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Controllable Microfluidic Synthesis of Multiphase Drug-Carrying Lipospheres for Site-Targeted Therapy

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Abstract

We report the production of micrometer-sized gas-filled lipospheres using digital microfluidics technology for chemotherapeutic drug delivery. Advantages of on-chip synthesis include a monodisperse size distribution (polydispersity index (σ) values of $<5\%$) with consistent stability and uniform drug loading. Photolithography techniques are applied to fabricate novel PDMS-based microfluidic devices that feature a combined dual hydrodynamic flow-focusing region and expanding nozzle geometry with a narrow orifice. Spherical vehicles are formed through flow-focusing by the self-assembly of phospholipids to a lipid layer around the gas core, followed by a shear-induced break off at the orifice. The encapsulation of an extra oil layer between the outer lipid shell and inner bubble gaseous core allows the safe transport of highly hydrophobic and toxic drugs at high concentrations. Doxorubicin (Dox) entrapment is estimated at 15 mg mL^{-1} of particles packed in a single ordered layer. In addition, the attachment of targeting ligands to the lipid shell allows for direct vehicle binding to cancer cells. Preliminary acoustic studies of these monodisperse gas lipospheres reveal a highly uniform echo correlation of greater than 95%. The potential exists for localized drug concentration and release with ultrasound energy.

Introduction

Recent advances in molecular biology and genetic research have made possible the creation of more powerful and effective cancer therapeutics, bringing about the realization of the century-old concept of “magic bullets” that can carry therapeutic drugs to target sites with high specificity.¹ Efficient carrier-based systems are of increasing importance due to the growing number of active pharmaceutical ingredients (API) with low bioavailability.² One of the biggest limitations with current cancer therapy with chemotherapeutics has been the systemic toxicity involved, especially with the intravenous or oral administration route. A lack of selectivity to tumor tissues impedes the therapeutic potential of anticancer drugs.³ Advances in chemotherapeutics include encapsulating these cytotoxic drugs in a liposome to minimize systemic effects.^{4, 5} Liposomes consist of single or multiple concentric lipid layers (lamellae) that encapsulate an inner aqueous core. Hydrophilic drugs can be carried in the aqueous compartment of a liposome and hydrophobic drugs can be incorporated in the lipid bilayer. Polymers such as polyethylene glycol (PEG) can be attached to the surface for stabilization and increase liposome residence time in the blood circulation, and particular ligands such as antibodies or peptides can be attached to increase specificity for target sites.⁶

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By tailoring the size, material characteristics, or shell components of the liposome, researchers have been able to achieve some specificity for where these vehicles accumulate, preferentially in tumors.⁷ Several major FDA-approved cytotoxic liposomal formulations (e.g. DaunoXome®, DepoCyt®, Doxil®, and Myocet®) have been in the market since the 1990s. Liposome-based products however suffer from relatively nonspecific biodistribution after injection. Accumulation of liposomes by size selection or molecular targeting is a slow process.⁸ It is desirable to minimize non-specific drug-carrier accumulation due to the toxicity involved with most chemotherapeutic agents.

Although an API can passively diffuse into a liposome, encapsulation yields are often small, especially when the API possesses some degree of membrane permeability. Active encapsulation methods involving for example a gradient in pH or chemical potential⁹ have improved yields, but release of the API in a controlled manner is often a challenge. Release mechanisms range from passive means such as liposome disintegration or diffusion-driven leakage to more active release methods triggered by external or environmental stimuli such as a change in pH, temperature, or enzymatic degradation.^{10–12} Regardless of a passive or active mechanism, an unpredictable release profile will lead to a lack of control in drug release.

One method that has shown initial promise for controlling particle localization and disruption is ultrasound.¹³ Standard liposomes are not acoustically active because their density and compressibility are similar to the surrounding blood. Microbubble carriers are uniquely suited for ultrasound-enhanced local drug delivery because they can be selectively concentrated and disrupted at the region of the acoustic focus.¹⁴ Additionally, the rapid mechanical oscillation of microbubbles in an acoustic field has been shown to enhance the delivery of compounds across cell membranes¹⁵ as well as result in local increases in vascular permeability.^{16–17} These stabilized gas microbubbles are used in the clinic today as ultrasound contrast agents to enhance the reflectivity of perfused tissues in applications spanning cardiology¹⁸ and radiology.¹⁹ Although molecularly targeted agents have not yet been applied clinically, preliminary studies have demonstrated application in vascular inflammation and angiogenesis, where researchers have shown the effectiveness of targeted lipid microbubbles as a diagnostic tool in detecting tumors and metastatic spread by assessing the degree of new blood vessel growth.^{20–22} However the thin microbubble shell and gas core each have limited drug-carrying capacity. Researchers have recently created a new drug delivery vehicle by mounting the liposomes on microbubble shells.²³ This new vehicle possesses the drug payload capacity of liposomes yet can still be concentrated with ultrasound radiation force and disrupted with the higher energy acoustic pulses.

The utilization of multi-layer gas lipospheres capable of delivering bioactive substances at high concentrations is an interesting prospect for the development of cancer treatments.²⁴ As shown in Figure 1, such lipospheres possess a layer with drug-carrying capacity (such as oil) and a gas core with significantly different density and compressibility than the surrounding liquid media, providing contrast for ultrasound imaging. These vehicles can be steered, concentrated, and disrupted with ultrasound, both *in vitro* and *in ex vivo* microvasculature.²⁵ Gas lipospheres however are challenging to produce in a controlled manner due to current manufacturing processes. Conventional production techniques involve the use of mechanical agitation²⁶ or sonication²⁷, both of which create a highly polydisperse population. Thus, the drug dosages within each vehicle are also not consistent, and microscopy often shows a percentage of vehicles without any gas at all, leaving them acoustically inactive. The size distribution of the vehicle is extremely important since parameters such as the destruction threshold, amount of radiation force experienced, resonant frequency (important for imaging), and biodistribution are significantly affected by the vehicle diameter.^{14, 28, 29}

To control particle synthesis and drug encapsulation, our manufacturing strategy utilizes microfluidics technology. The versatility in function and application of the microfluidic platform has provided unique solutions to healthcare problems within a span of just a few years.^{30–35} Microfluidics is one of the few technologies that is able to produce fluid emulsions with high precision.³⁶ Microfluidic techniques have already shown to be powerful technologies for the generation of highly controlled droplet dispersions^{37–40} and microbubbles.^{41–44} An additional major advantage of microfluidics is that the potential also exists for manipulating the chemistry and material characteristics of droplets or particles within the devices so that different functional properties are added for a given application. Using microfluidic flow-focusing, we recently demonstrated the production of lipid-based microbubbles with a much narrower size distribution than commercially available for use as ultrasound contrast imaging agents.⁴⁴ A monodisperse microbubble population leads to a more consistent echo response and the potential for higher sensitivity in molecular imaging.

In this work, we report the ability to generate functionalized multi-layer gas lipospheres with precisely controlled size and drug carrying capacity. Previous work in the literature have demonstrated the application of multi-layer lipospheres as drug delivery agents, but those vehicles were produced with techniques that resulted in size and loading inconsistency. This is the first such paper that describes a system for providing a high level of manufacturing control in the production of these complex (three-phase) biocompatible particles. Our microfluidic device in Figure 2 combines two hydrodynamic flow-focusing regions together and features expanding nozzle geometry to generate monodisperse gas-filled lipospheres. The same design may be used to generate single or multi-layer gas lipospheres.

Materials and Methods

Photolithography and Fabrication

Fabrication of devices followed standard soft lithography techniques.⁴⁵ Briefly, fluidic channel geometries were designed in Illustrator (Adobe, Inc.) and printed at 20,000 dpi onto transparency masks by CAD/Art Services (Bandon, OR). In a class 10,000 clean room, a 3 in. silicon wafer was first oxidized in oxygen plasma for 10 min at 200 W and 100 mTorr and then spin-coated at 2000 rpm with a UV-curable epoxy (SU-8-25, MicroChem) to form a 25 μ m high layer. Exposure to UV light through the photomask containing the channel pattern cross-links the exposed areas of SU-8, which then remain on the wafer after development.

The wafer was used to cast a replica in the silicone elastomer polydimethylsiloxane (PDMS) (Sylgard 184, Dow Corning), consisting of a 10:1 prepolymer and curing agent ratio. PDMS as a stamp material has favorable properties such as optical transparency and wide solvent compatibility. The prepared mixture of PDMS was degassed under vacuum, poured onto the wafer with template, and cured for at least 4 hrs at 65° C in a temperature-controlled dry oven. The cured PDMS was peeled from the wafer in a laminar flow hood. Inlets and outlets were punched with a blunt 18 G needle, and the stamp was bonded to clean soda lime glass (Corning) after 90 s of air plasma (200 mTorr, 200 W) treatment with an expanded air plasma cleaner (Harrick Scientific, NY). To preserve the hydrophilicity of the PDMS after plasma preparation, channels were immediately filled with DI water.

Lipid Materials and Preparation

The lipid flow stream contains an aqueous glycerol/propylene glycol (GPW) mixture from Sigma (St Louis, MO) with the stabilizing lipids DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine, Avanti Polar Lipids) and polyethylene glycol (PEG) lipid conjugate DSPE-

PEG2000-Biotin (1,2-Distearoyl-sn-Glycero-3-Phosphoethanolamine-N-(Biotinyl(Polyethylene Glycol)2000), Avanti Polar Lipids) at a concentration of 0.5 mg mL⁻¹ DSPC. These lipid shell components are similar to those commonly used for formulation of lipid-encapsulated ultrasound contrast agents.⁴⁶

First, DSPC and DSPE-PEG2000-Biotin were combined at a 9:1 molar ratio and dissolved in chloroform (CHCl₃, Sigma) to create a homogenous mixture. The solvent was evaporated with a nitrogen stream, exposed to vacuum, and allowed to dry. DiI-C18 (1,1'-dilinoylel-3,3,3',3'-tetramethylindocarbocyanine perchlorate, Molecular Probes) was then added at 1 mol% for fluorescence microscopy studies of the outer lipid shell layer. Ultra-pure water was added to the vial containing the dry lipid mixture, sonicated at room temperature for 20 min, and combined with glycerol/propylene glycol to form a 10% GPW mixture. The solution was again sonicated during preparation and immediately before using at 37° C to prevent unwanted liposome formation. To ensure air saturation, the lipid solution was stirred overnight in a 1 atm air environment.

System Assembly

The PDMS microfluidic device was placed on an inverted fluorescence microscope (TE2000, Nikon) before external fluidic connections are made. Nitrogen (N₂, Airgas) for multi-layer gas lipospheres or octofluorocyclobutane (C₄F₈, Specialty Chemical Products) for single-layer gas lipospheres were supplied from pressurized tanks via clear Tygon® tubing and delivered into the gas inlet of the microfluidic chamber using a homemade micro flow meter. The continuous liquid (lipid and oil) phase mixtures in 3 cc Becton-Dickinson syringes were pumped into the microfluidic device via 23 G hypodermic tubing at constant flow rates using two digitally controlled syringe pumps (Pico Plus, Harvard Apparatus). A high-speed camera (Fastcam PCI-10K, Photron Ltd.) was used to capture monochrome still images and record movies. A file viewer (PFV, Photron Ltd.) and image analysis program (ImageJ, NIH) were used for data processing and measurements. The polydispersity index $\sigma = \delta/d_{avg} \times 100\%$ was calculated from the average vehicle size d_{avg} and standard deviation δ , determined by measuring the sizes of at least 100 gas lipospheres from recorded images.

Cell Culture

Minimum essential medium (MEM), sodium pyruvate, sodium bicarbonate, trypsin/EDTA, gentamicin, and fetal bovine serum (FBS) were obtained from Invitrogen (Carlsbad, CA). Phosphate Buffered Saline (PBS) and Collagen type IV was obtained from Sigma (St Louis, MO). The human metastatic breast cancer cell line MDA-MB-231 was donated and originally purchased from American Type Culture Collection (Manassas, VA).

The cells were cultured in MEM supplemented with 10% FBS and sodium pyruvate, sodium bicarbonate, gentamicin at 37° C in a 5% CO₂ incubator. Cells were grown on cover slips coated with collagen type IV (100 µg mL⁻¹) to provide an adherent surface for cells. Labeling of the cytoskeleton was performed on live adherent cells in culture plates using TubulinTracker Green reagent (T34075) from Invitrogen (Carlsbad, CA). Mitochondria were stained with MitoTracker Deep Red 633 (M-22426) from Invitrogen (Carlsbad, CA), and the nucleus was stained blue with Hoechst 33342 (H1399) from Invitrogen (Carlsbad, CA). Labeling occurred predominately on the exposed surface.

Biotin (EZ-Link Sulfo-NHS-LC-Biotin, Pierce) binds with high affinity to avidin, and was used for labeling of cell surface proteins. MDA-MB-231 cells adherent to glass cover slips were washed with ice-cold PBS three times to avoid amine-containing components competing and quenching the reaction to cell surface proteins. Cells were suspended at a concentration of $\sim 25 \times 10^6$ cells mL⁻¹ in PBS (pH 8.0) and then incubated in 1.0 mg mL⁻¹

biotin/PBS solution (results in ~2 mM biotin reagent) for 30 min at room temperature before rinsing with a PBS and 100 mM glycine solution.

Imaging and Binding

Gas lipospheres in the form of a foam were collected using a glass pipette from the outlet reservoir for imaging of the stained lipid membranes. Aliquots were transferred onto glass slides and secured with plastic cover slips to reduce fluid flow and contain the lipospheres. The sample was positioned on the microscope stage and detection of fluorescence occurred at $\lambda = 569$ nm due to DiI labeling.

For visualization of the inner oil layer, triacetin oil (Glyceryl triacetate, Sigma), capable of carrying bioactive substances, was premixed with Oil Blue N dye from Sigma (St Louis, MO) at a concentration of 0.01 mg mL^{-1} . The viscosity of triacetin (28.0 cP) is considerably less compared to other oils, making it ideal for use at the desired liquid flow rate regimes of $0.25\text{--}0.50 \text{ }\mu\text{L s}^{-1}$ in the microchannels. Doxorubicin (Dox) from Sigma (St Louis, MO) was used as the model antitumor drug due to the intrinsic fluorescence properties of the molecule. Dox was mixed with triacetin at a concentration of 1 mmol L^{-1} . Drug concentration of Dox (ex $\lambda = 470$ nm; em $\lambda = 585$ nm) within the oil layer was visualized by fluorescence microscopy with rhodamine optics.

For gas liposphere-cell binding studies, $10 \text{ }\mu\text{L}$ of Avidin (ImmunoPure Avidin, Pierce) was added to $500 \text{ }\mu\text{L}$ of biotinylated vehicles. After 5 minutes, the vehicle solution was washed with DI water several times. The mixture was collected with a glass pipette and applied directly to the adherent cells on collagen type IV-coated cover slips and imaged with an Olympus BX-41 microscope and CytoViva imaging system consisting of a high resolution illuminator, dual mode fluorescence module, and Exfo fluorescence light source. Gentle movement of the cover slips was sufficient to achieve some degree of binding between the floating gas lipospheres and the adherent cells at the bottom.

Acoustic Response

The acoustic response of $15 \text{ }\mu\text{m}$ multi-layer gas lipospheres was measured as they were pumped at a low concentration through a 200 micron acoustically-translucent tube suspended in a water bath on the inverted microscope. Vehicles were excited with a 1 cycle, 2.25 MHz acoustic pulse at approximately 200 kPa using a spherically focused ultrasound transducer (V305, Panametrics), energized with an arbitrary waveform generator (AWG2021, Tektronix) and amplifier (3200L, ENI). The transducer was positioned in the water bath and the acoustic focus was aligned with the translucent tube. Echo signatures for each gas liposphere were received using a second 2.25 MHz transducer, amplified by 40dB (BR640, Ritec) and then recorded using a 14-bit, 100 MHz digitizer (PDA14, Signatec) through a LabView (National Instruments Corporation, Austin, TX) interface. Offline processing in Matlab (The Mathworks Inc, Natick, MA) was used to calculate the average correlation between echoes. The size distribution for the polydisperse microbubbles used as a control was similar to the size distribution of Definity® (Lantheus Medical Imaging, North Billerica, MA), an ultrasound contrast agent approved by the US Food and Drug Administration (FDA).

Results and Discussion

Device Parameters

Liposphere size and production rate are highly prone to downstream pressure conditions in microfluidic channels. Thus channel geometry and the liquid and gas flow rates are important parameters for stable production and precise control of the liposphere sizes.

Combining two hydrodynamic flow-focusing geometries together into a single region proved less sensitive to gas pressure than in double flow-focusing geometry where the two flow-focusing regions are separated by an arbitrary distance. This was critical to the stable formation of smaller sized multi-layer gas lipospheres. The gas inlet distance from the orifice region was minimized to reduce gas diffusion and reduction in the widths of the liquid and gas inlet channels to 30 μm and 20 μm respectively enabled the use of lower liquid flow rates and gas delivery pressures to generate micron-sized lipospheres. To reduce bubble contact, the outlet reservoir immediately followed the expansion chamber. The liposphere volume V depends on the ratio of the gas pressure P and overall liquid flow rate Q_t , which is a combination of the oil and lipid/water flow rates Q_o and Q_w . V increases linearly with P for a fixed Q_t . An increase in Q_t results in a decrease in V .

For the production of gas lipospheres, a pressure drop must occur along the longitudinal axis of the device until the tip of the gas stream breaks at the orifice. We found that the most stable production regime occurs when the oil and lipid/water phase form an interface of equal pressure upstream of the orifice. Due to the large viscosity difference between triacetin oil and water (28.0 cP vs. 1.0 cP), the oil flow rate Q_o was minimized as much as 30-times less in comparison to the lipid/water flow rate Q_w . Channels were also designed to minimize the contact time and angle between the oil and gas flow streams just prior to the orifice. Since PDMS has the characteristics of high diffusion coefficients to gases such as nitrogen, reducing the distance between the gas inlet and orifice results in a lower P necessary to produce the same V for a fixed Q_t .

Generation and Stability

By simple adjustments in flow parameters, differently sized gas lipospheres were produced using the same microfluidic flow-focusing device. The flow condition relationships can be found in Figure 3a. Stable production rates of 6×10^4 multi-layer gas lipospheres per minute have been recorded from a single chamber, requiring several hours to achieve an estimated 2×10^7 vehicles necessary for imaging and drug delivery. This relatively slow production rate currently limits our microfluidics system for use as a viable bulk manufacturing process. The PDMS devices are however robust enough for consistent production of gas lipospheres, and runtimes that last more than six hours have frequently been observed. Multiplexing several flow-focusing circuits together will also lead to higher throughput and time and cost savings. A ten-fold scale-up of microbubble contrast agent production in our lab has been achieved without a significant change in dimensions of the device. We feel that future device design improvements will likely allow for large-scale production of these vehicles.

As can be seen in Figure 3b, the smaller sized ($7.5 \pm 0.2 \mu\text{m}$) gas lipospheres were produced at a lipid/water flow rate of $0.33 \mu\text{L s}^{-1}$ and a gas pressure of 1.0 psi, while the larger sized ($> 20 \mu\text{m}$) vehicles were produced at a lipid/water flow rate of $0.5 \mu\text{L s}^{-1}$ and gas pressure of 1.6 psi and higher. The oil flow rate Q_o was set at a constant $0.016 \mu\text{L s}^{-1}$. We found that the smaller lipospheres undergo dissolution to a terminal size where the shell achieves very low surface tension as they were rising to the top. These lipospheres remain stable over the experimental period of 3 h. Increasing the gas and liquid flow rates however decreases the distance between exiting lipospheres. These contact interactions cause them to coalesce due to the lower shell resistance in a high flow velocity environment of the microfluidic expansion chamber, destroying the desired monodispersity of the bubble population. Growth in size was also seen for many of the larger ($> 20 \mu\text{m}$ in diameter) vehicles after they reached the outlet reservoir, typical of Ostwald ripening.⁴⁷ Since the gas lipospheres rise to the surface producing a highly concentrated layer known as a cream, reducing vehicle size (a function of flow parameters) or increasing the viscosity of the continuous lipid/water phase will delay the rise time and creaming. Varying the percentages of glycerol/propylene glycol will change the viscosity of the solution, and can therefore be used to optimize microbubble

stability. With sufficient time, vehicles that reach their terminal size form a fully compressed lipid monolayer shell that has reached a near zero surface tension.⁴⁸

We are currently exploring improved methods of stabilizing these drug delivery vehicles. Possible solutions to improve long-term stability may include adding polaxamers, a different PEG group, or photopolymerizable lipids. However, it may not be necessary to substantially increase shelf life if the vehicles can be manufactured at the point-of-care. Clinical (FDA-approved) lipid contrast agents that are formed via mechanical agitation are injected immediately into the patient, eliminating the need for long-term stability. Testing *in vivo* will be necessary to determine circulation lifetime, as the blood pressure and dissolved gas concentration may affect stability.

Structure Visualization

The outer DSPC lipid shell stabilizes the vehicle and the inner triacetin oil layer can contain dissolved therapeutics. Using a lipid dye, DiI, fluorescence microscopy images of these monodisperse vehicles were taken to confirm the lipid coating on the vehicle surface. The inner dark blue layer confirms the existence of the additional triacetin oil layer. The volume fraction of the oil layer can be adjusted using flow rates for varying the chemotherapeutic drug concentrations. The gas interior makes the vehicle acoustically active to ultrasound pulses. Low polydispersity values (<5%) demonstrate the advantage of the microfluidic method of manufacture. In contrast, multi-layer gas lipospheres capable of carrying consistent drug payloads cannot be reliably made with standard mechanical agitation techniques. Such lipospheres have high polydispersity values of >50%.²⁴

Microfluidic-generated multi-layer gas lipospheres are visible in brightfield after being collected on the cover glass, as seen in Figure 4a. A 10 μm gas liposphere contains approximately 0.5 pL of drug-carrying capacity. Based on an analysis of fluorescence measurements, standard curves of Dox dissolved in triacetin were used to estimate the drug content in the gas lipospheres. Dox is a naturally fluorescent anthracycline antibiotic and anticancer drug. It has been prepared in liposomal formulations as Doxil® and Myocet® to treat kaposi's sarcoma (KS) and metastatic breast cancer. The pattern of Dox fluorescence did not change remarkably in the vehicle suspensions after 1 h of monitoring. As seen in Figure 4b, the sharp boundary is due to the partitioning of Dox preferentially at the boundary of the lipid/oil layer. This is expected as Dox favors the lipid layer, and the drug has not been observed to leach out spontaneously. At the 1 mmol L^{-1} concentration of drug used in this study, the total amount of Dox entrapment is estimated at 15 mg mL^{-1} of packed particles. In a dual-layer vehicle, a large volume fraction of the oil phase can be used and other chemotherapeutic drugs such as Paclitaxel, which exhibits good solubility in triacetin (70 mg mL^{-1}), can be incorporated at high concentrations. At this concentration, approximately 2.5 mL of suspension would be necessary for an average dose in a human patient. Until now, the ability to accurately control the amount of drug in each vehicle has not been possible.

Cell Targeting

To demonstrate the targeted binding of monodisperse gas lipospheres to cancer cells and potential for localized chemotherapeutic drug delivery, we functionalized our vehicles by incorporating biotinylated lipid into the shell. Many current targeted molecular imaging methods involve using the avidin-biotin interaction.⁴⁹ The avidin-biotin interaction is the strongest non-covalent interaction between a protein and ligand. Without the conjugation, we have seen that the gas lipospheres will float on the top of the solution and will not adhere to the anchored biotinylated cells at the bottom. Upon collection of the functionalized gas lipospheres, it was necessary to perform gentle movement of the cover slips to achieve some

degree of binding. The live, labeled MDA-MB-231 breast cancer cells remained viable for up to 5 days of culturing under constant perfusion of media, but died in a matter of hours on cover slips from stress due to experimental conditions.

As shown in Figure 5, biotinylated gas lipospheres with the avidin linker bound to biotinylated MDA-MB-231 breast cancer cells are visible in fluorescence microscopy. It is possible to have multiple gas lipospheres bound to a single cell, and the binding efficiency depends largely on liposphere size. Gas lipospheres < 10 μm in diameter were found to more favorably target the cells than larger vehicles. The vehicles remained bound through cell death, approximately 1 h into the experiment. Due to the possibility of an immunogenic response with avidin or Streptavidin, we anticipate an alternative conjugation method besides avidin-biotin will be used for future targeted drug delivery to tumor vasculature or other target sites.

Acoustic Response

From an m-mode ultrasound image of 12 echoes, as shown in Figure 6a, the acoustic response of 15 μm multi-layer gas lipospheres can be determined. Average correlation was measured to be approximately 0.97, indicating that the echo signatures were nearly identical. This is in contrast to previous measurements of commercially-available polydisperse contrast agents, which exhibit echo correlations on the order of 0.7. A comparison can be found in Figure 6b. The nearly identical signatures mean that we can optimize ultrasound concentration and drug-release parameters to affect all gas lipospheres similarly. In contrast, for a polydisperse drug delivery vehicle, ultrasound may be able to fragment a 5 μm vehicle but not a 10 μm vehicle due to the different acoustic parameters required to affect vehicles of different size and corresponding different resonant frequencies. Future experiments will study the acoustic parameters required to concentrate the vehicles with ultrasound radiation force and locally release drug payloads at the target site.

Conclusions

With microfluidics technology, we can produce monodisperse multi-layer gas lipospheres in a suitable size range for local injection into tumor sites. Their composition allows them to be both ultrasonically active and able to carry a large and precise drug payload to the target site. Precision engineered multilayer gas lipospheres are much more consistent in their response to ultrasound radiation force. We expect this to directly translate into uniform drug release and improved imaging characteristics compared with polydisperse vehicles.

Vehicles can be further developed by having very specific targeting ligands on the shell surface. As proof-of-concept, we produced vehicles containing biotin groups with avidin linkers on the shell for attachment to biotinylated breast cancer cells, and have demonstrated loading with a model drug Doxorubicin. Conjugating a cyclic-RGD (cyclo[Arg-Gly-Asp]) peptide to the shell to enhance vehicle retention in tumor vasculature that over express the $\alpha_v\beta_3$ integrin is a possibility for targeted delivery. Upon binding, the narrow size distribution will enable consistent vehicle disruption at the lowest Mechanical Index (MI) possible.

The controlled production of multi-layer microbubble lipospheres using the microfluidic platform provides exciting opportunities for cancer research. We believe similar technology can be applied to generate vehicles that carry hydrophilic drugs as well. The incorporation of a small gas bubble within water or hydrophilic core contained within a secondary shell would make an acoustically-active drug delivery vehicle for hydrophilic molecules. A nice prospect is that when using these vehicles with ultrasound, we can expect to not only deliver and release chemotherapeutic-containing vehicles for therapeutic purposes with site-

specificity and low toxicity, but the acoustic activity of the vehicles will also allow imaging of their distribution within the vasculature and estimation of tumor blood perfusion.

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Figure 1. Schematic of a multi-layer gas liposphere with targeting capability, carrying therapeutics in the lipid-oil complex.

Figure 2.

Geometry of device for gas liposphere production. (a) Schematic view of the microfluidic flow-focusing device. All channels have a rectangular cross section and a height of 25 μm . (b) Main functional area with a 25 μm orifice and filtering channels. The arrows indicate direction of flow. (c) 3D view of assembled PDMS device on an inverted microscope.

Figure 3.

Flow rate vs. size relationships. (a) The optimum flow rate regime (highlighted) to produce $<15 \mu\text{m}$ multi-layer lipospheres, with five different flow condition points (filled diamonds) that produce mean liposphere sizes of $\{11, 7, 17, 22, 30 \pm 0.2 \mu\text{m}\}$ respectively. (b) High-speed images showing changes in gas liposphere size upon application of a low lipid/water flow rate of $0.33 \mu\text{L s}^{-1}$ (left) and a high lipid/water flow rate of $0.5 \mu\text{L s}^{-1}$ (right). The widths of the liquid, oil, and gas inlet channels are $30 \mu\text{m}$, $25 \mu\text{m}$, and $20 \mu\text{m}$ respectively, and the orifice size is $25 \mu\text{m}$. (c) Images showing close-up of two multi-layer lipospheres clearly showing the inner blue oil layer (top) and monodisperse multi-layer gas lipospheres visible in brightfield (bottom).

Figure 4. Microscopy images of multi-layer gas lipospheres containing Doxorubicin. (a) Brightfield image of lipospheres. Scale bar is 15 μm . (b) Fluorescence image of lipospheres showing Dox fluorescence within the lipid-oil complex. Scale bar is 15 μm .

Figure 5. Several monodisperse gas lipospheres capable of carrying drug (circled in white) attached to human metastatic breast cancer cells (MDA-MB-231). The cells are labeled at low passage density with MitoTracker Red, and the nucleus stained with Hoechst 33342. Scale bar is 100 μm.

Figure 6. Acoustic response of 15 μm multi-layer gas lipospheres. (a) M-mode ultrasound image of 12 gas liposphere echoes, with a mean echo correlation of 0.97. (b) Echoes of individual gas lipospheres were more correlated for a monodisperse population produced with microfluidics compared with a standard polydisperse microbubble population produced with mechanical agitation.