrast agents such as metallic nanoparticles and organics dyes such as indocyanine green (ICG) can be used to enhance the contrast of a photoacoustic image [4–6]. Over the past decades, PAT has been developed tremendously and adapted to a variety of potential preclinical and clinical applications including breast cancer [7–10], inflammatory arthritis [11], and sentinel lymph node [12].

Indocyanine green (ICG), also known as cardigreen, is an U.S. Food and Drug Administration (FDA)-approved, nontoxic, organic dye used for medical diagnostics. Since

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its introduction in 1957, ICG has been widely used in medical imaging ranging from ocular imaging in ophthalmology [13, 14] to fluorescent, tissue imaging [15, 16]. The absorption spectrum of ICG depends upon its concentration as well as the nature of its environment, i.e., the solvent medium into which it is dissolved [17]. In blood, ICG binds with plasma proteins, causing a change in its absorption spectrum [17]. These spectral behaviors of ICG make it difficult to use ICG as a stable blood pool agent and quantify the concentration or optical absorption spectrum of ICG in complex in vivo environment by using optical imaging technologies [18].

Perfluorocarbon (PFC) emulsions are being studied as targeted drug delivery systems, since the drug release from these emulsions can be controlled via ultrasound [19–24]. Upon exposure to ultrasound above a certain threshold pressure, the emulsions are converted into gas bubbles [21, 25–28], a process termed acoustic droplet vaporization (ADV). Due to the extreme hydrophobicity and lipophobicity of the PFCs used in these emulsions [29], therapeutic agents can be incorporated into the emulsions via the inclusion of a secondary immiscible phase, as in the case of double emulsions [23–25]. Diagnostic ultrasound can also be used to monitor the therapeutic effects of PFCs emulsions, such as the accumulation of extravagated nanodroplets within a tumor or the formation of gas bubbles upon ADV [22]. However, diagnostic ultrasound is unable to directly quantify the amount of drug released or delivered upon ADV.

Here we propose a new technique of using photoacoustic tomography to quantify the release of ICG, triggered by ADV, from a PFC double emulsion. This technique makes use of the change in the photoacoustic spectrum due to the binding of ICG with plasma proteins in blood. From the spectral changes obtained before and after ADV, it is possible to quantify the concentration of released ICG and hence the released amount of drug which would also be co-encapsulated with the dye. This technique has potential application in tracking ADV-triggered drug release and delivery within targeted regions. Moreover, by encapsulating ICG in PFC double emulsion, the ICG keeps very stable optical absorption spectrum which will not be changed by the materials in the environmental medium, and hence could be a better blood pool agent for optical imaging. In this paper, we first present a procedure to make a stable ICG-loaded double emulsion. Then, the technique to monitor drug release and delivery is validated using a point target and two dimensional image measurements. This paper is organized as follows. Section 2 provides a detailed account of formulating the ICG-loaded double emulsion. A description of the experimental setup for the photoacoustic spectral measurement and also the two dimensional photoacoustic imaging setup is given in section 3. The results are presented and discussed in section 4. Finally, conclusions and future work are presented in section 5.

2. Materials and methods

The ICG-loaded, PFC double emulsion was prepared as follows: 6 mg of fluorousurfactant copolymer was dissolved in 1.1 g of perfluoropentane (PFP) using a Vortex mixer (VWR Scientific, USA). Next, 0.55 mL of a 22.9 mg/mL solution of ICG (Sigma-Aldrich, USA) in distilled water was added to the surfactant-PFP solution. The mixture was placed into an ice bath and then emulsified with a sonicator (model 450, 20 kHz, 3.2 mm tip diameter, Branson, Danbury, CT, USA) for 40 seconds at 150 W/cm² (based on manufacturer supplied output measurement) to obtain the primary emulsion. The primary emulsion (W₁/PFC) was added slowly into a 20 mL glass vial that contained 3.5 mL of Pluronic F68 (Sigma-Aldrich, USA) in normal saline (50 mg/mL). The Pluronic solution was stirred magnetically during the addition of the primary emulsion. The glass vial was cooled by placing it in an ice bath to avoid vaporization of PFP while stirring. The solution was continuously stirred for about 10 minutes at the maximum spinning speed of the magnetic stirrer. This procedure yielded a double emulsion of the form W₁/PFC/W₂ where ICG was contained in the W₁ phase. As measured optically, the resulting double emulsion contained particles up to 300 μm in diameter. The double emulsion was stirred for an additional hour to reduce the particle size. The emulsion was then diluted with 2 mL of normal saline and large droplets, which had
sedimented after 2 minutes, were removed. The remaining emulsion was then diluted with 5 mL of normal saline and centrifuged at 500 rpm for 10 minutes at 15°C. The supernatant, containing non-encapsulated ICG, was removed and 8 mL of normal saline was added to resuspend the pellet. The emulsion was washed two additional times using the aforementioned procedure. The size distribution of the droplets in the ICG-loaded double emulsion was measured using Coulter counter (Multisizer 3, Beckman Coulter Inc., Fullerton, CA, USA) and is given in Fig. 1. The average droplet diameter calculated was 4.4 μm, with diameters greater than 10 μm being 6.5% by number and 71.1% by volume. A micrograph of the ICG-loaded emulsion, diluted in normal saline, is given in Fig. 2. The dark green color in the image shows the encapsulated ICG within the droplets.

Fresh, canine blood was acquired using a protocol approved by the University of Michigan Committee on the Use and Care of Animals, and was used as blood medium. Whole canine blood was centrifuged at 4000 rpm for 40 minutes and the supernatant was isolated and used as plasma medium. Samples were made by diluting the emulsion with solvents such as normal saline, plasma or whole blood. The concentration of emulsion in the samples was 3 μL per mL of the solvent. After the sample was injected into a phantom vessel made of transparent and soft polyethylene tubes (0.58 mm inner diameter, BD Intramedic, Sparks, MD, USA), its optical absorption spectrum can be quantitatively measured with our spectroscopic photoacoustic imaging system.

Fig. 1. The size distribution of the ICG-loaded double emulsion. The mean droplet diameter is 4.4 μm.
3. Experimental procedure

The schematic of the experimental setup used for the photoacoustic measurements is given in Fig. 3. Figure 3(a) is the setup used for the point target measurement. In this setup, the laser was incident on the sample vessel and a cylindrically focused transducer was used to measure the generated photoacoustic signal. The measurements were carried out in a wide range of wavelengths from 700 to 900 nm with a step size of 20 nm. A tunable OPO laser (Vibrant B, Opotek Inc, Carlsbad, CA, USA) pumped by the second harmonic output of an Nd:YAG laser (Brilliant B, Quantel, Bozeman, MT, USA), with pulse repetition rate of 10 Hz was used to illuminate the sample in the negative z-direction. The sample was contained in the vessel placed along the x-direction. The energy density of the laser at the illuminating spot was adjusted to 3 mJ/cm$^2$ at 740 nm. For all other wavelengths in the range of wavelengths, the laser energy density was slightly less than 3 mJ/cm$^2$, all much lower than the ANSI safety limit. It should be noted that the photoacoustic spectrum of a fluorescent dye such as ICG is different from its optical absorption spectrum. This is because, optical energy absorbed by a fluorescent dye is converted into radiative (i.e., fluorescence) and nonradiative (i.e., thermal) energy, and the photoacoustic signal results from the nonradiative part only.

A calibrated, 3.5 MHz single element transducer (1.9 cm diameter, 3.81 cm focal length, A381S, Panametrics, Olympus NDT Inc., Waltham, MA, USA) (Transducer 1) was focally aligned in the z-direction in order to generate ADV of the emulsion contained within the vessel. The transducer was placed on a translation stage with its direction of movement parallel to the vessel. During ADV, the 3.5 MHz transducer was moved along the tube for approximately 20 mm (i.e. 10 mm on either side of the center of the laser beam irradiation) in order to maximize the release of ICG in the target region. Electric pulses sent into Transducer 1 were obtained using a master function generator (33120A, Agilent Technologies, Palo Alto, CA, USA) gated by a secondary function generator (3314A, Agilent Technologies, Palo Alto, CA, USA). The gated output signal was sent to a power amplifier (60 dB, model 350, Matec, Northborough, MA, USA). ADV was generated using the following acoustic parameters: 0.286 μs pulse duration, 1 ms pulse repetition period, 5.2 MPa peak rarefractional pressure,
and 12.2 MPa peak compressional pressure measured using a calibrated fiber optic hydrophone [30]. The ADV was confirmed by the visual observation of the gas bubbles generated due to ADV [31]. The presence of gas bubbles generated by ADV can also be confirmed using B-mode ultrasound [26].

Fig. 3. Schematic of the photoacoustic experimental setup used: (a) for single point measurement and (b) for 2-D imaging. NDF - neutral density filters, FG - function generator.
Photoacoustic signal emanating from the sample was collected along the y-direction by using a 10 MHz focused transducer (6.35 mm diameter, 1.9 cm focal length (V312, Panametrics, Olympus NDT Inc., Waltham, MA, USA) (Transducer 2). The output current of the transducer was amplified by a pulser/receiver (PR5072, Panametrics, Olympus NDT Inc., Waltham, MA, USA) and then was received by a 500 MHz oscilloscope (TDS 540B, Tektronix). The sample vessel and transducers were immersed in a water bath at room temperature to provide acoustic coupling. A portion of the laser beam that was reflected by a glass plate was measured using a photodiode to monitor the laser energy and the laser energy fluctuation. The output of the photodiode was sent into the oscilloscope. The oscilloscope was triggered by the firing of the laser. At each wavelength, the signal was averaged for 30 laser pulses. The data acquired by the oscilloscope was collected by a computer for further analysis.

Figure 3(b) shows the experimental setup used to acquire two dimensional, photoacoustic images of two parallel vessels (numbered 1 & 2). The two vessels were the same as the one used in the single point measurements and were separated by 1 cm. In order to illuminate both vessels, the OPO laser beam was expanded by a combination of a diverging lens and an optical diffuser. To avoid the blockage of the acoustic signals from the second vessel by the first vessel, the plane containing the two vessels was at an angle of about 15 degrees with respect to the probe transducer plane. A 2-D image was acquired using a commercial ultrasound unit (z.one, Zonare Medical Systems, Inc., Mountain View, CA, USA) with a linear array probe (P4-1; Zonare Medical Systems, Inc., Mountain View, CA, USA). The images were acquired at two different laser wavelengths, 800 and 860 nm. ADV was generated in vessel 1 by the transducer 1, and then the photoacoustic image was acquired again. The raw data was acquired by a computer and processed subsequently to obtain the image.

4. Results and discussion

The photoacoustic spectra of non-emulsified or free ICG in saline and in plasma is shown in Fig. 4. The spectra were the averages of eight independent measurements. The spectrum of each individual measurement was obtained by taking the ratio of the peak-to-peak values of the photoacoustic signal and the corresponding peak value of the photodiode signal. Since the peak or the maximum value of the spectrum changes across different measurements, we normalized each data set by fitting one data set against another by using the principle of linear least square procedure and then summing them to find the average and the standard deviation. In this way, the error is distributed uniformly over all the data points. As seen in Eq. (1), if \( f(\lambda) \) is the true, normalized spectrum, then the following equation can be written,

\[
y_{i,j} = a_j f(\lambda)
\]

where \( i \) represents the wavelength and \( j \) is the data set number and \( a_j \) is the normalization constant.

If the first set of data \((j=1)\) is normalized, then \( a_1 = 1 \); and for the second set of data \((j=2)\) it can be written as,

\[
y_{i,2} = a_2 y_{i,1}
\]

The best value of \( a_2 \) can be obtained by minimizing the sum of the squares of the error between the left and the right hand sides of Eq. (2) by the principle of least squares i.e.,

\[
\frac{\partial}{\partial a_2} \sum_{i=1}^{\infty} (y_{i,2} - a_2 y_{i,1})^2 = 0
\]

where \( n \) is the total number of wavelengths.

Solving Eq. (3) for \( a_2 \):
\[ a_2 = \frac{\sum_{i=1}^{n} y_{i,2} y_{i,4}}{\sum_{i=1}^{n} y_{i,2}^2} \]

\[ a_j = \frac{\sum_{i=1}^{n} y_{i,j} y_{i,1}}{\sum_{i=1}^{n} y_{i,j}^2}, \quad j = 2, \ldots, n \]

(4)

\( a_2 \) is the normalization constant for data set 2. Similarly for other data sets, the normalization constant can be written as,

The spectra were normalized by dividing their normalization constant. Then the mean and the standard deviations were estimated.

Figure 4 shows the spectral differences of free ICG in saline and that in plasma. We can see clearly that the optical absorption spectrum of ICG presents a big change at the presence of plasma. That is, the ratio of the photoacoustic signal intensities of ICG at 860 nm and 800 nm, decreases from 0.89 in saline to 0.34 in plasma, a change by a factor of 2.6. In Fig. 5, the photoacoustic spectra of the ICG-loaded emulsion in saline and in plasma are plotted. For comparison, the photoacoustic spectrum of free ICG in saline, is also overlaid in Fig. 5. First we can see that the spectrum of the ICG-loaded emulsion in saline is very similar to the spectrum of the free ICG in saline, which demonstrates that the encapsulation procedure does not induce significant change of the ICG absorption spectrum. Second, it is apparent that the spectrum of the ICG-loaded emulsion in plasma is very close to the spectrum of ICG-loaded emulsion in saline, which indicates that, by encapsulating ICG molecules in the emulsion, its optical absorption spectrum can be stabilized and will not change at the presence of plasma. In Fig. 6, the photoacoustic spectra of the ICG-loaded emulsion, diluted in plasma, before and after ADV are shown. As seen in Fig. 6, it becomes evident that the PA signal intensity at 860 nm decreases from 0.93 to 0.27, a factor of 3.4, as compared to the intensity at 800 nm, where
there is no change, due to ADV. Additionally, the spectrum after ADV is similar to the spectrum of free ICG in plasma, which is plotted as dashed green lines in the figure. This suggests that the majority of ICG was released, due to ADV, and thus able to bind with plasma proteins, similar to non-emulsified ICG.

Figure 7 shows the photoacoustic spectra of the ICG-loaded emulsion diluted in whole blood before and after ADV. Oxygen gas was introduced into the blood samples in order to make the blood 100% oxygenated. Unlike in the transparent “plasma medium”, hemoglobin in the “whole-blood medium” also contribute to the photoacoustic spectrum, in addition to the contribution from the ICG. As measured, the added emulsion increased the absorption coefficient by about 60% at 800 nm. Other than the experimental measurements obtained before and after ADV, we have also presented the simulated spectra. The simulation was carried out by taking the linear combination of the spectrum of pure blood with 100% oxygen saturation and the spectrum of the ICG-loaded emulsion in plasma (red curve), or the spectrum of the free ICG in plasma (green curve). As seen in Fig. 7, the simulated spectra match well with their corresponding experimentally measured spectrum, which shows the linearity of the system. After ADV, the photoacoustic spectrum changes as a result of the releasing of ICG into the plasma containing medium. In comparison with the result in Fig. 6, this spectrum change in Fig. 7 is not as pronounced due to the background photoacoustic signal from the blood. As seen in Fig. 7, the PA signal intensity, at 860 nm, decreases from 1.11 to 0.75 due to the ADV - a change of about 32%, as compared to the intensity at 800 nm, due to ADV.
In order to improve the contrast-to-noise ratio in imaging, the magnitude of the spectral change can be enhanced by increasing the concentration of emulsion in blood. However, for in vivo and ultimately for clinical applications, the maximum volume of emulsion that can be safely administered is limited by bioeffects related to elevated particulate and perfluorocarbon loading within the vascular space [32]. Alternatively, the concentration of ICG used to make the emulsion can be increased (i.e., enhance the loading), thus increasing the concentration of emulsified ICG and the amount of ICG released and reducing the required emulsion concentration required for the PA measurement.

In the 2-D imaging experiments, it was confirmed first, as seen in Fig. 8, that ultrasound pulses used to generate ADV, in the absence of the emulsion, did not cause a change in the photoacoustic signal of oxygenated blood. The vessels were then filled with ICG-loaded emulsion and oxygenated blood, and photoacoustic images were acquired, which are seen in Fig. 9. Figure 9(A) is the image of the two vessels at 860 nm, normalized by the average of the PA signal values of their respective vessel at 800 nm, before ADV. Figure 9(B) is the corresponding image after ADV. It is evident in Fig. 9(B) that ADV within vessel 1 caused the signal intensity within the vessel to decrease significantly relative to vessel 2, where no ADV occurred. This is consistent with results obtained in the point target measurements.
Fig. 7. The PA spectra of the ICG emulsion in canine blood before (■) and after (▲) ADV. The red dashed line represents the linear combination of the spectrum of whole blood and the spectrum of ICG emulsion in plasma. The green dashed line represents spectrum of whole blood and the spectrum of free ICG in plasma.

Fig. 8. 2-D, photoacoustic images of two vessels containing oxygenated canine blood at 860 nm before (a) and after (b) exposure to ultrasound (no emulsion present). Only vessel 1 was exposed to ultrasound pulses used to generate ADV.
Overall, the aforementioned point target and 2-D imaging results demonstrate that there is a significant change in the PA spectrum due to ADV of the ICG-loaded emulsion. Also, it was found, that the photoacoustic spectrum of the free ICG or emulsified ICG in blood is a linear combination of their respective, individual spectrum. As shown in Fig. 7, the overall photoacoustic spectrum of a sample containing ICG/ICG emulsion and blood, matches well with the linear combination of the spectra of the individual components. Due to the linearity of the system, the degree of spectral change caused by ADV should be proportional to the amount of ICG released by ADV. The absolute value can be obtained by a calibration chart. With an ADV-triggered drug delivery system, gas bubbles are created and can be visualized via the backscattering of ultrasound by the bubbles. However, a quantitative measurement of the amount of drug released or delivered is difficult to achieve using ultrasound alone. Since this new technique can be used to quantitatively estimate the amount of ICG released by ADV, the same technique can be used to monitor drug release when the desired drug is added to W₁ phase of the emulsion. Quantitative estimation of drug release and delivery has potential applications in targeted drug treatments. Moreover, the large change in the photoacoustic spectrum can also be exploited as contrast in differential photoacoustic imaging. For example, the acquisition of photoacoustic images of a sample before and after ADV may enable the development of a differential image that presents the distribution of ICG emulsion contrast agent in the sample without being affected by the background signals from other absorbing materials.

5. Conclusions

We have successfully encapsulated ICG in an ultrasound-triggerable, perfluorocarbon double emulsion (W₁/PFC/W₂) that prevents ICG from binding with plasma proteins. The emulsion was found to be stable in both plasma and in whole canine blood, thus hindering the ability of ICG – contained in the W₁ phase – to bind with plasma proteins. Also, it was found that the photoacoustic spectrum of the free (non-emulsified) or emulsified ICG in blood was a linear combination of the spectra of the individual respective components.
measurements on a point target as well as 2-D images of phantom vessels revealed that the photoacoustic spectrum changes significantly in blood when the ICG-loaded emulsion undergoes ADV. This ADV-induced spectral change of the ICG-emulsion in blood and the linearity of the spectral dependence suggests that the amount of ICG released is proportional to the degree of the spectral change. Since the spectral change can be used to measure the amount of ICG released, the same technique can be used to monitor drug release in targeted treatments, such as cancer therapy, where the desired drug is solubilized in the W_1 phase of the emulsion. Moreover, the spectral change due to ADV might be exploited as contrast from a focally targeted vascular bed in differential photoacoustic imaging to obtain a background-free photoacoustic image.

Future work will focus on applying the described techniques in vivo. Currently, the ICG-loaded emulsion has a significant volume fraction of particles with diameters greater than 10 μm. This emulsion is suitable for intra-arterial administration; the number of larger droplets (>10 μm) will need to be minimized in order for the emulsion to be suitable for intravenous injection. The loading of ICG within the emulsion will also need to be increased in order to minimize the amount of emulsion that will need to be injected in vivo. This can be accomplished by either increasing the initial ICG concentration used when preparing the emulsion or choosing another biocompatible solvent in which ICG has a higher solubility than water.

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