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Delivery of Chlorambucil Using an Acoustically-Triggered, Perfluoropentane Emulsion

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Abstract

Ultrasound-mediated delivery systems have mainly focused on microbubble contrast agents as carriers of drugs or genetic material. This study utilizes micron-sized, perfluoropentane (PFP) emulsions as carriers for chlorambucil (CHL), a lipophilic chemotherapeutic. The release of CHL is achieved via acoustic droplet vaporization (ADV), whereby the superheated emulsion is converted into gas bubbles using ultrasound. Emulsions were made using an albumin shell and soybean oil as the CHL carrier. The ratio of the PFP to soybean oil phases in the droplets, as well as the fraction of droplets that vaporize per ultrasound exposure were shown to correlate with droplet diameter. A 60-minute incubation with the CHL-loaded emulsion caused a 46.7% cellular growth inhibition, whereas incubation with the CHL-loaded emulsion that was exposed to ultrasound at 6.3 MHz caused an 84.3% growth inhibition. This difference was statistically significant ($p < 0.01$), signifying that ADV can be used as a method to substantially enhance drug delivery.

Keywords

ultrasound; acoustic droplet vaporization; perfluorocarbon; superheated; drug delivery; emulsion; droplets; chemotherapy; chlorambucil

Introduction and Literature

Perfluorocarbon (PFC) colloids, either microbubbles or emulsions, are being studied in diagnostic and therapeutic applications of ultrasound (US). The use of these colloids as delivery systems, either for therapeutic agents or genetic material, is motivated by their spatially and temporally-selective activation via US. Recent reviews (Hernot and Klivanov 2008; Pua and Zhong 2009; Tinkov et al. 2009) highlight examples of drug and gene delivery using microbubbles. The incorporation of the drug or gene is typically accomplished using one of the following methods: attachment (covalent or non-covalent) to the shell either directly or using a secondary carrier, such as a liposome or nanoparticle (Kheirilomoom et al. 2007; Lentacker et al. 2006); intercalation within the shell (Borden et al. 2007; Eisenbrey et al.

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2009); or incorporation within a fluid inside the shell. The last technique is employed in acoustically active lipospheres (AALs) (Kooiman et al. 2009; May et al. 2002), and is thus amenable to the dissolution of lipophilic drugs, including chemotherapy agents such as paclitaxel (Tartis et al. 2006; Unger et al. 1998); alternatively, hydrophilic or lipophilic agents can be incorporated into US activated echogenic liposomes ((Kopechek et al. 2008). The ability to localize the release of a chemotherapeutic agent via US could be used to increase the local drug concentration at the intended, target site while minimizing the overall systemic toxicity. This could expand the therapeutic window of many chemotherapeutics that possess a narrow distinction between efficacy and unacceptable toxicity (Priestman 2008).

Though not as echogenic as microbubble-based delivery systems, unless phase-transitioned or accumulated due to targeting, PFC liquid emulsions are also being studied as delivery systems due to their increased circulation half-life relative to microbubbles (Lanza and Wickline 2001). Additionally, compared to microbubbles, stable PFC emulsions can be formulated with mean diameters of about 200 nm (Unger et al. 2004). These nanoparticles can extravasate in the microvasculature of tumors due to the presence of large inter-endothelial gaps (Shenoy et al. 2005). Formulation approaches, similar to those used for microbubble delivery systems, have been used for PFC emulsions (Fang et al. 2007, 2009; Hwang et al. 2009; Rapoport et al. 2009) due to the hydrophobicity and lipophobicity of the dispersed, PFC phase (Riess 2001). These PFC emulsions can be activated (i.e. phase transitioned from a liquid to a gas) either thermally upon systemic injection (Rapoport et al. 2007), or via US, a mechanism termed acoustic droplet vaporization (ADV) (Apfel 1998; Kripfgans et al. 2000; Kawabata et al. 2005). ADV is a threshold phenomenon where the phase-transition can only take place if the acoustic amplitudes are greater than a particular threshold value. In the case of ADV, the resulting gas bubbles have been used *in vivo* to selectively reduce cerebral (Kripfgans et al. 2002) and renal (Kripfgans et al. 2005) perfusion.

This study focuses on the characterization and *in vitro* performance of an ADV-triggered emulsion, termed a dual-phase emulsion, that contains both chlorambucil (CHL) and PFC. CHL is a lipophilic nitrogen mustard derivative that, similar to other alkylating agents, is predominately non-cell cycle specific. CHL has been used clinically to treat chronic lymphatic leukemia and lymphoma as well as advanced ovarian and breast cancers (Price and Sikora 2002). The use of CHL is limited, despite its good oral bioavailability (Leukeran, GlaxoSmithKline, London, United Kingdom), by its toxic side effects that include myelosuppression and neurological toxicity (Perry 2008). Therefore, the ability to localize the effects of CHL – by encapsulation and subsequent release using US - could be used to minimize systemic toxicity while increasing the local concentration of CHL at the target site. Additionally, incorporation of CHL into an emulsion has been shown to improve the pharmacokinetic profile of the drug and increase its therapeutic activity relative to a CHL solution (Ganta et al. 2008b). The presented PFC emulsion is micron-sized - unlike the submicron-sized, PFC droplets investigated by other groups (Fang et al. 2007, 2009; Hwang et al. 2009; Rapoport et al. 2007, 2009; Unger et al. 2004). The ADV-triggered drug release from this emulsion could potentially be coupled with ADV-induced occlusion (Kripfgans et al. 2002, 2005).

This study is composed of three main sections. First, the micron-sized, PFC emulsion is prepared and characterized, with a focus on emulsion morphology and its effect on the ADV threshold. Second, US-activated drug release from the emulsion is studied, in terms of growth inhibition, in chambers containing Chinese hamster ovary (CHO) cells. Third, the relationship between droplet size, ADV efficiency (i.e. the fraction of droplets that vaporize per ultrasound exposure), and drug release is analyzed.

Materials and Methods

Emulsion preparation

All chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise noted. Albumin droplets were prepared by modifying a previously established protocol (Kripfgans et al. 2000). Briefly, 750 μ L of 4 mg/mL bovine albumin in normal saline (0.9% w/v, Hospira Inc., Lake Forest, IL, USA) was added to a 2 mL glass vial (Shamrock Glass, Seaford, DE, USA). Chlorambucil (CHL) was dissolved in soybean oil at a concentration of 25 mg/mL. The soybean oil and perfluoro-n-pentane (PFP, 29°C normal boiling point, Alfa Aesar, Ward Hill, MA, USA) were added gravimetrically to produce a final volume fraction of 12.5% for each component. The vial was sealed with a rubber stopper (Shamrock Glass) and metal cap (Shamrock Glass). The vial was then shaken for 45 seconds at 4550 cycles per minute using an amalgamator (VialMix, Lantheus Medical Imaging, Billerica, MA, USA).

The shaken vials were refrigerated (5°C) overnight prior to use. The droplets were diluted in normal saline that had been filtered with a syringe filter (0.22 μ m, Millex GV, Millipore, Billerica, MA, USA) and sized using a Coulter counter (Multisizer III, Beckman Coulter Inc., Fullerton, CA, USA) with a 50 μ m aperture. For *in vitro* experiments with cells, all materials used in the droplet formulation procedure were autoclaved and all solutions were filtered using a 0.22 μ m filter to yield sterile vials of emulsion.

Optical characterization of the emulsions

To determine the location of the oil (i.e. drug laden phase) within each droplet, a fluorescent dye – Vybrant DiI (Invitrogen, Eugene, OR, USA) - was used as a surrogate for CHL. The utilization of a lipophilic dye to mimic an oil-soluble compound (such as a therapeutic agent) and to confirm where such an oil-soluble compound would reside in a microbubble or droplet has been used extensively in microscopy or drug release studies (Ganta et al. 2008a; Kooiman et al. 2009; Lee et al. 2002; Tartis et al. 2006; Unger et al. 1998). The resulting emulsions were diluted in normal saline and samples were aliquoted onto a hemacytometer (Brightline, Hausser Scientific, Horsham, PA, USA). Visible and fluorescent micrographs of the droplets were taken with a microscope (Leica DMRB, Bannockburn, IL, USA) and camera (Spot FLEX, Diagnostic Instruments Inc., Sterling Heights, MI, USA) using Spot Advanced Software (Diagnostic Instruments Inc.).

The acquired images were analyzed in MATLAB (The Mathworks Inc., Natick, MA, USA). The pixel resolution within each image was calibrated using the hemacytometer grid spacing. Since the emulsions contained two immiscible, dispersed phases, the inner and outer diameters for each droplet were manually sized. Magnification limitations restricted this manual sizing to droplets with an outer diameter greater than or equal to 1.5 μ m.

ADV threshold measurements

The ADV threshold was determined using identical methodologies as previously described (Fabiilli et al. 2009). Briefly, diluted emulsions were exposed to acoustic pulses from a calibrated, 3.5 MHz single-element transducer (1.9 cm diameter, 3.81 cm focal length, A381S, Panametrics, Olympus NDT Inc., Waltham, Ma) while in a flow tube setup. The increase in echogenicity, recorded via B-mode US, was used to detect the presence of bubbles generated by ADV and hence the ADV threshold.

Evaluation of CHL loading in emulsion

The CHL concentration in the drug-loaded emulsion was determined via high performance liquid chromatography (HPLC) using a Hitachi 7000 series HPLC system (Pleasanton, CA, USA). EZChrom Elite software (Agilent Technologies, Santa Clara, CA, USA) was used to

control the system as well as acquire and process data. The mobile phase, filtered through a 0.22 μm filter (Durapore GS, Millipore) consisted of methanol (CHROMASOLV Plus for HPLC) and 0.1% (w/v) ammonium acetate (65:35 (v/v)) and was pumped at 1 mL/min through a Luna column (C5, 150 $\times$ 4.6 mm, 5 μm , Phenomenex, Torrance, CA, USA). Samples were manually injected using a 20 μL injector loop. The column was kept at ambient temperature and samples were analyzed at a wavelength of 254 nm. The CHL peak eluted at approximately 4.1 minutes.

CHL standards were prepared, via serial dilution, by dissolving CHL in a solution of methanol and 0.1% (w/v) ammonium acetate (80:20 (v/v)). The HPLC method was validated for linearity in the range of 1 to 500 $\mu\text{g/mL}$ ($R^2 > 0.999$) using the CHL peak area. Triplicate injections of each standard yielded peak area variabilities of less than 3% relative standard deviation. The ability to extract CHL from the soybean oil was validated by adding CHL solubilized in soybean oil to 2 mL glass vials containing 1 mL of extraction solvent – methanol and 0.1% (w/v) ammonium acetate (80:20 (v/v)). The vial was capped, sealed, and then shaken for 45 seconds at 4550 cycles per minute using an amalgamator in order to facilitate the extraction of CHL. The standard was then centrifuged at 3000 rpm for 10 minutes in order to separate the oil from the solvent. The solvent phase was then injected onto the HPLC. It was confirmed that complete extraction of the CHL from the oil was achieved in this sample processing and that any solubilized soybean oil did not chromatographically interfere with the CHL peak.

Emulsion samples were processed similarly to the CHL-laden soybean oil samples by adding an aliquot of emulsion to methanol. The emulsion was broken by shaking the sample, which was evident by the presence of precipitated albumin. The samples were centrifuged and the solvent phase was injected onto the HPLC. It was validated that this sample processing yielded complete extraction of CHL from the emulsion. Also, it was confirmed that the presence of any solubilized albumin within the solvent did not chromatographically interfere with the CHL peak.

Cytotoxicity of CHL in solution

Chinese hamster ovary cells (CHO, American Type Culture Collection, Manassas, VA, USA) were cultured in RPMI 1640 growth media (Invitrogen) supplemented with 10% (v/v) defined fetal bovine serum (Hyclone, Logan, UT, USA), 100 U/mL penicillin, and 100 $\mu\text{g/mL}$ streptomycin (Invitrogen). The cells were grown in a humidified 5% carbon dioxide environment at 37°C. The cells were plated at approximately 2.0×10^4 cells/mL, determined via Coulter counter, in 6-well microtiter plates (Fisher Scientific, Pittsburgh, PA, USA) 24 hours prior to treatment. A CHL solution was prepared by dissolving CHL in dimethyl sulfoxide (DMSO, Sigma-Aldrich). The cells were then incubated with varying concentrations of CHL solution for either 15 or 60 minutes. The concentration of DMSO within each well did not exceed 1% (v/v) in any experimental group, which consisted of 6 wells per CHL concentration as well as a control group (without DMSO or CHL) and a group treated with DMSO alone. Following the incubation period, the medium from each well was removed and the cells were washed in triplicate with warmed (37°C) Dulbecco's phosphate buffered saline (DPBS, with calcium chloride and magnesium chloride, Invitrogen). Fresh medium was added to each well and the cells were grown for an additional 48 hours post treatment. After 48 hours, the control cells had reached approximately 80-90% confluence, which was visibly estimated using an inverted microscope (Leica DMIL). The cells were then harvested using trypsin-EDTA (Invitrogen), centrifuged at 1200 rpm for 3 minutes, resuspended in new medium, and counted using a Coulter counter with a 100 μm aperture. Particles larger than 10 μm were counted as intact cells and used for subsequent calculations (Freshney 2006; Hussain-Hakimjee et al. 2006; Lopes et al. 1993).

The percent growth inhibition (%GI) for each experimental group was determined as follows:

$$\%GI = 100 - 100 \left(\frac{N_{\text{exp},t_f} - N_{\text{exp},t_o}}{N_{\text{control},t_f} - N_{\text{control},t_o}} \right) \quad (1)$$

where N_{control,t_o} is the initial number of cells seeded in the control group, N_{exp,t_o} is the initial number of cells seeded in the experimental group, N_{control,t_f} is the final number of cells in the control group, and N_{exp,t_f} is the final number of cells in the experimental group. The %GI data, as a function of CHL concentration in DMSO, was fit using a sigmoidal curve:

$$y = m_1 + \frac{m_2 - m_1}{1 + \left(\frac{x}{m_3} \right)^{m_4}} \quad (2)$$

where m_1 is the maximum y-value, m_2 is the minimum y-value, m_3 is the x-value at the midpoint of y, and m_4 is the slope at the y-midpoint.

Ultrasound triggering of CHL-loaded emulsion

OptiCell™ (Thermo Fisher Scientific, Rochester, NY, USA) chambers were coated with 20 $\mu\text{g}/\text{cm}^2$ collagen (type I, BD Biosciences, Bedford, MA, USA) 24 hours prior to plating approximately 4.0×10^4 cells/mL CHO cells within the chambers. The cells, seeded 24 hours prior to treatment, were grown using the same conditions as previously described for the 6-well plate experiments. Figure 1 displays the experimental setup and exposure conditions. For experimental groups treated with droplets, the emulsion was diluted with DPBS and introduced into the OptiCell™, yielding a concentration of 7.0×10^7 droplets/mL within the chamber. The chamber was gently and repeatedly inverted to homogenously mix the droplets, then inverted to allow the droplets to settle onto the bottom window; the chamber was then placed into the OptiCell™ holder which was located within a tank ($40 \times 60 \times 27$ cm) that contained degassed, deionized water heated to 37°C (Ex 7, ThermoNESLAB, Newington, NH, USA). The surface of the tank water, located 5 cm above the OptiCell™, was covered with air-filled plastic balls (Cole-Parmer Inc., Vernon Hills, IL, USA) to minimize regassing and heat loss as well as reduce the planar reflection of the US at the air/water surface. A linear array (10L probe, Logiq 9, GE Healthcare, Milwaukee, WI, USA) operating at the following conditions – 6.3 MHz, contrast mode, single focus at 2.5 cm, 1.57 MPa peak rarefactional focal pressure, 0.8 μs pulse duration per scan line, 12 kHz scan line pulse repetition frequency, 10 Hz frame rate – was positioned below the chamber such that the 2.5 cm focus was at the bottom window of the chamber. The array was rastered in a minimally-overlapping manner, via a computer-controlled positioning system, at 2 mm/s across the chamber surface in order to vaporize the emulsion. Only two rasters of the linear array were required to insonify the entire chamber (approximately 75 seconds to scan the entire chamber). Following the exposure, the chamber was removed from the tank, inverted so that the cells were once again on the bottom window, and incubated for 60 minutes. During the incubation, any droplets that did not vaporize remained in contact with the cells; bubbles, and hence PFP gas, generated due to ADV were not in contact with the cells due to their buoyancy and size. The chamber was then washed using similar methods as for the 6-well plate experiments and the cells were grown for another 48 hours post washing. The cells were counted as previously described and the %GI was calculated for all experimental groups listed in Table 1. Due to the fluid movement into and out of the OptiCell™ during washing, a relatively large shear force was exerted on the cells adjacent to the OptiCell™ injection port, resulting in their deplating. The %GI for each chamber was normalized by the deplated area to account for this loss. All control groups received the same handling during the exposure and washing processes. The same CHL concentration was used for groups receiving

CHL in DMSO or emulsified CHL. Analysis of variance (ANOVA) was used to establish the significance between the different experimental groups. The Tukey-Kramer method was applied to differentiate significant differences between the groups.

ADV efficiency

The ADV efficiency in the OptiCell™, using the experimental setup and acoustic parameters from the previous section, was determined using chambers without cells. The emulsion was introduced into the chamber, exposed to US, and the droplets remaining post exposure were counted with the Coulter counter. The droplet sample was briefly over-pressurized in a syringe and then diluted prior to counting, thereby decreasing the likelihood that bubbles were still present in solution. The ADV efficiency was defined as the ratio of the droplet concentration remaining in the chamber post US exposure to the initial droplet concentration (i.e. before US exposure). The experiment was repeated using 1, 2 and 5 passes of the US array across the chamber surface.

Results

Emulsion characterization

Figure 2 displays the visible and fluorescent images of the droplets, where two distinct phases are clearly seen within the droplets. The dye is contained within the oil phase due to the lipophilicity of the dye and the lipophobicity, and also hydrophobicity, of the PFP phase (Riess 2001). The oil phase surrounds the PFP core due to the relative hydrophobicities of the two phases; fluorocarbons are significantly more hydrophobic than hydrocarbons (Riess 2001). A similarly structured emulsion, with a fluorocarbon surrounded by oil, was observed when silicone was emulsified with a perfluoropolyether and water (Lee et al. 2002).

Based on the micrographs, Figure 3 displays the ratio of the inner droplet diameter (PFP) to the outer droplet diameter (PFP plus soybean oil) for the emulsion. Microscopy magnification limitations prevented the optical sizing of droplets with outer diameters smaller than 1.5 μm . Even though these smaller droplets could not be sized, it is evident that as the outer droplet diameter increases, the ratio of the inner to outer diameter also increases. Therefore, larger droplets tend to have a larger volume percentage of PFP relative to smaller droplets and a tighter distribution of the ratio. It also appears that there is a cutoff for each droplet diameter that limits how small the ratio may be for a given size. The ratio distribution has a slight positive skew with a mean ratio of 0.45. The actual outer diameter size distribution, as obtained by the Coulter counter, is displayed in Figure 4.

Characterizing the ratio of the inner and outer droplet diameters may enable a clearer interpretation of the ADV threshold for the dual-phase emulsion, which is seen in Figure 5. Previous findings using PFP droplets (Fabiilli et al. 2009) demonstrated that the ADV threshold was inversely proportional to the mean droplet diameter below 2.5 μm and relatively constant for mean droplet diameters larger than 2.5 μm ; the shell material, either albumin or lipid, had a negligible effect on the ADV threshold. The ADV thresholds for the dual-phase droplets as a function of their outer droplet diameter (solid squares) is plotted along with the ADV threshold for single-phase droplets (x's) from previous work (Fabiilli et al. 2009). Using the results from Figure 3, the mean inner diameter of the dual-phase droplets can be estimated based on mean outer diameter so that the plotted diameters are the PFP core diameters (open squares).

CHL cytotoxicity on CHO cells

Figure 6 displays the cytotoxicity of CHL initially dissolved in DMSO on CHO cells for 15 and 60-minute exposures. The group treated with DMSO alone yielded a %GI that was not

statistically different ($p > 0.01$) than the control group (without DMSO or CHL). Based on the sigmoidal curve fits ($R^2 > 0.99$ in both cases), the CHL concentration required to produce 50% GI was 167 μM and 57 μM , respectively, for 15 and 60-minute exposures. By comparison, the IC_{50} – the concentration required to kill 50% of cultured cells – was 40 μM for CHO cells incubated with CHL for 48 hours (Yamazaki et al. 2007).

Figure 6 also displays the linear correlation ($R^2 > 0.94$) between the mean CHO cell diameter 48 hours post-incubation and the %GI for the CHL in DMSO data. The increase in mean cell diameter can be used as an indicator of CHL exposure. Cell volume, and hence diameter, is known to increase in response to exposure to a variety of chemotherapeutic agents, including CHL (Detke et al. 1980; Ross 1981). Though not fully understood, the increase in cell volume is likely due to the continued protein synthesis by the cell despite the disruption in DNA synthesis and function. If the %GI is due to CHL and additional factors, as will be discussed below, the change in mean cell diameter should only be used as a qualitative indicator of whether CHL was or was not a contributor to %GI.

Ultrasound triggering of the CHL-loaded emulsion

Figure 7 displays the %GI for the eight experimental groups 48 hours post treatment and Table 2 lists the results of Tukey's test for the groups. As per the definition of %GI (Eqn. 1), the control group (group 1) has a mean %GI of zero. The application of US (group 4) yielded a %GI that was not statistically different from the control group.

The blank emulsion alone (group 2) and the combination of US with the blank emulsion (group 6) yielded $18.6 \pm 3.4\%$ GI and $22.6 \pm 2.4\%$ GI, respectively. Inverting group 2 during the 60-minute incubation period (i.e. droplets were not in direct contact with the cells – see Figure 1) resulted in a $17.0 \pm 7.6\%$ GI, indicating that merely the presence of sham droplets within the chamber caused GI. It was found that the %GI experienced by group 2 was a result of increased detachment of the CHO cells during the washing of the chamber when droplets were introduced. Cell detachment in the OptiCell™ can be an issue due to the deformable nature of the chamber growth surfaces, unlike the rigid nature of conventional microtiter plates. Therefore, OptiCells™ can be coated with fibronectin or collagen, as was done in this manuscript, to minimize cell detachment. Despite coating the chambers with collagen, cell detachment was still appreciable during the vigorous washing step. The washing step was critical in ensuring that the cells were not incubated with drug-carrying emulsion after the 1-hour treatment. This detachment was confirmed, via microscopy and using the Coulter counter, by adding the blank emulsion to the chamber and then immediately washing the chamber. The droplets became attached to the cells following their introduction into the chamber, possibly due to an electrostatic interaction. During the washing of the chamber, the adherence of the droplets to the cells coupled with the vigorous movement of droplets during the procedure caused this %GI. Different emulsion shells or different cell types could influence the amount of droplet attachment, and therefore the amount of cells that are depleted. All of these effects lie outside of the intended demonstration of drug release by ADV and were considered as correction factors in the final examination of drug release described below. Additionally, it is not known whether these depleted cells would have been viable had they not been depleted.

Groups 2 and 6 were not statistically different ($p > 0.05$), indicating the ADV process (group 6) did not cause any additional GI when compared to the droplets alone (group 2). The %GI experienced by group 6 may include two potentially offsetting phenomena: 1) the reduction in the number of droplets due to ADV, and thus the decrease in cell detachment due to a decrease in droplet shearing; 2) %GI induced by the ADV process itself. This second phenomena is clearly obtained when the chamber is not inverted prior to US exposure (see Figure 1). When the droplets are resting on the cells and they are vaporized, there is a $74.9 \pm 8.5\%$ GI. However

again, the viability of these detached cells is unknown since they are removed from the OptiCell™ during the washing.

The groups treated with CHL in DMSO (group 3) and CHL in DMSO with US (group 7) yielded %GI values of $83.3 \pm 2.5\%$ and $77.7 \pm 1.5\%$, respectively, which is similar to the results obtained at the same concentration in Figure 6. Additionally, groups 3 and 7 are not statistically different ($p > 0.05$), which is expected assuming a lack of appropriately-sized cavitation nuclei within the medium lacking droplets. The groups treated with emulsified CHL (group 5) and emulsified CHL with US (group 8) experienced $46.7 \pm 7.6\%$ and $84.3 \pm 3.8\%$ GI, respectively. Groups 5 and 8 are statistically different (see Table 2) indicating that the application of US did cause an increase in CHL release from the emulsion. Since group 5 is statistically different from both groups 3 and 7, and group 8 is not statistically different from groups 3 and 7, it can be surmised that the retention of CHL within the emulsion reduces its cytotoxicity until US is applied. Upon insonation, the cytotoxicity of emulsified CHL is equivalent to non-emulsified CHL (i.e. CHL in DMSO, group 3). It should be noted that a portion of the %GI for groups 5 and 8 was due not only to CHL release (via leakage or US), but also the droplet-cell adhesion described above for groups 2 and 6. To determine the %GI due to CHL release, the effects of the droplets and ADV must be estimated. One option is to subtract the approximate 20% GI experienced by groups 2 and 6 from groups 5 and 8 in Figure 7. Alternatively, groups 5 and 8 could be scaled by a fraction of 20% GI, considering that some of the cells exposed to CHL will also be removed during the washing of the chamber.

Figure 8 displays the mean cell diameter for the eight experimental groups. As indicated in Figure 6, the average cell diameter can be used to assess whether CHL exposure contributed to GI. The level of CHL exposure can only be estimated from Figure 6 if no other factors contributed to the %GI. Groups that were not exposed to CHL – 1, 2, 4, and 6 – possessed mean diameters that were not statistically different (Table 2), when comparing any of the two groups. Groups 3 and 8 are statistically different, suggesting that the GI observed in group 8 is not solely due to the presence of CHL but from other factors such as droplets or ADV. Additionally, groups 3 and 7 are statistically different, though only at a 0.05 level. Group 5 displayed a smaller average cell diameter than group 8, but the groups are not statistically different ($p > 0.05$), implying that the dominant cause of GI in group 8 is caused by CHL exposure. Overall, the %GI obtained via cell counts is a more robust metric, when compared to using the mean cell diameter, though the latter metric can be used to gain insight into the mechanism driving the GI experienced by each group.

ADV efficiency

Figure 9 displays the ADV efficiency – the fraction of droplets that vaporize – as a function of droplet diameter. The ADV efficiency was measured with a Coulter counter using the same setup described in Figure 1. A sigmoidal relationship exists between the fraction of droplets vaporized and the droplet diameter, with the ADV efficiency relatively constant above $12 \mu\text{m}$. The direct correlation between droplet diameter and ADV efficiency, at least between 2 and $12 \mu\text{m}$, is qualitatively consistent with a probability of ADV stemming from the droplet distribution (i.e. larger droplets have larger PFP cores (Figure 3) that are more likely to possess nuclei that could facilitate ADV).

Repeated passes of the US array across the chamber cause further ADV of remaining droplets, though the incremental difference in efficiency is larger going from 0 to 1 passes (where it was shown that no recordable droplet vaporization occurred by merely adding the droplets in the chamber and subsequently withdrawing them) than from 1 to 2 passes. This supports the possibility that for each pass of the US array, a constant fraction of the remaining droplets undergo ADV.

Discussion

This study focuses on the development of a micron-sized, drug delivery vehicle - containing a superheated PFC phase - which releases the drug payload upon ADV of the PFC. Other groups formulating drug-carrying PFC emulsions have focused on submicron sized droplets (Fang et al. 2007, 2009; Hwang et al. 2009; Rapoport et al. 2007, 2009). The use of micron-sized droplets that are transpulmonary enables the coupling of ADV-induced drug delivery and localized occlusion. Localized occlusion results from relatively large microbubbles blocking perfusion at the capillary level. This selective generation of transient, vascular occlusion has been previously demonstrated *in vivo* (Kripfgans et al. 2002, 2005). ADV-induced occlusion can complement ADV-induced drug delivery in two ways. First, the ischemic conditions created could increase the residence time of therapeutic material at the intended site, resulting in greater diffusion into the target tissue. A simple example of this phenomenon is obtained by comparing the 50%GI concentration for CHO cells when incubated with CHL for either 15 minutes (167 μ M) or 60 minutes (57 μ M). Second, the ischemic conditions can generate hypoxia that could be used to activate bioreductive prodrugs (McKeown et al. 2007) that may be encapsulated within the emulsion. Thus, the proof-of-concept studies presented here serve as a foundation in developing future therapies that incorporate ADV.

The emulsion discussed in this work was prepared using a single emulsification step with only an aqueous-soluble surfactant, similar to other drug-carrying PFC emulsions (Fang et al. 2007, 2009) and contrast agents (Unger et al. 1998; Tartis et al. 2006; Eisenbrey et al. 2009). In contrast, double emulsions are typically prepared in two stages with two types of surfactants, where the innermost droplets are first emulsified followed by their subsequent emulsification in a secondary fluid (Goubault et al. 2001). The presence of natural emulsifiers within soybean oil, including phospholipids (Sonntag 1988) may help stabilize the PFP core.

The effect on the ADV threshold of encapsulating the PFP core within a layer of oil is currently unknown, though from Figure 5 it does not appear to be as significant for smaller droplets as larger droplets. In the case of AALs, the presence of an oil layer causes the pulse length required for contrast agent destruction to increase at least five-fold relative to contrast agent without an oil shell (May et al. 2002). The thickness of the oil-lipid layer in AALs is 500 to 1000 nm and 300 to 700 nm for AALs containing triacetin and soybean oil, respectively (May et al. 2002). By comparison, the mean oil-albumin layer thickness for the dual-phase droplets described in this study, measured using optical microscopy, is 1390 ± 720 nm. It is possible that the oil layer could inhibit or dampen the expansion of any gas nuclei generated within the PFP core during the ADV process, possibly even causing a recondensation of the PFP at lower rarefactional pressures. This is similar to results where the ADV threshold of PFP droplets increased as the viscosity of the bulk fluid containing the droplets increased (Fabiilli et al. 2009).

Concerning the %GI observed for group 2, PFC emulsions are known to cause cellular growth inhibition, but only in phagocytic cell lines (Bucala et al. 1983; Centis et al. 2007). While this %GI was well explained by droplet-cell adhesion and subsequent detachment from the OptiCell™ during washing, it was also hypothesized that the %GI experienced by group 2 might be attributable to the fact that PFC emulsions can modulate the oxygen content of media due to their high gas dissolving capabilities (Lowe et al. 1998; Riess 2001). Based on the amount of PFP injected into each chamber and the solubility of oxygen in PFP – 80% (v/v) (Johnson et al. 2009) - the maximum change in the oxygen concentration within the chamber, assuming that the PFP did not initially contain any oxygen, is 10%, by weight. Hypoxic conditions can decrease the growth rate of CHO cells, but only when the cells are exposed to an oxygen concentration less than 3.5%, relative to the atmospheric oxygen concentration, for prolonged periods (i.e. > 10 hrs) (Lin and Miller 1992). By comparison, after injecting the emulsion into the OptiCell™, the measured change in dissolved oxygen concentration was

negligible (i.e. less than 1%) over one hour. This is likely due to the gas permeable nature of the OptiCell™ windows and that the PFP was partially saturated with oxygen prior to injection due to atmospheric contact.

The cellular bioeffects of ADV are currently unknown, though some insights can be obtained from the presented studies. The cell detachment due to the ADV process may be a result of droplet displacement prior to vaporization, where velocities up to 20 m/s have been recorded (Kripfgans et al. 2004). Additionally, the rapid consumption of the liquid PFP and expansion of the resulting bubble during the ADV process (Haworth and Kripfgans 2008), combined with bioeffects stemming from cavitation (Dalecki 2004), could cause cell detachment. It is unknown whether ADV could cause cell detachment *in vivo* or if the vaporization process could cause increased cell or vascular permeability, similar to results observed with microbubble cavitation (Hernot and Klibanov 2008; Pua and Zhong 2009).

Since the intended mechanism of drug release from the dual-phase emulsion is US, the ADV efficiency is directly related to the amount of oil, and hence CHL, that could be potentially available for cellular exposure. As seen in Figure 5, an inverse trend exists between the ADV threshold and the droplet diameter. Though this trend is confounded by the use of polydisperse droplets, it is hypothesized that droplets with a lower ADV threshold will also display a higher ADV efficiency for a given acoustic exposure. The inverse relationship between ADV threshold and droplet diameter is similar to the relationship between the thermal vaporization temperature of PFP droplets and droplet diameter. Using the Laplace pressure and Antoine equations, the diameter above which PFP droplets will undergo thermal vaporization at 37°C is 6.4 µm and 4.0 µm for surface tensions of 50 mN/m and 30 mN/m, respectively (Rapoport et al. 2009); shell effects beyond surface tension reduction are ignored in these estimates. Therefore, the distinct ADV thresholds and defined ADV efficiencies of the emulsions, coupled with the observation that PFP emulsions are thermally stable up to 60°C (Kripfgans et al. 2000), indicate these emulsions are relatively free of nuclei that enable the emulsion to stably exist in a superheated state.

Table 3 lists the fraction of total droplets vaporized in terms of both number and volume weighted distributions. The droplets in the 1 to 6 µm range are transpulmonary, and thus amenable to intravenous administration, whereas the droplets in the 6 to 30 µm range are amenable to intra-arterial administration. The number and volume-weighted fractions (without volume correction) were estimated by using the respective distributions and ADV efficiency in Figure 9. The volume corrected fractions were estimated by correlating the ratio of the inner to outer diameter to the outer droplet diameter (Figure 3). This correction was then applied to determine the volume of oil, and thus CHL, released upon ADV. The correction has a more significant impact on the larger droplets that, as seen in Figure 3, have a larger PFP core. Thus, for a single pass of the US array – which was used in the cell experiments – approximately 50% of the oil is released upon ADV. Assuming an equal distribution of CHL within the oil, the actual concentration of CHL released via ADV in the chamber is 50 µM, which causes a 51% GI (based on Figure 6). In Figure 7, group 8 displayed an 84.3% GI, which when the %GI caused by the emulsion alone (groups 2 and 6) is subtracted, the %GI from released CHL is approximately 64.3%. This is qualitatively consistent with the %GI predicted by the ADV efficiency.

In summary, a stable, superheated, micron-sized emulsion has been developed that carries a lipophilic, therapeutic agent. The emulsion is triggered via US to produce gas bubbles and enhance the release of the encapsulated agent, as demonstrated with cultured cells. Current efforts are focused on increasing the drug loading of the dual-phase emulsions, minimizing the effect of non-US related drug release, and controlling the size distribution of the emulsions.

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Figure 1.

Left: Experimental setup used to perform ADV experiments with OptiCells™. Right: Exposure conditions utilized during experiments. The emulsion was added to the chamber containing adherent CHO cells and subsequently exposed to US to cause ADV of the introduced droplets.

Figure 2.

A visible image (left) and its corresponding fluorescent image (right) of the dual-phase emulsion containing PFP and soybean oil stained with a fluorescent dye. The spacing between the two horizontal lines on the hemacytometer is 200 μm and a 20 μm scale is included in each image. Although large droplets appear in this figure, droplets larger than 10 μm in diameter account for only 4.9% (by number) of total droplets. Refer to Figure 4 for a size distribution, obtained via Coulter counter, for the emulsion.

Figure 3.

Left: For $n = 338$ droplets, the ratio of the inner to outer droplet diameter is plotted as function of the outer droplet diameter. The raw data is plotted using open, gray circles and the averaged, binned (1 μm) data is presented as closed, black circles. Right: A histogram of the raw data.

Figure 4.

The emulsion size distribution, obtained using a Coulter counter, plotted as the number percent of total droplets. The 50 μm aperture enables the sizing of particles whose diameters are between 1 and 30 microns. The mean droplet diameter is $3.06 \pm 0.21 \mu\text{m}$ with 4.9% (by number) of the droplets larger than 10 μm in diameter. The CHL loading in the droplets is $3.12 \pm 0.01 \text{ mg/mL}$ emulsion.

Figure 5.

A comparison of the mean ($n = 3$) ADV thresholds, plotted with standard deviations, for single-phase and dual-phase droplets as a function of average droplet diameter. The single-phase ADV thresholds are taken from a previous study (Fabiilli et al. 2009). The dual-phase droplets with a mean diameter of $6.96 \mu\text{m}$ were obtained by centrifuging the emulsion. The data labeled 'dual-phase (shifted)' has been corrected for the estimated mean PFP core diameter, using Figure 3. The horizontal error bars were obtained based on the standard deviation in Figure 3 (right). The following experimental conditions were used: degassed water at 37°C , 3.5 MHz single element transducer, 83 Hz pulse repetition frequency, and 13 cycles.

Figure 6.

Left: Cytotoxicity of CHL initially dissolved in DMSO on CHO cells for 15 and 60-minute exposures. Right: The mean CHO cell diameter as a function of the %GI. For both the left and right plots, each data point is the average of six wells, from three independent experiments. Additionally, error bars are standard deviations of the means.

Figure 7.

Mean ($n = 5$) %GI and standard deviation for each of the eight experimental groups. The presence (+) or absence (-) of each parameter – droplets, CHL, and US – is indicated above each group number. The same exposure procedure, acoustic parameters, droplet concentration, and CHL concentration ($100 \mu\text{M}$ whether in DMSO or emulsified) was used for all groups.

Figure 8.

Mean ($n = 5$) cell diameter and standard deviation for each of the eight experimental groups. The presence (+) or absence (-) of each parameter – droplets, CHL, and US – is indicated above each group number. The same exposure procedure, acoustic parameters, droplet concentration, and CHL concentration (100 μM whether in DMSO or emulsified) was used for all groups.

Figure 9.

The number and volume weighted distributions for the dual-phase droplets are plotted along with the mean ($n=3$) fraction of droplets vaporized, as a function of droplet size, for different number of exposure passes by the ultrasound array. The standard deviation is plotted for the single pass case, with similarly sized standard deviations obtained for the 2 and 5 pass cases. The small decrease in efficiency between 1 and 2 μm is due to the subtraction errors between the treated (with US) and untreated (without US) cases.

Table 1

Groups used in the OptiCell™ experiments. For each group, the positive (+) and negative (-) symbols indicate the presence or absence, respectively, of each parameter. In the case of group 5 or 8, the CHL is present within the dispersed phase of the emulsion.

Group	Droplets	CHL	US
1	-	-	-
2	+	-	-
3	-	+	-
4	-	-	+
5	+	+	-
6	+	-	+
7	-	+	+
8	+	+	+

Table 2

Results of Tukey's test on %GI (upper right triangle) and mean diameter (lower left triangle) data. An 'x' indicates groups that are not statistically different at a 0.01 or 0.05 level. Statistically significant differences at a 0.01 or 0.05 level are indicated by '0.01' and '0.05', respectively.

Group	1	2	3	4	5	6	7	8
1		0.01	0.01	x	0.01	0.01	0.01	0.01
2	x		0.01	0.01	0.01	x	0.01	0.01
3	0.01	0.01		0.01	0.01	0.01	x	x
4	x	x	0.01		0.01	0.01	0.01	0.01
5	0.01	0.01	0.01	0.01		0.01	0.01	0.01
6	x	x	0.01	x	0.01		0.01	0.01
7	0.01	0.01	0.05	0.01	0.05	0.01		x
8	0.01	0.01	0.01	0.01	x	0.01	x	

Table 3

The fraction of total droplets vaporized, expressed in terms of number and volume fraction. The data without volume correction is the fraction of droplets vaporized, based on Figure 9. The volume corrected data uses the relationship between the inner and outer droplet diameters (Figure 3) to estimate the fraction of oil released.

Diameter Range	Counts	Volume corrected	Number of Passes				
			1	2	5		
1 – 6 µm	Number	No	0.08	0.11	0.10		
	Volume	No	0.01	0.02	0.02		
	Volume	Yes	0.01	0.02	0.02		
6 – 30 µm	Number	No	0.06	0.08	0.08		
	Volume	No	0.72	0.85	0.83		
	Volume	Yes	0.49	0.58	0.57		