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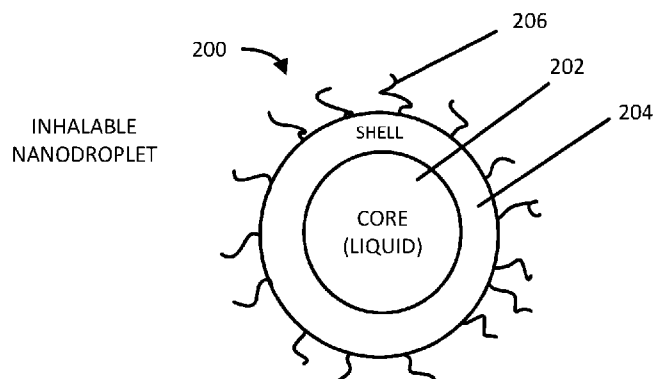


FIG. 2A

(57) Abstract: A method for evaluating respiratory function or structure of a subject includes providing a mixture including a liquid and ultrasound contrast agent particles, each of the inhalable ultrasound contrast agent particles comprising a core, a shell surrounding the core, and a mucus-targeting material in or on the shell, wherein the mucus-targeting comprises a material that binds with or adheres to respiratory mucus. The method further includes nebulizing the mixture to make the ultrasound contrast agent particles inhalable. The method further includes allowing a subject to inhale the nebulized mixture. The method further includes applying ultrasound energy to image the ultrasound contrast agent particles within the subject. The method further includes using results of the imaging to evaluate respiratory function or structure of the subject.



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ULTRASOUND CONTRAST AGENT FOR BINDING WITH OR ADHERING
TO RESPIRATORY MUCUS AND METHODS FOR USING SAME

FUNDING STATEMENT

5 This invention was made with support under Grant Number CAUGHE23G0 awarded by the Cystic Fibrosis Foundation.

PRIORITY CLAIM

10 This application claims the priority benefit of U.S. Provisional Patent Application Serial No. 63/531,151, filed August 7, 2023 and U.S. Provisional Patent Application Serial No. 63/650,474, filed May 22, 2024, the disclosures of which are incorporated herein by reference in their entireties.

TECHNICAL FIELD

15 The subject matter described herein relates to ultrasound contrast agents. More particularly, the subject matter described herein relates to an ultrasound contrast agent with a mucus targeting ligand and/or a cationic surface charge optimized for mucus adhesion and methods for using the ultrasound contrast agent for respiratory evaluation of a subject, including
20 evaluating mucociliary clearance of the subject and evaluation of pulmonary structures such as mucosal thickness, airway edema and mucus hypersecretion.

 The subject matter described herein also relates to a method for measuring lung function and structure using ultrasound and contrasting
25 agents targeted to the lung. More specifically, the subject matter described herein describes administering via inhalation an ultrasound contrast agent optimized for mucus adhesion and then applying ultrasound imaging techniques to evaluate lung structure, pulmonary structures such as mucosal thickness, airway edema and mucus hypersecretion, and
30 quantifying mucociliary clearance of the subject.

 The subject matter described herein also relates to a method and a system for delivering an acoustically activatable agent to the respiratory tract

of a subject to disrupt mucus in the respiratory tract and/or to deliver a therapeutic agent to the respiratory tract of the subject.

BACKGROUND

5 Evaluation of lung function is important in testing efficacy of treatments for respiratory diseases, such as cystic fibrosis. One lung function that is typically evaluated is mucociliary clearance (MCC). Mucociliary clearance is the mechanism by which the lungs expel particles and foreign pathogens from the airways. Currently, mucociliary clearance is evaluated using gamma
10 scintigraphy, which requires the subject to inhale and clear radioactive particles from the lungs while the lungs are imaged to monitor decrease in radioactivity as the gamma particles are expelled. While gamma scintigraphy is effective, repeated exposure of the lungs to ionizing radiation is undesirable. Accordingly, there exists a need for an improved inhalable contrast agent that
15 avoids the need to expose the subject to ionizing radiation. Airway evaluation is also important for risk stratification of burn inhalation injury, the leading cause of all fire-related fatalities. The current method for airway evaluation is bronchoscopy, which is an invasive procedure that is not easily performed at the scene of injury, particularly where first responder skill levels may vary.

20 Accordingly, there exists a need for rapid, noninvasive, and accessible imaging technologies to aid clinical decision making and management of burn inhalation injury.

SUMMARY

25 An inhalable ultrasound contrast agent for evaluation of respiratory function or structure of a subject includes a particle of material comprising a core. The inhalable ultrasound contrast agent further includes a shell surrounding the core. The inhalable ultrasound contrast agent further includes a mucus-targeting ligand located in or on the shell, wherein the mucus-
30 targeting ligand binds with or adheres to respiratory mucus.

According to another aspect of the subject matter described herein, the core comprises a liquid phase at room temperature and atmospheric pressure.

According to another aspect of the subject matter described herein, the core comprises a perfluorocarbon.

According to another aspect of the subject matter described herein, the shell comprises a lipid.

5 According to another aspect of the subject matter described herein, the mucus-targeting ligand comprises chitosan.

According to another aspect of the subject matter described herein, the mucus-targeting ligand comprises hyaluronan.

10 According to another aspect of the subject matter described herein, the mucus-targeting ligand comprises a material with a cationic surface charge.

According to another aspect of the subject matter described herein, the particle comprises a nanodroplet.

According to another aspect of the subject matter described herein, the particle comprises a microbubble.

15 According to another aspect of the subject matter described herein, a method for evaluating respiratory function or structure of a subject is provided. The method includes providing a mixture including a liquid and ultrasound contrast agent particles, each of the ultrasound contrast agent particles comprising a core, a shell surrounding the core, and a mucus-targeting
20 material located in or on the shell, wherein the mucus-targeting material comprises a material that binds with or adheres to respiratory mucus. The method further includes nebulizing the mixture to make the ultrasound contrast agent particles inhalable. The method further includes allowing a subject to inhale the nebulized mixture. The method further includes applying
25 ultrasound energy to image the ultrasound contrast agent particles within the subject. The method further includes using results of the imaging to evaluate respiratory function or structure of the subject.

According to another aspect of the subject matter described herein, the material that binds with or adheres to respiratory mucus comprises a mucus-
30 targeting ligand.

According to another aspect of the subject matter described herein, the material that binds with or adheres to respiratory mucus comprises a material with a cationic surface charge.

5 According to another aspect of the subject matter described herein, the material with the cationic surface charge comprises 1,2-distearoyl-3-

According to another aspect of the subject matter described herein, applying the ultrasound energy includes applying the ultrasound energy with a mechanical index that induces stable cavitation of the ultrasound contrast agent particles.

10 According to another aspect of the subject matter described herein, the mechanical index is about 0.19.

According to another aspect of the subject matter described herein, applying the ultrasound energy includes applying the ultrasound energy at a frequency corresponding to a resonant frequency of the ultrasound contrast agent particles.

15 According to another aspect of the subject matter described herein, using the results of the imaging to evaluate respiratory function or structure of the subject includes using results of the imaging to evaluate mucociliary clearance of the subject.

20 According to another aspect of the subject matter described herein, using the results of the imaging to evaluate respiratory function or structure of the subject includes using results of the imaging to evaluate lung damage caused by trauma or disease.

25 According to another aspect of the subject matter described herein, applying the ultrasound energy includes applying the ultrasound energy to the ultrasound contrast agent particles to disrupt respiratory mucus within the subject.

30 According to another aspect of the subject matter described herein, a method for disrupting mucus in a respiratory tract of a subject is provided. The method includes providing a mixture including a liquid and energy-activatable agent particles. The method further includes nebulizing the mixture to make the energy-activatable agent particles inhalable. The method further includes

administering the energy-activatable agent particles into the respiratory tract of the subject. The method further includes applying energy to the energy-activatable agent particles within the respiratory tract of the subject, causing the energy-activatable agent particles to cavitate and disrupt respiratory
5 mucus.

According to another aspect of the subject matter described herein, a method for delivering a therapeutic agent to a respiratory tract of a subject is provided. The method includes providing a mixture including a liquid and acoustically-activatable agent particles, where at least some of the particles
10 carry a therapeutic agent. The method further includes nebulizing the mixture to make the acoustically-activatable agent particles inhalable. The method further includes administering the acoustically-activatable agent particles into the respiratory tract of the subject. The method further includes applying acoustic energy to the acoustically-activatable agent particles, causing the
15 particles to cavitate, burst, and deliver the therapeutic agent into the respiratory tract of the subject.

According to another aspect of the subject matter described herein, a system for implementing the methods of either of the preceding two paragraphs is provided.

20 According to another aspect of the subject matter described herein, a system for evaluating respiratory function or structure of a subject is provided. The system includes a mixture including a liquid and ultrasound contrast agent particles, each of the ultrasound contrast agent particles comprising a core, a shell surrounding the core, and a mucus-targeting material located in or on
25 the shell, wherein the mucus-targeting material comprises a material that binds with or adheres to respiratory mucus. The system further includes a nebulizer for nebulizing the mixture to make the ultrasound contrast agent particles by a subject. The system further includes an ultrasound imaging system including at least one ultrasound transducer for applying ultrasound
30 energy to the ultrasound contrast agent particles within the subject, detecting ultrasound energy scattered by the ultrasound contrast agent particles, and generating, from the detected ultrasound energy, an image of the ultrasound

contrast agent particles usable to evaluate respiratory function or structure of the subject.

According to another aspect of the subject matter described herein, applying the ultrasound energy includes applying the ultrasound energy to the
5 ultrasound contrast agent particles to disrupt respiratory mucus within the subject.

The subject matter described herein can be implemented in software in combination with hardware and/or firmware. For example, the subject matter described herein can be implemented in software executed by a
10 processor. In one exemplary implementation, the subject matter described herein can be implemented using a non-transitory computer readable medium having stored thereon computer executable instructions that when executed by the processor of a computer control the computer to perform steps. Exemplary computer readable media suitable for implementing the subject
15 matter described herein include non-transitory computer-readable media, such as disk memory devices, chip memory devices, programmable logic devices, and application specific integrated circuits. In addition, a computer readable medium that implements the subject matter described herein may be located on a single device or computing platform or may be distributed
20 across multiple devices or computing platforms.

BRIEF DESCRIPTION OF THE DRAWINGS

Exemplary implementations of the subject matter described herein will now be explained with reference to the accompanying drawings, of which:

25 Figure 1 is an image of gamma scintigraphy of the lungs;

Figure 2A is a schematic diagram of a particle of an ultrasound contrast agent comprising a nanodroplet, and Figure 2B is a schematic diagram a particle of an ultrasound contrast agent comprising a microbubble;

Figure 3 is an image of a ligand conjugated microbubble or nanodroplet
30 contrast agent suspended in liquid inside of an aerosolized particle;

Figure 4 is a graph illustrating particle deposition fraction versus aerosolized particle size;

Figures 5A-5C are images illustrating and ex vivo porcine trachea in agar (Figure 5A), cross-sectional imaging of the trachea before exposure to a prototype nebulized contrast agent that did not include mucus targeting ligands (Figure 5B), and cross-sectional imaging of the trachea after exposure to the prototype nebulized contrast agent, where the imaging was performed using a Siemens Sequoia ultrasound platform with contrast pulse sequencing (CPS) mode;

Figure 6 is a graph illustrating cytotoxic responses in cultured human bronchial epithelial cells exposed to 25 μ l of undiluted contrast agent for a duration of 24 hours, which far exceeds the exposure time needed for imaging purposes;

Figure 7 illustrates ultrasound images of a mouse trachea following inhalation of a prototype contrast agent (contrast in trachea shown in yellow), compared to an anatomical slice of the mouse trachea;

Figure 8A is an ultrasound image of a mouse lung following inhalation of a prototype ultrasound contrast agent without mucus targeting ligands;

Figure 8B is an ultrasound image of a human lung obtained without using a contrast agent;

Figure 9 is an image of Racetrack assay for mucociliary transport rate;⁴⁴

Figure 10 is an example of superharmonic ultrasound imaging in which individual contrast agent bubbles are tracked on a racetrack assay;

Figures 11A and 11B illustrate orientation and distribution of TAP microbubbles (Figure 11A) and neutral microbubbles(Figure 11B), relative to human nasal mucus sample (400x objective). When large (4 μ m) TAP microbubbles were suspended in saline and induced to flow across human respiratory mucus plated on a microscope slide, the microbubbles oriented in a spatial arrangement in proximity to the mucus (Figure 11A). By comparison, our standard, neutrally charged microbubbles demonstrated no affinity for mucus (Figure 11B), and freely flowed as a bulk liquid on the microscope slide, in a uniform distribution with no clustering around the mucus sample. Unlike TAP-microbubbles, these neutrally charged microbubbles appeared to float

above the mucus layer on a different plane, with no interaction or adhesion to the sample;

Figures 12A and 12B illustrate time-lapse movement of fluorescently labeled TAP microbubbles on human bronchial epithelial cell culture. Figure 12A illustrates a panel from a transmitted light video, and Figure 12B illustrates a panel from epifluorescence video. The panels show the same field of view, with fluorescently labeled particles similar in size and motion to those seen in the transmitted light images. The scale bars in each panel are 20 μm ;

Figure 13 illustrates mucociliary transport rate measured across 8 fields of view by tracking the motion of TAP microbubble fluorescence versus motion of visible debris on human mucociliary transport cultures.

Figure 14 illustrates cytotoxic (lactate dehydrogenase secretion) and proinflammatory response (interleukin-8 release) induced by exposing human bronchial epithelial cell cultures to TAP microbubble contrast agent, TAP microbubble vehicle, and saline. The abbreviations used in Figure 14 are as follows: LDH = lactate dehydrogenase, IL-1 = interleukin-8, TAP = 1,2-distearoyl-3-trimethylammonium-propane, IL-1B = interleukin-1 beta, TNF α = tumor necrosis factor alpha. The error bars illustrate standard deviations;

Figure 15 illustrates a comparison of *in vivo* tracheal ultrasound following oropharyngeal aspiration of saline versus TAP microbubble. Panels: Anatomical slice of mouse trachea (panel a). Ultrasound b-mode (panel b and panel d) and contrast mode (panel c and panel d) of a mouse trachea post-saline (panel b and panel c) and post inhalation of TAP microbubbles (panel d and panel e). Images acquired using a FUJIFILM VisualSonics Vevo F2;

Figure 16 illustrates randomly selected representations of mouse lung histology, 7-days post exposure to 50 μL of TAP microbubble contrast agent or saline control, via oropharyngeal aspiration to the lung. Pulmonary tissues stained with hematoxylin and eosin (H&E) and show no indication of inflammation or injury. Pink shapes are blood vessels, purple rings are cross-sections of airways. Images magnified to 10x objective. The scale bar in Figure 16 is 300 μm ;

Figure 17 is a block diagram of a system for evaluating respiratory function or structure of a subject;

Figure 18 is a flow chart illustrating an exemplary process for using ultrasound contrast agents with mucus-targeting material to evaluate
5 respiratory structure and/or function of a subject;

Figure 19 is a flow chart illustrating exemplary steps of a method for disrupting mucus in the respiratory tract of a subject; and

Figure 20 is a flow chart illustrating an exemplary process for delivering a therapeutic agent to a respiratory tract of a subject.

10

DETAILED DESCRIPTION

Highly effective modulator therapy (HEMT) has revolutionized the clinical management of cystic fibrosis (CF). Although the optimum timing to initiate HEMT has not been established, consensus opinion acknowledges a
15 potential window of opportunity in early childhood; beyond which initiation of modulator therapies may have less effect on the course of lung injury.¹ Detractors to an early initiation strategy cite high costs of HEMT and the difficulty of evaluating efficacy in very young patients.¹ The assessment of lung structure and mucociliary clearance in preschool age patients by
20 established diagnostic tests is challenging and reliant upon technologies that require sedation or anesthesia, or expose children to ionizing radiation.² Thus, developing novel, safe, and physiologically meaningful tools to evaluate the influence of *CFTR* modulation on lung function and MCC is of particular importance and an area with high priority to the Cystic Fibrosis Foundation.

25 Each year, nearly 500,000 Americans present to the emergency department with burn injuries. The majority of fire-related deaths (80% - 90%) are due to inhalation injuries. Inhaled smoke and steam induce inward swelling of the airway walls (*i.e.*, airway edema) and overproduction of mucus, which eventually occludes airflow, leading to respiratory failure and death.
30 Rapid identification and risk stratification of burn inhalation injury is crucial, and ideally performed at the scene of injury. While bronchoscopy is the current method for assessing inhalation injury, it is an invasive procedure requiring

sedation and intubation. Tracheal intubation and bronchoscopy are not always advisable, particularly at the scene of injury, where first responder skill levels may vary. These invasive procedures carry the risk of tracheospasm, hemorrhage, and arrhythmia. Thus, there is a clinical need for a noninvasive,
5 accessible, and portable imaging technology for at-the-scene evaluation of lung and airway pathology.

As described above, gamma scintigraphy is the current method for assessing MCC, which is quantified visually by the percent clearance of technetium-99m labeled sulfur colloid, a radiolabeled particle.³ Instead of
10 using ionizing radiation, the subject matter described herein includes an inhalable ultrasound contrast agent for MCC evaluation. Ultrasound is accessible, inexpensive, non-ionizing, and suitable for pediatric use, but is underutilized in pulmonology applications due to poor image quality.^{4,5} The subject matter described herein includes a nebulized contrast agent for
15 ultrasound imaging applications, which is inhaled to coat the airway linings for visualization of the pulmonary structures.⁶ An inhalable ultrasound contrast agent without mucus targeting ligands or cationic surface charge was preliminarily evaluated the *in vitro* toxicity and *in vivo* tolerability, with no significant bioeffects noted. The subject matter described herein includes
20 optimized microbubble and nanodroplet ultrasound contrast agents for mucosal adhesion, using targeting ligands informed by drug delivery research,⁷ and / or cationic surface charges used in inhaled drug delivery research (reference: Fromen et al)⁵², and the use of such contrast agents for a non-ionizing, clinical diagnostic test for assessment of MCC and pulmonary pathology.

25 According to one aspect of the subject matter described herein, an *in vitro* assay will be used to evaluate mucociliary clearance of the ultrasound contrast agents. An established *in vitro* assay will be used to quantify mucociliary transport rate in human airway cultures derived from normal and CF donors. Ciliary movement of bubbles of the ultrasound contrast agent will
30 be quantified visually by tracking individual bubbles by superharmonic ultrasound imaging. It is believed that contrast agent bubbles will travel with

the mucus, without cross-linking to the cilia. It is also believed that the mean mucociliary transport rate will be slower in CF human airway cultures.

According to another aspect of the subject matter described herein, the tolerability of the mucus-targeting ultrasound contrast agents will be evaluated
5 using a study involving mice. In the study, healthy mice will be exposed to inhaled ultrasound contrast agents and observed for acute effects during ultrasound imaging and for 7 days post-exposure. It is believed that the inhalable ultrasound contrast agents will be well tolerated, *in vivo*.

According to another aspect of the subject matter described herein, the
10 assessment of mucociliary clearance using the inhalable contrast agent will be validated using gamma scintigraphy. Nasal MCC, defined by the percent clearance of ultrasound contrast agent over 10 minutes, will be compared to percent clearance of technetium-99m labeled sulfur colloid on gamma scintigraphy, in healthy and affected mice with moderate or severely impaired
15 ciliary function. It is believed that nasal MCC assessed by contrast-enhanced ultrasound will correlate well with gamma scintigraphy. It is also believed that a graded response in the duration of visualized contrast bubbles will be observed for mice with normal, moderately impaired, and severely impaired ciliary function.

20 As stated above, the assessment of lung structure, function, and mucociliary clearance in preschool age pediatric populations by established techniques is challenging. Multi-breath washout testing (*i.e.*, lung clearance index) is an alternative to spirometry, but has not been incorporated into routine clinical practice, likely due to cost and uncertainty surrounding
25 clinically meaningful thresholds.² Diagnostic imaging tests, such as magnetic resonance imaging (MRI) of the lung, require sedation or anesthesia in this age group, while computed tomography (including “ultralow dose” CT) and gamma scintigraphy carry radiation exposure risks. The challenges of quantifying clinical endpoints by ionizing imaging modalities are compounded
30 by the difficulty in recruiting appropriate controls for pediatric CF groups, as the majority of institutional review boards prohibit exposing healthy children to ionizing radiation for research purposes.⁸

Impaired mucociliary clearance is a distinguishing sign of CF,⁹ and can be quantified visually by 60- or 90-minute percent clearance of technetium-99m labeled sulfur colloid on lung gamma scintigraphy (Figure 1). Technetium99m-sulfur colloid, a radiopharmaceutical, is delivered to the lung by a nebulizer, and once inhaled, remains suspended in the mucus layer of the respiratory tract.³ Although technetium99m-sulfur colloid particles are submicronic (0.3 μm), the nebulized form (*i.e.* particles carried within a saline droplet) spans 5 μm , facilitating deposition in the large airways.⁸ Thus, the 60- or 90-minute percent clearance of technetium-99m is interpreted as the action of the mucociliary escalator in the large airways. MCC assessment by gamma scintigraphy has been shown reproducible and is an accepted clinical trial endpoint for the evaluation of response to therapies in CF.¹⁰ However, despite standardization of gamma scintigraphy protocols, radioisotope exposure remains an important clinical consideration.¹¹ Although CF was historically only a disease of childhood, the life expectancy has now increased to 48 years,¹² which demands consideration of cumulative radiation exposure and its long-term effects. Across a 17 year period from 1992 – 2009, the mean cumulative effective dose of radiation exposure per patient with CF increased from 0.39 mSv / year to 1.67 mSv / year, with thoracic imaging accounting for 47% of the total exposure.¹³ A 6-fold increase in the use of computed tomography was also reported over this time interval.¹³ These observations underscore the clinical need to develop a sensitive, reproducible, non-ionizing assessment of lung function and MCC, particularly for longitudinal evaluations requiring recurrent testing.

Ultrasound as a non-ionizing alternative for MCC assessment

Ultrasound is accessible, inexpensive, non-ionizing, and suitable for pediatric use, but is underutilized in pulmonology applications due to poor image quality.^{4,5} Sonographic image quality can be improved by intravenously-administered microbubble contrast agents, which are currently FDA-approved for diagnostic cardiac imaging by ultrasound. Outside of the United States, microbubble contrast agents are also used to evaluate the pancreas, liver, kidney, and breast.¹⁴ However, no ultrasound contrast agents

have been developed to enhance pulmonary imaging, nor has an inhalable route of delivery been previously described. Our team has been working on technologies to enhance airway imaging by ultrasound, using an inhalable contrast agent.⁶ Our prototype technology uses a nebulizer to disperse
5 stabilized microbubbles, which are then inhaled and coat the airway linings. The result is a several fold increase in contrast and substantial improvement in visualization of the tracheobronchial airways. We have preliminarily evaluated *in vitro* toxicity of our prototype contrast agent, and the *in vivo* tolerability, with no significant bioeffects, and no acute or long-term adverse
10 health effects noted. These preliminary data provide strong evidence that with further development and optimization, we can design innovative and impactful technology for ultrasound airway imaging. The contrast agent described herein is believed to be a safe, inhalable, ultrasound contrast agent that will adhere to the respiratory mucus layer to facilitate non-invasive and non-
15 ionizing monitoring of MCC in patients with recurrent need for thoracic imaging.

Technical Background

Overview: Microbubble and nanodroplet ultrasound contrast agents

20 Ultrasound microbubble contrast agents are composed of a lipid or albumin outer shell filled with an inert, high molecular weight gas such as sulfur hexafluoride or octafluoropropane (both of which are FDA approved),¹⁴ or alternatively, decafluorobutane (pending FDA approval). The gas core is highly compressible and oscillates when exposed to an ultrasound pulse,
25 causing contraction or expansion of the microbubble diameter (in the range of 1-5 microns) and effective scattering of the ultrasound beam. Phase-change nanodroplets, composed of liquid perfluorocarbon encapsulated in a polymer shell, have also been developed as for use as ultrasound contrast agents.¹⁵ To date, none have been FDA approved, although at least one company is
30 moving them towards regulatory approval. The phase change nanodroplets can also be encapsulated in a lipid shell. The phase change nanodroplets may be stable with the core primarily in liquid form at room temperature (25

degrees Celsius) and atmospheric pressure. When exposed to ultrasonic energy, the nanodroplets undergo a phase transition from the liquid to gaseous state, expanding 3-6 times the original diameter (300 nm), with ultrasound signal enhancement comparable to that of microbubbles.¹⁶ The
5 inhalable ultrasound contrast agent described herein may be formed of microbubbles, phase change nanodroplets, or both.

Microbubble and nanodroplet stability

One benefit of nanodroplets is that they are substantially more stable
10 than microbubbles. Stability of the contrast agent is likely to be an important consideration for the development of an MCC imaging protocol, which will infer ciliary clearance of the contrast agent based on the elapsed time until the imaging signal disappears. Exposing contrast bubbles to acoustic pressure induces stable cavitation (*i.e.*, oscillating swelling and contraction), the
15 mechanism by which nonlinear echoes are produced to generate a contrast signal. However, with excessive acoustic pressure, transient cavitation is induced, causing bubbles to rupture. To minimize loss of contrast signal due to transient cavitation, the ultrasound system's mechanical index is typically lowered from 0.4 to ~0.19. A mechanical index of 0.4 is well within the FDA
20 approved range for diagnostic imaging and does not cause bioeffects but is sufficient to destroy contrast bubbles. Nondestructive subharmonic imaging techniques, whereby the ultrasound system's transmission frequency is in sync with the contrast bubbles' oscillating resonant frequency, are also used to prevent transient cavitation.¹⁷

25

Surface chemistry

Intravenously-administered microbubble contrast agents have been functionalized by conjugating adhesion ligands onto the outer shell.^{18,19} With this technique, vascular endothelial growth factors, such as VEGFR2, P- and
30 E-selectin, have been successfully targeted to image regions of angiogenesis or vascular endothelial dysfunction.^{14,20} A inhalable contrast agent targeting the respiratory mucus layer has not been previously developed but may be particularly effective for sonographic pulmonary evaluation and assessment

of MCC. The epithelium of the large (but not small) airways is coated by gel-forming mucus at the air-epithelium interface.²² In aerosolized drug delivery research, adhesion ligands such as chitosan (or alternatively hyaluronan⁷) have been developed to target the mucus layer.²³ Adhesion to the respiratory surfaces has also been achieved for aerosolized compounds with either cationic or anionic surface charges.⁵² When applied to an inhalable ultrasound contrast agent, we anticipate that this strategy will increase retention of the contrast agent to the airway mucus layer.

Thus, a particle of an inhalable contrast agent may include a core, a shell surrounding the core, and one or more mucus-targeting ligands located in or on the shell. Figure 2A is a schematic diagram of a particle of an ultrasound contrast agent comprising a nanodroplet, and Figure 2B is a schematic diagram a particle of an ultrasound contrast agent comprising a microbubble. Referring to Figure 2A, an inhalable ultrasound contrast agent nanodroplet particle **200** includes a core **202**, a shell **204** surrounding the core, and one or more mucus targeting materials **206** located in or on shell **204**. Core **202** may be a perfluorocarbon that is stable at room temperature and atmospheric pressure. Shell **204** may be a lipid, a polymer, or other suitable material. Mucus targeting material **206** may be a material designed to adhere to respiratory mucus. Examples of materials suitable for material **206** include ligands, such as chitosan or hyaluronan, or a material with a cationic surface charge. Because muco-adhesion is amplified by positive charge,⁵³ another optimization strategy is to synthesize contrast agent bubbles with cationic lipid shells, such as with the use of TAP (1,2-distearoyl-3-trimethylammonium-propane), a method for creating positively charged ultrasound microbubbles.⁵⁴

Referring to Figure 2B, an inhalable contrast agent microbubble particle **208** includes a core **210**, shell **204**, and mucus targeting ligands **206**. Core **210** may be a gaseous material, such as a perfluorocarbon, that has changed phase and increased in diameter in response to application of ultrasound energy. Shell **204** may be of the same composition described above for nanoparticle **200**. Mucus targeting material **206** may likewise be of the same material described above with regard to nanoparticle **200**. It is understood that

microbubble **208** may be formed from nanoparticle **200** after nanoparticle **200** is inhaled and exposed to ultrasound energy. In another example, microbubble **208** may be inhaled as a microbubble instead of a nanodroplet that changes phase inside of the body of the subject.

5

Aerosolized delivery methods for an inhaled ultrasound contrast agent

According to the subject matter described herein, microbubble or nanodroplet contrast agents will be suspended in liquid droplets (illustrated in Figure 3) generated by a nebulizer. The deposition of an inhaled particle is primarily determined by its MMAD (mass median aerodynamic diameter), which influences impaction, sedimentation, and diffusion properties.²⁴ Particles with nanoscale MMADs are transported through the airways by Brownian motion.^{24,25} In contrast, the movement and deposition of particles with micron-scale MMADs is driven by laminar or turbulent airflow, inertial impaction, gravity.

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Ligand conjugated microbubble or nanodroplet contrast agent suspended in liquid inside an aerosolized particle induced sedimentation, or by particle-particle interaction.²⁴ Small inhaled particles in the <1-3 μm range deposit in the alveoli deep in the lung tissue, while particles >8 μm deposit by inertial impaction in the large upper airways.²⁶ Interestingly, aerosolized particles in the 0.3 – 0.5 μm range have the lowest deposition in either the large, tracheobronchial airways or small, alveolar regions (Figure 4).²⁷ As described in the Methods section, we will deliver microbubble and nanodroplet contrast agents within an aerosolized droplet generated by a nebulizer. The aerosolized droplet size (MMAD) can be modulated by the nebulizer system, to optimize transport and deposition of our inhalable contrast agents. Large, aerosolized droplets are expected to impact more readily against the inner surfaces of the upper airways, due to inertial impaction.²⁴ Upon surface impact, the aerosolized carrier droplets will rupture, releasing the microbubble or nanodroplet payload onto the airway surface, as we previously demonstrated using ex vivo pig tracheas⁶ (described in the next section). The deposition of aerosols is also influenced by airflow dynamics, which can be modified by patient instruction. For example, tachypnea (i.e., fast and shallow

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breathing) increases the deposition and impaction of aerosolized particles in the extrathoracic airways (e.g., trachea), while limiting deposition in the more distal airways and lung.²⁸

Thus, in one example, microbubble or nanodroplet contrast agents with mucus targeting ligands can be provided to the subject using a nebulizer, which generates aerosolized liquid (e.g., water) droplets, each of which encapsulate and carry one or more particles of the contrast agent. The subject can then inhale the aerosolized liquid droplets including the particles of the contrast agent.

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Preliminary Results

Prototype nebulized contrast agent enhances ultrasonic imaging of an ex vivo trachea

To evaluate the feasibility of enhancing airway imaging with a prototype nebulized contrast agent, we constructed an imaging phantom by encasing a fresh ex vivo porcine trachea in agar (Figure 5A). Contrast microbubbles were formulated from distearoylphosphatidylcholine (DSPC) and distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (DSPE-PEG2000) and filled with inert, high molecular weight decafluorobutane gas. The microbubbles were activated by mechanical agitation for 45 seconds and nebulized using a custom-modified Pari LL nebulizer.²⁹ Imaging was performed using a Siemens Sequoia ultrasound system, with contrast pulse sequencing (CPS) mode selected to optimize signal enhancement. The mechanical index was set to 0.19 to prevent destruction of the contrast microbubbles by acoustic energy.³⁰ As described,⁶ the ex vivo trachea was imaged before and after administering our prototype contrast agent as an aerosol through the lumen. There was no discernable delineation of the tracheal walls on CPS imaging mode prior to exposure (Figure 5B), but the near wall of the trachea was easily visualized post-exposure (Figure 5C), demonstrating adhesion of the nebulized contrast agent to the luminal surface. There was no contrast-enhanced visualization of the far wall, which is consistent with poor ultrasound beam transmission through the air column. However, the complete structure of the trachea was easily

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visualized by obtaining images from the frontal, right lateral, and left lateral imaging planes. Because the trachea is easily imaged by ultrasound without obstruction from bone, we anticipate that it will be the clinical target for future imaging protocols to evaluate MCC, which will monitor the percentage of cleared contrast bubbles over time. In this situation, a single imaging plane of the trachea is likely to be sufficient for MCC assessment. Alternatively, existing technologies for 3D ultrasound could be used, by rendering imaging views from the frontal and 2 lateral imaging planes of the trachea.

10 *Prototype contrast agent is not toxic to human bronchial epithelial cells*

We preliminarily assessed the toxicity of our prototype contrast agent to primary human bronchial epithelial (hBE) cells collected from 3 donor lungs and differentiated at an air-liquid interface on 12 mm Transwell permeable support membranes to a mucociliary phenotype. Lactate dehydrogenase (LDH) was quantified in the basolateral medium following a 24-hour apical exposure to 25 μ L of undiluted prototype contrast agent with the microbubbles activated by mechanical agitation for 45 seconds, 25 μ L of undiluted vehicle control without mechanical agitation (non-activated), or a no treatment control. One Transwell from each donor was treated with 4% Triton X-100 to lyse cells and provide a positive (maximal LDH release) control. Each exposure condition was normalized to the basolateral LDH content in the positive control condition. Neither exposure to the activated prototype contrast agent nor vehicle control produced a cytotoxic response, as shown in Figure 6.

25 *Prototype contrast agent enhances in vivo airway imaging in a mouse model*

We also preliminarily assessed tracheobronchial image enhancement by our prototype contrast agent, using an *in vivo* murine model. A 1:1 dilution of prototype contrast agent (without mucus targeting ligands) to saline was inhaled into the lung via an established oropharyngeal aspiration technique.³¹ The mouse was anesthetized by isoflurane (indicated by depressed breathing and testing for righting reflex) and was suspended with the tongue extended from the mouth and hung to the side. A controlled amount (50 μ L) of prototype

contrast agent was instilled onto the oropharynx (back of the throat), using a gel loading micropipette tip. Immediately following oropharyngeal aspiration, the mouse was imaged using a 3D volumetric SonoVol Vega ultrasound platform. Contrast-enhanced delineation of the trachea is shown in Figure 7.

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Prototype contrast agent enhances in vivo lung parenchyma imaging in a mouse model

At 2 minutes post-inhalation, the prototype contrast agent became apparent in the lung parenchyma Figure 8A. Because the mouse was positioned laterally during aspiration, sedimentation of the contrast agent and resulting image enhancement was observed in the left lung. The mouse was closely observed for acute signs of respiratory distress (*i.e.*, gasping, suspended breathing) during the imaging procedure, and was also monitored for signs of physical distress up to 4 weeks following exposure. No acute or long-term adverse sequelae were noted. For comparison, we show our contrast-enhanced murine lung ultrasound image next to a conventional lung ultrasound from a human patient. In its current state, clinical lung ultrasound is suboptimal and limited to nonspecific interpretations of air-tissue artefact lines³² (Figure 8B), rather than direct visualization of the airway or lung structures. Our encouraging preliminary results evaluating a prototype inhalable contrast agent demonstrate that we can achieve pulmonary image quality far exceeding that which is currently used in clinical practice, and supports further development of an optimized contrast agent for noninvasive and non-ionizing MCC in children with CF.

The following section describes proposed experiments to validate the use of the microbubble and nanodroplet ultrasound contrast agents with mucus-targeting ligands to evaluate mucociliary clearance.

Validate *in vitro* mucociliary transport of microbubble and nanodroplet ultrasound contrast agents functionalized with muco-adhesive targeting ligands and/or cationic surface charge.

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The formulation and functionalization of nanodroplet and microbubble contrast agents is described in various publications.^{15,33–37} To optimize adhesion of our contrast agents to the respiratory mucus layer, we will conjugate microbubble and nanodroplet formulations with targeting ligands informed by inhalable drug delivery research^{23,28,38–40} and/or formulate contrast agent bubbles with a cationic surface charge^{52–54} (references: Fromen, Christensen). Based on its extensive development in drug delivery research, we propose to use chitosan as the muco-adhesive targeting ligand, or alternatively hyaluronan.⁷ To decorate the outer lipid shells of nanodroplet and microbubble contrast agents, a chitosan-lipid bioconjugate will be formulated.^{41,42} As described, the bioconjugate will be added to a vial which will be sealed, with the headspace of the vial purged with 50 mL of perfluorocarbon gas.³³ The colloidal dispersion will then be agitated and cooled, to create functionalized microbubbles or nanodroplets.³³ Because muco-adhesion is amplified by positive charge,⁵³ we propose to synthesize microbubbles with cationic lipid shells using TAP (1,2-distearoyl-3-trimethylammonium-propane), a method for creating positively charged ultrasound microbubbles.⁵⁴

***In vitro* MCC assay:** Mucociliary transport of our functionalized contrast agents will be verified using an established *in vitro* assay.⁴³ The so-called “racetrack assay” is created by affixing a central ring to a Millicell culture insert, forming a circular track around the perimeter. First passage primary human airway epithelial cells are cultured and then plated on the Millicell insert and allowed to grow for 2-3 weeks until confluent. When grown on the patterned substrate (*i.e.*, “racetrack”),⁴⁴ human airway epithelial cultures achieve continuous directional mucociliary transport (MCT). The MCT rate can then be quantified by imaging the movement of mucus over time, using high frame rate video microscopy. A controlled concentration of mucus is used for the assay, with fluorescent beads added to the mucus to aid visualization of motion (Figure 9). Individual bead trajectories are measured automatically with a custom Python script to quantify the MCT.⁴³

Experimental Plan: To ascertain the feasibility of using ultrasound contrast bubbles to quantify MCC, we will first conduct an *in vitro* validation, by demonstrating that our candidate contrast agents travel with the mucus layer on racetrack assays without forming cross-links to the cilia. A total of 6 contrast agents will be tested: 1). conjugated microbubble and 2). conjugated nanodroplet (*i.e.*, both functionalized with a mucus targeting ligand); also 3). unconjugated microbubble and 4). unconjugated nanodroplet (*i.e.*, no targeting ligand), 5). Microbubble with cationic surface charge, and 6). Nanodroplet with cationic surface charge. Each of the 6 agents will be tested in racetrack assays cultured from 6 human donors (N=3 healthy, and N=3 with cystic fibrosis) We will quantify the MCT rate for each contrast bubble formulation, using superharmonic ultrasound imaging techniques (tracking the motion of contrast agent bubbles along the racetrack assay), which will be compared with the MCT rate quantified by video microscopy (tracking the motion of fluorescent beads), as an established control. We will also compare the mean MCT rate for healthy donor assays vs. CF assays, with the expectation that mucociliary transport will be slower in assays derived from CF cultures. The human airway cells used to create the racetrack assays will be procured from the UNC Marsico Lung Institute, an activity deemed to be “Not Human Subjects Research” by our IRB.

Superharmonic Ultrasound Imaging: For each racetrack assay, a controlled amount (1 μ L) of activated candidate contrast agent will be diluted with 1 μ L of saline and added to the mucus layer. Once deposited, the motion of individual contrast bubbles will be tracked using high frame rate superharmonic ultrasound. This technology achieves resolution beyond the diffraction limit of conventional coherent imaging and can resolve 25-50 micron single bubble displacements over 1 second. Our team member (Dr. Dayton) has extensive experience in the localization and tracking of ultrasound contrast agents by this technique.^{37,45} We have preliminarily ascertained the feasibility of this approach, by imaging the movement of non-conjugated microbubble contrast agent for 8 seconds, as shown by the arc in

Figure 10. In this preliminary work, the contrast bubbles were observed to move with the mucus layer, without forming cross-links to the cilia.

Hypothesis, Potential Pitfalls, & Solutions: Our preliminary racetrack assay demonstrated that prototype contrast agent moved with the mucus layer without epithelial absorption, supporting the feasibility of contrast-enhanced ultrasound for MCC assessment. When administered intravenously, ultrasound contrast agents are impermeable to the endothelium and remain in the circulation without entering the surrounding tissues. We expect that all 4 contrast agents will travel with the mucus layer, and that no formulations will cross-link with the cilia. We also expect that MCT rates will be slower in assays derived from CF cell cultures, compared to assays from healthy donors. Cross-links to the cilia are a potential concern with mucus-binding ligands. However, chitosan has been extensively evaluated in aerosolized drug delivery research and the likelihood of cross-linking to the cilia is thought to be low. If cross-linking is observed, we will eliminate chitosan-conjugated contrast agents from further consideration and will evaluate hyaluronan as alternative. If ciliary cross-links are observed with both types of mucus binding ligands, we will abandon mucus-targeting optimization of our contrast agents and our focus for Aim 2 will instead be the evaluation of non-conjugated formulations.

Analytical Plan: For both imaging modalities (*i.e.*, video microscopy and superharmonic ultrasound), the mucociliary transport rate will be calculated across a 10 second interval, with measurements made in triplicate. Consistency in the measured mucociliary transport rate will be analyzed by calculating the coefficient of variation,⁴⁶ grouping the 3 video microscopy measurements with 3 measurements for each candidate contrast agent to assess assay-specific variability in the MCT rate measured by the 2 modalities. We expect a similar MCT rate for microscopy and superharmonic ultrasound imaging, irrespective of the contrast agent used, and expect all contrast agent formulations will move with the mucus without forming ciliary cross-links. With a sample size of 42 triplicate-averaged assay runs (6 tracking candidate contrast bubbles and 1 tracking fluorescent beads, across 6

separate donor assays) we expect 88% power (2-sided $\alpha=0.05$) to detect a statistically significant proportion with ciliary cross-links, if observed in a minimum of 2 or more of the assay runs. We will also compare mean MCT rate for CF vs. normal control assays. Previous research suggests that the mean MCT rate in normal mucus is $74.7 \pm 15.7 \mu\text{m/s}$.⁴³ With this assumed standard deviation and a sample size of 30 triplicate-averaged assay runs (15 from CF assays and 15 from normal donor assays), we expect 82% power (2-sided $\alpha=0.05$) to detect a minimum mean difference in MCT rate of $17 \mu\text{m/s}$ or greater between the 2 groups.

Limitations: The resolution demonstrated by superharmonic ultrasound imaging of the racetrack assays will not be possible *in vivo*. This is because there is a trade-off between imaging depth and imaging resolution inherent with ultrasound. For our *in vivo* and future clinical imaging protocols, we will instead rely upon the elapsed time until contrast signal clearance, which is similar to the approach used by gamma scintigraphy (described later in Aim 3). By validating our assessment of MCC by ultrasound contrast agents both *in vitro* (tracking individual contrast bubbles) and *in vivo*, we expect to develop a sensitive, safe, and physiologically meaningful diagnostic test for MCC.

Verify *in vivo* safety and tolerability of candidate contrast agents optimized for muco-adhesion, using in healthy mice.

Contrast agent allocation: After validating ciliary clearance of our contrast bubbles *in vitro*, we will randomly assign 23 adult wild type C57BL/6 mice⁴⁷ (aged 8-12 weeks, weighing 20-25 g) to 3 exposure groups: 1). microbubble functionalized with chitosan [N=10], 2). nanodroplet functionalized with chitosan [N=10], and 3). saline control [N=3]. Mice will be anesthetized by intravenous injection and nebulized contrast agents (or saline control) will be administered via nose cone. To increase deposition in the upper airways, a nebulizer generating a large MMAD droplet (*i.e.*, Pari XLent, MMAD = $7.3 \mu\text{m}$) will be used. For each mouse, a controlled amount of activated candidate contrast agent ($50 \mu\text{L}$) or an equivalent dose of saline control will be released as an aerosol into the nose cone, for a duration of 30 seconds. Immediately

following dose inhalation, the nose cone will be removed, and an alcohol wipe will be used to remove any contrast agent that may have deposited on the fur surrounding the nose and head. Images of the upper/nasal airways, trachea, and lung will be acquired by whole-body ultrasound using a SonoVol Vega with nondestructive subharmonic imaging techniques.

Contrast-enhanced ultrasound: The elapsed time from appearance until dissipation of contrast signal will be recorded, within 3 distinct regions of interest: 1). Nasal airways, 2). Trachea, and 3). Lung. In our preliminary experiment using a non-conjugated prototype contrast agent, contrast signal dissipation was first observed in the trachea, then the upper/nasal airways, and lastly in the lung. We did not use nondestructive subharmonic imaging techniques in this preliminary work, and consequently the time until signal disappearance was likely to be truncated, providing an inaccurate estimate of ciliary clearance of the contrast agent. With nondestructive imaging techniques, we expect better preservation of the contrast agent bubbles and longer times until signal disappearance.

Evaluation of *in vivo* tolerability: The *in vivo* tolerability of our mucus-targeting, inhalable ultrasound contrast agents will be assessed by observing acute symptoms of respiratory distress (e.g., gasping, suspended breathing) during the ultrasound imaging session, as well as noting any post-exposure symptoms of malaise (e.g., lack of grooming or food refusal) within 7 days. The development of symptoms will be compared to the control group exposed to nebulized saline. We have selected healthy mice for the *in vivo* tolerability assessment, because symptomatic disease states (e.g., CF phenotype) would confound the attribution of adverse effects.

Hypothesis, Potential Pitfalls, & Solutions: We expect that mucus-targeting contrast agents will be well tolerated, both with the microbubble and nanodroplet formulation. Given the established safety profile of FDA-approved intravenous microbubble contrast agents and the extensive development of chitosan in drug delivery research, the risk of adverse events following exposure to mucus-targeting contrast agents is expected to be low. However, if an adverse event occurs, the candidate contrast agent will be eliminated

from consideration. In this situation, alternative formulations such as mucus-targeting by conjugated hyaluronan, or non-conjugated formulations will be substituted. In our preliminary work, our prototype contrast agent (*i.e.*, non-conjugated microbubble) was well tolerated *in vivo*.

- 5 **Analytical Plan and Power Calculation:** With a sample size of 23 mice and an assumed binomial distribution of events, we expect 87% power (2-sided alpha = 0.05) to detect a statistically significant adverse event if a minimum of 2 or more mice are observed to have acute effects.

10 **Validate MCC assessment by contrast enhanced ultrasound compared to gamma scintigraphy, in healthy and affected mice**

- Following the 7-day observation period, the 20 C57BL/6 mice that were imaged with contrast agent in Aim 2 will also be imaged by gamma scintigraphy, as a gold-standard comparison. Nasal MCC will be quantified using an established gamma scintigraphy protocol for mice.⁴⁸ The nasal
15 airways are selected for evaluation, because clearance of mucus in the upper and nasal airways is completely dependent on MCC, without confounding by other mechanisms such as coughing.⁴⁹ Our team has extensive experience with gamma scintigraphy and includes investigators (Drs. Bennett and Zeman) who pioneered the original imaging protocols that are in use today. Gamma
20 scintigraphy images will be acquired using an Intel Medical planar gamma camera and pinhole collimator. As described,⁴⁸ mice will be anesthetized by inhalational isoflurane, and 1 μ L of technetium99m-labeled sulfur colloid suspension will be delivered into the nose by inserting a catheter into the nostril. Mice will then be positioned under the pinhole collimeter for continuous
25 imaging by the planar gamma camera. The nasal MCC rate is not constant over time, with a rapid phase observed during the first 10 minutes, followed by a slower clearance rate from 10 to 60 minutes. We will quantify the radioactivity intensity (RI) from the image pixel intensity, by sampling every minute for up to 10 minutes, with pixel intensity quantified by image analysis
30 software (ImageJ). As described,⁴⁸ we will calculate the cumulative nasal MCC and the average nasal MCC rate, using established algorithms.

Potential Hazards to Research Personnel: Technetium99m-sulfur colloid is a radiopharmaceutical. Research personnel will be required to complete mandatory training in the safe handling and disposal of radionuclides, as required by UNC Division of Institutional Integrity and Risk Management and
5 Environment, Health and Safety.

Hypothesis, Potential Pitfalls, & Solutions: We expect that MCC by contrast-enhanced ultrasound will correlate with MCC by gamma scintigraphy. We also anticipate that the nanodroplet formulation will outperform the microbubble formulation, due to better stability and lower likelihood of spurious
10 contrast signal loss due to bubble rupture, rather than bubble clearance by the mucociliary escalator. However, if a comparable correlation between scintigraphy and ultrasound is observed for the microbubble and nanodroplet formulations, the microbubble will be selected for subsequent testing and development. This is because the microbubble (but not nanodroplet)
15 formulation has prior FDA approval for intravenous use, which may hasten translation of our inhalable, mucus-targeting contrast agent to clinical practice.

Analytical Plan: The relationship between nasal MCC measured by contrast-enhanced ultrasound versus gamma scintigraphy will first be visualized by scatterplots. We will then analyze the within-subject agreement in nasal MCC
20 measurements using Bland-Altman plots, and the within-subject correlation using Spearman or Pearson correlation coefficients. Separate plots and correlation coefficients will be calculated for nanodroplet and microbubble formulations, comparing each to gamma scintigraphy as the gold-standard comparator. The formulation with the strongest correlation and agreement
25 with gamma scintigraphy will be considered to have the best diagnostic performance.

Validation in Affected Mice with Impaired MCC: By first evaluating healthy C57BL/6 mice, we will select the best performing candidate contrast agent using 1) *in vivo* tolerability and 2) correlation and agreement with gamma
30 scintigraphy as the criteria. The best-performing contrast agent will then be tested in mice with MCC impairment ranging from moderate to severe. We propose the betaENaC-Tg and Dnaic1-/- mice, both of which were developed

at the University of North Carolina. The betaENaC-Tg mouse is a model of CF and expresses moderately impaired MCC.⁵⁰ The Dnaic1^{-/-} mouse is a model of primary ciliary dyskinesia, in which mucociliary action is completely abrogated.⁵¹ Ultrasound and gamma scintigraphy will be acquired in total of 10 affected mice (N=5 betaENaC-Tg; N=5 Dnaic1^{-/-}), following a protocol identical to the healthy C57BL/6 mice.

Hypothesis, Potential Pitfalls, & Solutions: We expect that MCC assessment by ultrasound will have a graded response, with the longest time to contrast signal clearance observed in the Dnaic1^{-/-} mice (MCC completely abrogated), followed by the betaENaC-Tg mice (MCC moderately impaired), and lastly the C57BL/6 mice (normal MCC function). Further, we expect quantification of MCC by ultrasound to correlate with MCC by gamma scintigraphy for all 3 mouse groups. In the event that our inhalable ultrasound contrast agent does not provide a sensitive assessment of MCC, we may consider modifications to the experimental approach, such as increasing the dose of administered contrast agent or using a nebulizer with a different MMAD to change its deposition properties.

Analytical Plan and Power Calculations: During the rapid phase (first 10 minutes), the mean clearance of technetium^{99m}-sulfur colloid in the mouse nose is reported to be 1.71% ± 0.17% per minute.⁴⁸ The relationship between MCC (10-minute percent clearance), as assessed by ultrasound versus gamma scintigraphy will be visualized by scatterplots, with the correlation quantified by Spearman or Pearson correlation. With a sample size of 20 paired assessments of MCC by contrast-enhanced ultrasound versus gamma scintigraphy (N=10 healthy mice, N=10 affected mice), we expect 88% power (alpha = 0.05) to detect a statistically significant correlation of 0.75 or higher.

In-Vitro Inhalable Ultrasound Contrast Agent Formulation Targeting Respiratory Tract Mucus Layer and Use for Pulmonary Ultrasonic Imaging

In this section, we describe the chemical formulation, *in vitro* evaluation (authentication, validation, safety), and *in vivo* assessment (tolerability and

imaging feasibility) of a mucus-targeting inhalable ultrasound contrast agent that may be used to enhance pulmonary imaging and measure mucociliary transport.

Methods

5 *Ethics statement*

In this investigation, human bronchial epithelial cell (hBEC) cultures and C57BL/6 mice were evaluated. Cell cultures originated from 4 adult, healthy, de-identified donors (2 male, 2 female). Donated cells were obtained from the Marsico Lung Institute Tissue Procurement Core facility at the
10 University of North Carolina. The use of hBEC cultures and associated respiratory mucus for analytic testing was deemed “Not Human Subjects Research” by the UNC Institutional Review Board. Mice were purchased from Jackson Laboratories, with all animal protocols approved by the UNC Institutional Animal Care and Use Committee (IACUC).

15 *Contrast agent design considerations*

To optimize pulmonary imaging, we designed an inhalable ultrasound contrast agent formulated to adhere to the airway inner surfaces. A gel mucus layer coats the large airways at the air-epithelium interface,²² and can be targeted by ligands (e.g., chitosan, hyaluronan)⁶¹ or by imparting a surface
20 charge onto aerosolized compounds.⁵² Because the mucus layer covering the epithelium has a mesh spacing of less than 300 nm,⁶² we designed a polydisperse contrast agent with diameters ranging from 1-5 μm , which is consistent with intravenously administered contrast agents that are used clinically.²⁰ The micron-scale diameter was selected to ensure the contrast
25 agent remained within the airway surface layer with minimal epithelial absorption.

Chemical formulation of cationic microbubble contrast agent

Mucus-targeting contrast agent was synthesized in house, using 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) and polyoxyethylene (40) stearate (PEG 40-S) to create the microbubble lipid outer shell. Because
30 muco-adhesion is amplified by positive charge,⁵³ the lipid mixture included 1,2-distearoyl-3-trimethylammonium-propane (TAP) to yield a cationic

surface.⁵⁴ The cationic lipid mixture (12 mol% TAP, 34 mol% PEG40-S, and 54 mol% DSPC; TAP-lipid)⁵⁴ was heated in propylene glycol (PG) at 60 C for 1 hour with sonication to create a dissolved suspension.⁶³ The aqueous lipid stock solution (4.67 mM total lipid concentration) contained 15% (v/v) propylene glycol and 5% (v/v) glycerol in phosphate-buffered saline (PBS) as the diluent (PBS-PGG). TAP-lipid solutions containing 1-, 2-, and 3-mM total lipid were created by diluting the stock cationic lipid solution with PBS-PGG. Lipid solutions (1.5 mL) were packaged into 3 mL serum vials, sealed with silicone septa, and the air in the headspace was removed and exchanged with perfluorobutane (C₄F₁₀), an inert, high molecular weight gas. Once prepared, TAP-lipid solutions and vials of lipid solution were stored at 4°C. To create gas-filled microbubble emulsions, sealed vials containing room-temperature TAP-lipid solutions and perfluorocarbon gas were shaken in a VialMix (Lantheus Medical Imaging, N. Billerica, MA; 4500 rpm, 45 seconds). The diameter and yield of TAP-microbubbles were determined for microbubbles prepared from 1, 2, and 3 mM TAP-lipid solutions using an Accusizer FX Nano system (Entegris, Billerica, MA).

Authentication of mucus-targeting

To verify the association of our cationic contrast agent with the respiratory mucus layer, we observed the movement and distribution of TAP microbubbles versus our standard, neutrally charged microbubbles relative to a target mucus sample. Our neutral microbubble contrast agent was formulated in-house, as previously described,⁶⁴ from a 1 mM lipid solution containing a (9:1) molar ratio of DSPC to 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (DSPE-PEG2000) to create a neutral lipid shell (DSPC/P2K) around a core of C₄F₁₀ gas. Cationic (TAP) or neutral contrast agents were enriched for larger diameter microbubbles to facilitate capturing photomicrographs of microbubble-mucus interactions at 400X magnification. TAP or neutral microbubbles were centrifuged (1-2 minutes, 160x g) in sealed 5-mL syringes. The smaller diameter microbubbles in the infranatant fluid were separated and discarded from the floating 'cake' of larger microbubbles. Larger microbubbles

were resuspended in PBS-PGG, with the centrifugal enrichment repeated 3 times before determining the final concentration and size distribution of 'large' microbubbles. Clean glass slides were plated with respiratory mucus from an anonymous, healthy donor and allowed to dry overnight. A 1% bovine serum albumin (BSA) solution in PBS was used to rehydrate the mucus and block the rectangular area surrounding the mucus sample. After blocking, 50 μ L of contrast solution was pipetted onto the BSA-blocked area. The slide was tipped to allow contrast to flow over the mucus area, then inverted to allow contrast to "float" up against the mucus sample. After a 1-minute inversion to allow flotation, the slide was dipped into PBS to remove unbound microbubbles. The slide was wiped to restore the rectangular wetted area around the region of interest, and PBS was added to couple a water-immersion objective lens optically. The spatial arrangement of microbubbles was observed microscopically at 400X, and photomicrographs were captured to document microbubble-mucus interactions.

Mucociliary transport

A mucociliary transport assay⁴³ was used to quantify the movement of TAP versus neutrally charged microbubbles on human bronchial epithelial cell cultures, with a fluorescent lipophilic cationic indocarbocyanine dye (Invitrogen, Waltham, MA, Vybrant™ DiO) used for labeling. As described,⁶⁵ the *in vitro* transport assay was created in house by affixing a central ring to a Millicell culture insert, which was etched to form circular "racetracks" around the perimeter. First passage primary hBECs were cultured and plated on the Millicell insert and allowed to grow for 2-3 weeks until confluent. As previously noted, hBEC cultures achieve continuous directional mucociliary transport when grown on the patterned substrate.⁶⁶ Mucociliary transport assays are coated by a mucus layer at the air surface interface, allowing direct, *in vitro* quantification of mucociliary transport.

To perform the transport assay, a culture was first incubated for 5 minutes with 1 ml of PBS with calcium and magnesium at 37°C to rehydrate the mucus. The surface fluid was then removed and 20 μ L volume of microbubble solution (either TAP or neutral contrast agent, concentrated to

200,000 microbubbles/ μ L), was pipetted onto one position in the center of the track. The culture was then placed in an OkoLab microscope incubator. The incubator was maintained at 37°C and humidified air with 5% CO₂ flowing through the culture chamber at 400 ml/min. The culture was allowed to

5 equilibrate for ten minutes. Videos were captured from eight equally spaced fields in the center of the track using a Nikon Eclipse TE2000 microscope (Nikon Instruments Inc, Melville, NY, USA) and a Basler acA1300-200 um camera controlled by SAVA software (version 2.0.8W, Ammons Engineering, Clio, MI, USA).⁶⁷ At each field of view, a transmitted light video was taken

10 using phase optics (20x ELWD objective) and a 120 frame/second capture rate. Then immediately following that video, a fluorescence video was taken of the same field of view with a 15 frames per second (fps) capture rate. The fluorescence excitation was provided by a Lumencor SOLA 5-LCR-VG LED source (Lumencor; Beaverton, OR) and the light path used a Nikon B-2E/C

15 filter block. To measure transport, a particle position was manually recorded at two locations in each video. To standardize the comparison of transport rates for our TAP and neutrally charged microbubbles, transport assays were cultured from the same healthy, human donor. As a validation, the calculated transport of fluorescence was compared with the transport rate of visible

20 debris within the same field of view, captured on transmitted light video at 15 fps. Evidence of cross-links between contrast agent and cilia was visually monitored, for each of the 8 fields of view, as a safety objective. Epithelial absorption was identified by fluorescence beneath the cilia, with the proportion quantified relative to the entire area for each field of view.

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Cytotoxicity and proinflammatory response

Potential biological effects of our TAP microbubble were tested using primary hBEC cells collected from 4 donor lungs, which were differentiated at an air-liquid interface on 12 mm Transwell permeable support membranes

30 (Corning, Glendale, AZ) to a mucociliary phenotype. Lactate dehydrogenase (LDH) and proinflammatory cytokine interleukin-8 (IL-8) were quantified according to manufacturer instructions (CyQUANT LDH Cytotoxicity Assy,

Invitrogen and OptEIA Human IL-8 ELISA, BD Biosciences, respectively) in the basolateral medium following a 24-hour apical exposure to either 25 μ L of undiluted TAP-contrast (with the microbubbles activated by mechanical agitation), 25 μ L of undiluted vehicle control (TAP lipids), 25 μ L saline control, or a no-treatment control. As positive controls, one Transwell insert from each donor was treated with 4% Triton X-100 to lyse cells and provide maximal LDH release, and one Transwell insert was treated with 10ng/mL of each IL-1 beta and tumor necrosis factor alpha (TNF alpha) to produce maximum IL-8 proinflammatory response. Donor-specific release of LDH and IL-8 were quantified in triplicate for each exposure condition and averaged. Potential biological effects of TAP-microbubble contrast agent were statistically analyzed by comparing each exposure condition with both the negative and positive control conditions, using paired t-tests.

15 *In vivo safety, tolerability, and imaging feasibility*

To examine the *in vivo* tolerability, safety, and pulmonary imaging feasibility of our inhaled ultrasound contrast agent, we evaluated six C57BL/6 mice (8-12 weeks, all female). Mice were administered either 50 μ L of TAP microbubble contrast agent (n=3 mice), or sterile saline control (n=3). Because mice are obligate nose breathers, a controlled dose was administered to the lung using an established oropharyngeal aspiration technique.³¹ Mice were anesthetized by isoflurane (indicated by depressed breathing and testing for righting reflex) and positioned supine on a recumbent imaging stage, in reverse Trendelenburg orientation, with the tongue extended from the mouth and hung to the side. A controlled amount of contrast agent or saline was instilled onto the oropharynx, using a gel loading micropipette tip. Immediately following oropharyngeal aspiration, mice were imaged in contrast mode using a FUJIFILM Vevo F2 ultrasound machine interfaced with a 15.0 – 29.0 MHz transducer, capturing at a frame rate of 30 fps and dynamic range of 57.65 dB. The trachea was imaged in longitudinal orientation, with the ultrasound transducer positioned by a stereotactic clamp. Mice were observed during the procedure for signs of distress, and for 7 days post-procedure by

monitoring weight, grooming, and body condition score. After monitoring for acute effects for 7 days, mice were sacrificed. The trachea and lungs were harvested and preserved in formalin, with tissues fixed with hematoxylin and eosin (H&E) stain. Histology was evaluated by an independent, veterinary pathologist who was blinded to the exposure condition.

Results

TAP-microbubbles, emulsified from C₄F₁₀ gas and a 1 mM aqueous solution of lipids containing 12 mol% TAP, 34 mol% PEG40-S, and 54 mol% DSPC, yielded an average microbubble concentration of $1 \times 10^9/\text{mL}$. Microbubble diameters were polydisperse, ranging from ~ 0.27 to $10 \mu\text{m}$. For evaluation, we selected TAP-microbubbles $> 0.5 \mu\text{m}$, which had a mean diameter of $0.78 \mu\text{m}$ when stored overnight at 4°C . When large ($4 \mu\text{m}$) TAP microbubbles were suspended in saline and induced to flow across human respiratory mucus plated on a microscope slide, the microbubbles oriented in a spatial arrangement in proximity to the mucus (Figure 11A). By comparison, our standard, neutrally charged microbubbles demonstrated no affinity for mucus (Figure 11B), and freely flowed as a bulk liquid on the microscope slide, in a uniform distribution with no clustering around the mucus sample. Unlike TAP-microbubbles, these neutrally charged microbubbles appeared to float above the mucus layer on a different plane, with no interaction or adhesion to the sample.

When imaged by high frame rate video microscopy on mucociliary transport assays, TAP-microbubbles were easily visualized as bright, fluorescent clouds on epifluorescence video (Figures 12A and 12B), with transport successfully tracked in 8 fields of view in the center of the track and evenly distributed along the full length of the track. By comparison, fluorescently tagged neutral microbubbles were faint and poorly visualized, and transport rates could not be quantified. The calculated transport rate of TAP-microbubble fluorescent clouds ranged from 90 to $150 \mu\text{m/s}$ and was in close correlation ($r = 0.94$; $P\text{-value} = 0.0006$) and agreement (6% fractional difference) with the calculated transport rate of visible debris on transmitted

light video (Figure 13). The mean ciliary beat frequency for the *in vitro* assay was 12.2 ± 0.8 Hz, with a mean active ciliated area of 29.2 ± 16 %, across the 8 fields of view. There was no observable evidence of cross-linking between TAP-microbubbles and the cilia, in any of the 8 fields of view. Epithelial
5 absorption was observed to minimal and temporary, averaging at 0.8% across all fields of view during the 60 second observation period.

Exposing human bronchial epithelial cell (BEC) cultures to TAP-microbubbles for a duration of 24 hours did not induce cytotoxicity or inflammation, as assessed by the cellular release of LDH (a biomarker of
10 cytotoxicity) or IL-8 secretion (a biomarker of proinflammatory response) which were normalized to the positive control conditions (Figure 14). Normalized concentrations of LDH were not significantly increased when comparing TAP microbubble exposure to saline control (3.8% vs. 3.7%; $P = 0.12$) or when comparing to vehicle exposure to saline control (3.8% vs.
15 3.7%; $P = 0.13$). In the event that saline itself may induce inflammation, we also compared LDH release following exposure to saline versus no-exposure control; again, with no significant difference (3.7% vs. 3.5%; $P = 0.6$). As expected, the positive control (Triton X-100) induced significant cytotoxicity (100%). The 24-hour exposure to TAP microbubbles demonstrated no effect
20 on secretion of proinflammatory cytokine IL-8, either. Normalized concentrations were comparable for TAP microbubble exposure versus saline (12% vs. 9%; $P = 0.4$) and for vehicle exposure versus saline (15% vs. 9%; $P = 0.3$). There was also no statistically significant difference in normalized IL-8 concentration when comparing saline to a no-exposure control (9% for both).
25 Again, as expected, the positive control (IL-1 β and TNF α) did induce IL-8 production ($P=0.02$), compared to the saline control.

When we performed pulmonary ultrasound of mice *in vivo*, the imaging procedure was well tolerated by all mice, irrespective of allocation to TAP microbubbles or saline control. Administration of saline control had no
30 demonstrated effect on image enhancement or pulmonary structure delineation. By comparison, the entire length of the trachea was brightly visualized following oropharyngeal administration of TAP microbubbles

(Figure 15), with clustered contrast signal appearing to move up the mucociliary escalator. No acute, adverse effects were observed during the 7-day, post-exposure monitoring period, for allocation to either contrast agent or saline. Similarly, there were no signs of inflammation or injury by histology (Figure 16).

Discussion

In this experimental work, we have formulated a novel ultrasound contrast agent that is optimized for muco-adhesion, which may have future applications for evaluation of lung function and mucociliary clearance. We were able to show that our TAP-microbubble contrast agent associated with mucus and traveled with the mucus layer *in vitro*, by evaluating mucociliary transport assays composed of human bronchial epithelial cells. The microbubbles mixed into the air-surface layer, without absorption into the epithelium, promoting the feasibility of TAP contrast-enhanced ultrasound imaging as a means to evaluate mucociliary transport. Our TAP-microbubble contrast agent demonstrated no cytotoxic or proinflammatory response to human bronchial epithelial cells cultures with 24hr of exposure, nor were any ciliary cross-links observed, which suggests feasibility for clinical use, pending further evaluation. When administered to mice, TAP-microbubble contrast agent enhanced tracheal ultrasound imaging and was well tolerated *in vivo*, with no adverse effects noted on lung histology.

One clinical application for our optimized contrast agent may be evaluation of mucociliary clearance, by quantifying the overall percent clearance of visible microbubbles across a specified time interval (similar to the approach used by gamma scintigraphy). Assessment of mucociliary clearance time could potentially be performed using commercially available equipment. If deemed safe for clinical use, the contrast agent could be administered to the patient as an aerosol using a medical grade nebulizer. We have previously shown that our microbubble contrast agent remains intact following the nebulization process, and that nebulized microbubbles can be visualized using clinical ultrasound scanners interfaced with commercial

contrast-mode imaging packages.⁶ In clinical scenarios, the trachea could be imaged without bone obstruction, providing a clear window to evaluate mucociliary clearance time. This could be quantified by the elapsed time for contrast signal to diminish, as microbubbles on the airway surfaces are cleared by the action of the mucociliary escalator. In future preclinical evaluations, we will compare signal clearance time in normal controls versus mice designed to express a cystic fibrosis phenotype,⁵⁰ and validate both against gamma scintigraphy.

A different approach to evaluate bulk mucociliary transport would be to quantify the “flash-replenishment” time, which could be accomplished by selectively destroying microbubbles in a region of interest and recording the time for contrast signal to return as distal microbubbles are transported up the mucociliary escalator. Flash-replenishment time is accessible with clinical scanners and commercially available image analysis packages,⁶⁸ but its safe use in pulmonary applications, and particularly in patients with CF, has not been evaluated. With this technique, microbubbles are destroyed by increasing the ultrasound system insonation power, rupturing the microbubbles through induced cavitation. It is possible that the violent destruction of microbubbles lodged within the mucus layer may adversely affect ciliary function. Conversely, it is also a possibility that microbubble cavitation may be therapeutic, by breaking apart the thickened mucus in patients with CF and facilitating its clearance from the respiratory tract. However, both scenarios are speculative and as of yet untested.

Rather than relying upon the bulk clearance (or replenishment) of contrast signal for assessment of mucociliary function, TAP contrast agent could potentially be used to measure mucociliary transport rate. The *in vitro* transport rates that we calculated for TAP microbubbles on microscopy ranged from 90 to 150 $\mu\text{m/s}$, velocities that are too low to be captured by conventional Doppler, but within the range quantifiable by acoustic angiography, a contrast-enhanced superharmonic ultrasound imaging technique.^{69,37} Acoustic angiography is feasible using commercially available equipment but requires a dual frequency transducer to selectively receive the

superharmonic echoes arising from contrast microbubbles. From the received contrast signals, pixel-wise mean intensities are plotted to map the microcirculation in high resolution, with perfusion rates quantified by the time for mean signal intensities to peak. Without substantial modification, this technique could potentially be adapted to mucociliary transport rate. Although currently undemonstrated, the mucociliary transport rate could be quantified from the time for contrast signal intensity to peak, using a flash-replenishment approach, or by quantifying the rate of contrast signal intensity decay as microbubbles are cleared by the action of mucociliary escalator.

Mucociliary transport rate could also be measured by tracking the motion of individual microbubbles along a specified distance in the trachea (similar to invasive particle tracking by fiberoptic bronchoscopy⁵⁵⁻⁵⁷ or intranasal optical coherence tomography⁵⁸). Super-resolution ultrasound imaging is capable of tracking individual microbubbles in the circulation, and may also have applications in pulmonary imaging. Although not performed by routine imaging equipment, super-resolution ultrasound imaging achieves resolution beyond the diffraction limit of conventional coherent imaging and can resolve 25-50 micron single bubble displacements over 1 second.^{33,34} In future preclinical work, we will evaluate the accuracy of super-resolution ultrasound imaging against video microscopy in mice with and without a CF phenotype. Current microscopic techniques are facilitated by removing the mouse jaw and the tissue covering the trachea, and by the continued beating of cilia for up to 30 minutes post-mortem.^{71,72} Unlike microscopy, super-resolution ultrasound acquires images transcutaneously, and is suitable for *in vivo* applications at depths up to 6 cm.⁷³ However, the technique is very sensitive to motion, and in clinical settings, preschool aged children would almost certainly require sedation or anesthesia to remain still for image acquisition. With this consideration, mucociliary clearance time or mucociliary transport rate measured by acoustic angiography may be more feasible imaging markers of mucociliary function than direct measurement of mucociliary transport rate by super-resolution ultrasound. These translational aspects remain to be evaluated in future work.

In conclusion, we have presented preliminary findings supporting the safety and feasibility of a mucus-targeting contrast agent that may be useful for pulmonary imaging applications.

Figure 17 is a block diagram of a system for evaluating respiratory function or structure of a subject. Referring to Figure 17, the system includes a mixture **1700** including a liquid and ultrasound contrast agent particles, where each of the inhalable ultrasound contrast agent particles comprises a core, a shell surrounding the core, and a mucus-targeting material located in or on the shell. The liquid may be water, and the mucus-targeting material comprises a material that binds with or adheres to respiratory mucus. Mixture **1700** can include any of the ultrasound contrast agent particle formulations described herein. The system further includes a nebulizer **1702** for nebulizing the mixture to make the particles of the ultrasound contrast agent inhalable by a subject **1704**. Nebulizer **1702** can be any suitable device for aerosolizing mixture **1700** to make mixture **1700** suitable for inhaling by subject **1704**. In the illustrated example, subject **1704** is a human subject. In an alternate implementation, subject **1704** can be a preclinical mammalian subject, such as a mouse.

The system further includes an ultrasound imaging system **1706** including at least one ultrasound transducer **1708** for applying ultrasound energy to the particles of the ultrasound contrast agent within the subject, detecting ultrasound energy scattered by the ultrasound contrast agent particles, and generating, from the detected ultrasound energy, an image of the ultrasound contrast agent particles usable to evaluate respiratory function or structure of the subject. In one example, ultrasound imaging system **1706** may be a super-resolution ultrasound imaging system of the type described in commonly-assigned, co-pending U.S. patent application publication no. 2022/0211350, filed October 27, 2021, the disclosure of which is incorporated herein by reference in its entirety. Briefly, the super-resolution ultrasound imaging system includes a dual-frequency ultrasound transducer with non-overlapping -6 dB bandwidths for transmit and receive. The system further includes a super-resolution processor capable of generating images including

the spatial locations of individual contrast agent particles. The super-resolution processor may be implemented using computer-executable instructions stored in a memory of ultrasound imaging system **1706** and executed by a processor of ultrasound imaging system **1706**.

5 Figure 18 is a flow chart illustrating an exemplary method for using ultrasound contrast agents with mucus-targeting ligands and/or cationic surface charges to evaluate respiratory function or structure of a subject. Referring to Figure 18, in step **1800**, the method includes providing a mixture including a liquid and ultrasound contrast agent particles, each of the
10 ultrasound contrast agent particles comprising a core, a shell surrounding the core, and a mucus-targeting material in or on the shell, wherein the mucus-targeting material comprises a material that binds with or adheres to respiratory mucus. In one example, the mucus-targeting material comprises a mucus-targeting ligand that binds with respiratory mucus. In another
15 example, the mucus-targeting material may include a material with a cationic surface charge.

 In step **1802**, the method further includes nebulizing the mixture to make the particles of the ultrasound contrast agent inhalable. In step **1804**, the method further includes allowing a subject to inhale the nebulized mixture.

20 In step **1806**, the method further includes applying ultrasound energy to image the particles of the ultrasound contrast agent within the subject. For example, ultrasound energy may be applied using an ultrasound transducer external to the subject at a frequency and power level that causes the ultrasound contrast agent to induce stable cavitation of the ultrasound contrast
25 agent.

 In addition to or instead of imaging, the ultrasound energy may be applied to produce a therapeutic response in the respiratory tract of the subject. For example, ultrasound energy may be applied to the mucus-targeting ultrasound contrast agent in the subject after the ultrasound contrast
30 agent has been inhaled and adhered to respiratory mucus to cause the ultrasound contrast agent particles to cavitate and disrupt a mucus plug.

In step **1808**, the method further includes using results of the imaging to evaluate respiratory function or structure of the subject. For example, the imaging results may be used to evaluate mucociliary clearance in subjects with cystic fibrosis or lung damage in burn victims.

5 According to another aspect of the subject matter described herein, a process for disrupting mucus in the respiratory tract of a subject is provided. Figure 19 is a flow chart illustrating exemplary steps of a process for disrupting mucus in the respiratory tract of a subject. Referring to Figure 19, in step **1900**, the process includes providing a mixture including a liquid and energy-
10 activatable agent particles. For example, the energy-activatable agent particles can be microbubbles, nanodroplets, or a combination of nanodroplets and microbubbles. The energy-activatable agent particles can include the contrast agent particles described above which include a mucus-targeting material located in or on the shells of the particles. In another
15 example, the particles may not include a mucus-targeting material.

 In step **1902**, the process further includes nebulizing the mixture to make the energy-activatable agent particles inhalable. In step **1904**, the method further includes administering the particles of the energy-activatable agent into a respiratory tract of the subject. For example, a physician or a
20 technician may allow a subject to inhale the nebulized mixture.

 In step **1906**, the process further includes applying energy to the energy-activatable agent particles within the respiratory tract of the subject, causing the particles to cavitate and disrupt respiratory mucus. For example, ultrasound energy may be applied using an ultrasound transducer external to
25 the subject at a frequency and power level that causes the ultrasound contrast agent to cavitate and decrease the viscosity of respiratory mucus, including mucus plugs. In another example, mechanical or thermal energy may be applied to the energy-activatable agent particles to cause the particles to cavitate and disrupt the respiratory mucus. The application of energy may
30 cause shear or shock waves in the particles of the energy-activatable agent. The energy delivered to the particles of the energy-activatable agent may be

delivered at a frequency in a range of 100 kHz to 3 MHz and/or with a mechanical index within a range from 0.2 to 3.0.

According to another aspect of the subject matter described herein, a process for delivering a therapeutic agent to a respiratory tract of a subject is provided. Figure 20 is a flow chart illustrating an exemplary process for delivering a therapeutic agent to a respiratory tract of a subject. Referring to Figure 20, in step **2000**, the process includes providing a mixture including a liquid and acoustically-activatable agent particles, where at least some of the particles carry a therapeutic agent. For example, the acoustically-activatable agent particles can be microbubbles, nanodroplets, or a combination of nanodroplets and microbubbles. The acoustically-activatable agent particles can include the contrast agent particles described above which include a mucus-targeting material located in or on the shells of the particles. In another example, the particles may not include a mucus-targeting material.

Examples of therapeutic agents that may be carried by the acoustically-activatable agent particles include mRNA or DNA for gene therapy, for example, to treat cystic fibrosis. An example of mRNA or DNA for gene therapy for cystic fibrosis includes mRNA or DNA that encodes a cystic fibrosis transmembrane conductance regulator (CFTR) modulator, such as a corrector, a potentiator, a stabilizer, or an amplifier. Such CFTR modulators must be repeatedly delivered to the airway of a CF subject to be effective, and acoustically-activatable agent particles are believed to be candidates for such repeated delivery. Another example of a therapeutic agent that may be carried by the acoustically-activatable contrast agent particles is a disulfide disrupter, such as tris(2-carboxyethyl)phosphine (TCEP). Such a therapeutic agent may be useful to treat patients with asthma or allergies who are at risk for mucus plugs.

In step **2002**, the process further includes nebulizing the mixture to make the acoustically-activatable agent particles inhalable. In step **2004**, the process further includes administering the particles of the acoustically-activatable agent into a respiratory tract of the subject. For example, a physician or a technician may allow a subject to inhale the nebulized mixture.

In step **2006**, the process includes applying acoustic energy to the acoustically-activatable particles within the respiratory tract of the subject to release the therapeutic agent within the respiratory tract of the subject. For example, an ultrasound transducer may apply ultrasound energy to the

5 acoustically-activatable particles causing the particles to inertially cavitate, burst or rupture, and deliver the therapeutic agent to the respiratory tract of the subject.

The subject matter described herein may also include a system for implementing the subject matter of Figure 19 or Figure 20. The system may

10 include the same or similar components to the system illustrated in Figure 17 except that the ultrasound contrast agent particles may be replaced with the energy-activatable particles to implement the process of Figure 18 or acoustically-activatable particles carrying the therapeutic agent to implement the process of Figure 19. Further, the ultrasound imaging system may be

15 replaced with an energy source of the appropriate type (ultrasound, thermal, or mechanical to implement the process of Figure 19 or an acoustic energy source (including ultrasound) to implement the process of Figure 20.

According to another aspect of the subject matter described herein, a method for evaluation of lung function or structure of a subject is provided.

20 The method includes first administering a contrast agent through inhalation so that the contrast agent is deposited within the lung of the subject. The method further includes acquiring ultrasound images of the distribution and/or movement of the contrast agent within the subject's lungs. The method further includes evaluating respiratory function or structure of the subject based on

25 the distribution or movement of the contrast agent.

According to another aspect of the subject matter described herein, the contrast agent comprises shelled particles, each of the particles comprising a core, a shell surrounding the core, and a mucus-targeting material located in or on the shell, where the mucus-targeting material comprises a material that

30 binds with or adheres to respiratory mucus.

According to another aspect of the subject matter described herein, the shells of the contrast agent particles comprise a lipid, a protein, or a polymer.

According to another aspect of the subject matter described herein, the shells of the contrast agent particles comprise a phospholipid

According to another aspect of the subject matter described herein, the shells of the contrast agent particles comprise polyethylene glycol

5 According to another aspect of the subject matter described herein, the shells of the contrast agent particles comprise albumin.

According to another aspect of the subject matter described herein, the shells of the contrast agent particles comprise a phospholipid with a cationic charge

10 According to another aspect of the subject matter described herein, the material imparting the cationic charge comprises 1,2-distearoyl-3-trimethylammonium-propane.

According to another aspect of the subject matter described herein, the cores of the contrast agent particles comprise a gas.

15 According to another aspect of the subject matter described herein, the gas in the cores in the contrast agent particles comprises a perfluorocarbon.

According to another aspect of the subject matter described herein, the gas in the cores of the contrast agent particles comprises a fluorinated compound.

20 According to another aspect of the subject matter described herein, the cores of the contrast agent particles comprise a liquid.

According to another aspect of the subject matter described herein, the liquid in the cores of the contrast agent particles comprises a perfluorocarbon.

25 According to another aspect of the subject matter described herein, the liquid in the cores of the contrast agent particles comprises a perfluorocarbon, which is a gas normally at room temperature and pressure, yet is metastable in a liquid state encapsulated within the shell of the contrast agent particles.

30 According to another aspect of the subject matter described herein, the cores of the contrast agent particles comprise perfluorobutane or perfluoropentane.

According to another aspect of the subject matter described herein, the cores of the contrast agent particles are a liquid when external to the lung, but convert into gas phase after administered into the lung.

5 According to another aspect of the subject matter described herein, the cores of the contrast agent particles convert into gas phase after being administered into the lung and exposed to ultrasound energy.

According to another aspect of the subject matter described herein, administering the contrast agent comprises nebulizing the mixture to make the particles of the ultrasound contrast agent inhalable; allowing a subject to
10 inhale the nebulized mixture; and allowing the inhaled contrast agent to distribute within the lung.

According to another aspect of the subject matter described herein, prior to the nebulization, the ultrasound contrast agent particles are suspended in an aqueous solution.

15 According to another aspect of the subject matter described herein, the aqueous solution comprises a salt.

According to another aspect of the subject matter described herein, the aqueous solution comprises glycerin and/or propylene glycol.

20 According to another aspect of the subject matter described herein, prior to the nebulization, the particles of the ultrasound contrast agent are dehydrated and exist as a dry powder.

According to another aspect of the subject matter described herein, applying ultrasound energy to image the particles of the ultrasound contrast agent within the subject comprises application of an ultrasound imaging
25 sequence specific for detection of the contrast agent and suppression of background signal from lung tissue.

According to another aspect of the subject matter described herein, the ultrasound energy is applied to the ultrasound contrast agent according to an imaging sequence after the ultrasound contrast agent has been inhaled by the
30 subject. The imaging sequence includes transmitting a pulse of ultrasound energy at a first frequency to induce the production of harmonic content from the ultrasound contrast agent microbubbles, where the harmonic content is at

a second frequency greater than the first frequency. The imaging sequence further includes receiving ultrasound scattered by the contrast agent microbubbles. The imaging sequence further includes filtering the frequency content of the received ultrasound energy to select the frequency energy at
5 the second frequency produced by the microbubbles, which results in reducing the background tissue signal and enhancing the microbubble signal.

According to another aspect of the subject matter described herein, the ultrasound transducer used to image the contrast agent particles in the subject's airway includes at least two different ultrasound transducer
10 elements, one for transmitting at the first frequency and another for receiving at the second frequency.

According to another aspect of the subject matter described herein, the ultrasound imaging sequence comprises transmitting two pulses of inverted phase, i.e., a first pulse with a first phase and a second pulse with a phase
15 that is offset by 180 degrees from the phase of the first pulse. The imaging sequence further includes receiving the ultrasound data from the subject and summing the received data from the two pulses, which cancels part of the signal from tissue and which results in reducing the background tissue signal and enhancing the microbubble signal.

According to another aspect of the subject matter described herein, the ultrasound imaging sequence comprises transmitting two pulses, a first pulse of an amplitude A and a second pulse of an amplitude $0.5A$, receiving the ultrasound data from the subject, and subtracting the received data from the pulse with the amplitude A from data from twice the amplitude of the data from
20 the pulse with the amplitude $0.5A$, or equivalently the data from the pulse with the amplitude A from twice the amplitude of data from the pulse with the amplitude $0.5A$, which results in reducing the background tissue signal and enhancing the microbubble signal.

According to another aspect of the subject matter described herein,
30 The inhalable contrast agent includes a core which comprises a gas phase at room temperature and atmospheric pressure stabilized by a shell, and a mucus-targeting ligand is located in or on the shell, wherein the mucus-

targeting ligand binds with or adheres to respiratory mucus. The disclosure of each of the following references is incorporated herein by reference in its entirety.

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10

It will be understood that various details of the subject matter described herein may be changed without departing from the scope of the subject matter .described herein. Furthermore, the foregoing description is for the purpose of illustration only, and not for the purpose of limitation, as the subject matter
15 described herein is defined by the claims as set forth hereinafter.

CLAIMS

What is claimed is:

1. An inhalable ultrasound contrast agent for evaluation of respiratory function or structure of a subject, the inhalable ultrasound contrast agent comprising:
 - a particle of material comprising a core;
 - a shell surrounding the core; and
 - a mucus-targeting ligand located in or on the shell, wherein the mucus-targeting ligand binds with or adheres to respiratory mucus.
2. The inhalable ultrasound contrast agent of claim 1 wherein the core comprises a liquid phase at room temperature and atmospheric pressure.
3. The inhalable ultrasound contrast agent of claim 2 wherein the core comprises a perfluorocarbon.
4. The inhalable ultrasound contrast agent of claim 1 wherein the shell comprises a lipid.
5. The inhalable ultrasound contrast agent of claim 1 wherein the mucus-targeting ligand comprises chitosan.
6. The inhalable ultrasound contrast agent of claim 1 wherein the mucus-targeting ligand comprises hyaluronan.
7. The inhalable ultrasound contrast agent of claim 1 wherein the mucus-targeting ligand comprises a material with a cationic surface charge.
8. The inhalable ultrasound contrast agent of claim 1 wherein the particle comprises a nanodroplet.
9. The inhalable ultrasound contrast agent of claim 1 wherein the particle comprises a microbubble.
10. A method for evaluating respiratory function or structure of a subject, the method comprising:
 - providing a mixture including a liquid and ultrasound contrast agent particles, each of the ultrasound contrast agent particles comprising a core, a shell surrounding the core, and a mucus-targeting

- material located in or on the shell, wherein the mucus-targeting material comprises a material that binds with or adheres to respiratory mucus; nebulizing the mixture to make the ultrasound contrast agent particles inhalable;
- 5 allowing a subject to inhale the nebulized mixture;
- applying ultrasound energy to image the ultrasound contrast agent particles within the subject; and
- using results of the imaging to evaluate respiratory function or structure of the subject.
- 10 11. The method of claim 10 wherein the core comprises a liquid phase at room temperature and atmospheric pressure.
12. The method of claim 11 wherein the core comprises a perfluorocarbon.
13. The method of claim 10 wherein the shell comprises a lipid.
14. The method of claim 10 wherein the material that binds with or adheres to respiratory mucus comprises a mucus-targeting ligand.
- 15 15. The method of claim 14 wherein the mucus-targeting ligand comprises chitosan.
16. The method of claim 14 wherein the mucus-targeting ligand comprises hyaluronan.
- 20 17. The method of claim 10 wherein the material that binds with or adheres to respiratory mucus comprises a material with a cationic surface charge.
18. The method of claim 17 wherein the material with the cationic surface charge comprises 1,2-distearoyl-3-trimethylammonium-propane.
- 25 19. The method of claim 10 wherein the ultrasound contrast agent particles comprise nanodroplets.
20. The method of claim 10 wherein the ultrasound contrast agent particles comprise microbubbles.
21. The method of claim 10 wherein applying the ultrasound energy includes applying the ultrasound energy with a mechanical index that induces stable cavitation of the ultrasound contrast agent particles.
- 30 22. The method of claim 21 wherein the mechanical index is about 0.19.

23. The method of claim 10 wherein applying the ultrasound energy includes applying the ultrasound energy at a frequency corresponding to a resonant frequency of the ultrasound contrast agent particles.
24. The method of claim 10 wherein using the results of the imaging to evaluate respiratory function or structure of the subject includes using results of the imaging to evaluate mucociliary clearance of the subject.
25. The method of claim 10 wherein using the results of the imaging to evaluate respiratory function or structure of the subject includes using results of the imaging to evaluate lung damage caused by trauma or disease.
26. The method of claim 10 wherein applying the ultrasound energy includes applying the ultrasound energy to the ultrasound contrast agent particles to disrupt respiratory mucus within the subject.
27. A method for disrupting mucus in a respiratory tract of a subject, the method comprising:
- providing a mixture including a liquid and energy-activatable agent particles;
 - nebulizing the mixture to make the energy-activatable agent particles inhalable;
 - administering the energy-activatable agent particles into the respiratory tract of the subject; and
 - applying energy to the energy-activatable agent particles within the respiratory tract of the subject, causing the energy-activatable agent particles to cavitate and disrupt respiratory mucus.
28. A method for delivering a therapeutic agent to a respiratory tract of a subject, the method comprising:
- providing a mixture including a liquid and acoustically-activatable agent particles, where at least some of the particles carry a therapeutic agent;
 - nebulizing the mixture to make the acoustically-activatable agent particles inhalable;

administering the acoustically-activatable agent particles into the respiratory tract of the subject; and

applying acoustic energy to the acoustically-activatable agent particles, causing the particles to cavitate, burst, and deliver the therapeutic agent into the respiratory tract of the subject.

5

29. A system for implementing the method of claim 27 or 28.

30. A system for evaluating respiratory function or structure of a subject, the system comprising:

10

a mixture including a liquid and ultrasound contrast agent particles, each of the ultrasound contrast agent particles comprising a core, a shell surrounding the core, and a mucus-targeting material located in or on the shell, wherein the mucus-targeting material comprises a material that binds with or adheres to respiratory mucus;

15

a nebulizer for nebulizing the mixture to make the ultrasound contrast agent particles by a subject; and

20

an ultrasound imaging system including at least one ultrasound transducer for applying ultrasound energy to the ultrasound contrast agent particles within the subject, detecting ultrasound energy scattered by the ultrasound contrast agent particles, and generating, from the detected ultrasound energy, an image of the ultrasound contrast agent particles usable to evaluate respiratory function or structure of the subject.

31. The system of claim 30 wherein the core comprises a liquid phase at room temperature and atmospheric pressure.

25

32. The system of claim 30 wherein the core comprises a perfluorocarbon.

33. The system of claim 30 wherein the shell comprises a lipid.

34. The system of claim 30 wherein the material that binds with or adheres to respiratory mucus comprises a mucus-targeting ligand.

30

35. The system of claim 34 wherein the mucus-targeting ligand comprises chitosan.

36. The system of claim 34 wherein the mucus-targeting ligand comprises hyaluronan.

37. The system of claim 30 wherein the material that binds with or adheres to respiratory mucus comprises a material with a cationic surface charge.
- 5 38. The system of claim 37 wherein the material with the cationic surface charge comprises 1,2-distearoyl-3-trimethylammonium-propane.
39. The system of claim 30 wherein the ultrasound contrast agent particles comprise nanodroplets.
40. The system of claim 30 wherein the ultrasound contrast agent particles comprise microbubbles.
- 10 41. The system of claim 30 wherein applying the ultrasound energy includes applying the ultrasound energy with a mechanical index that induces stable cavitation of the ultrasound contrast agent.
42. The system of claim 41 wherein the mechanical index is about 0.19.
43. The system of claim 30 wherein applying the ultrasound energy
15 includes applying the ultrasound energy at a frequency corresponding to a resonant frequency of the ultrasound contrast agent particles.
44. The system of claim 30 wherein applying the ultrasound energy includes applying the ultrasound energy to the ultrasound contrast agent particles to disrupt respiratory mucus within the subject.

20



FIG. 1

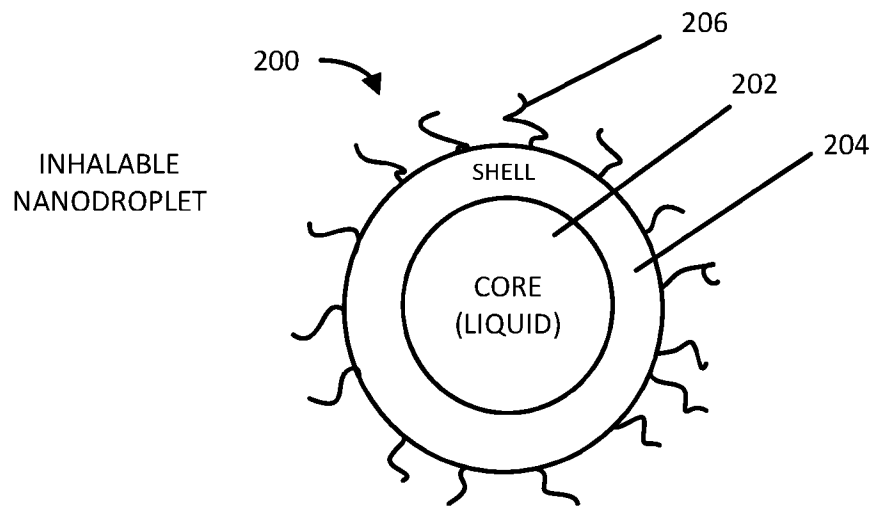


FIG. 2A

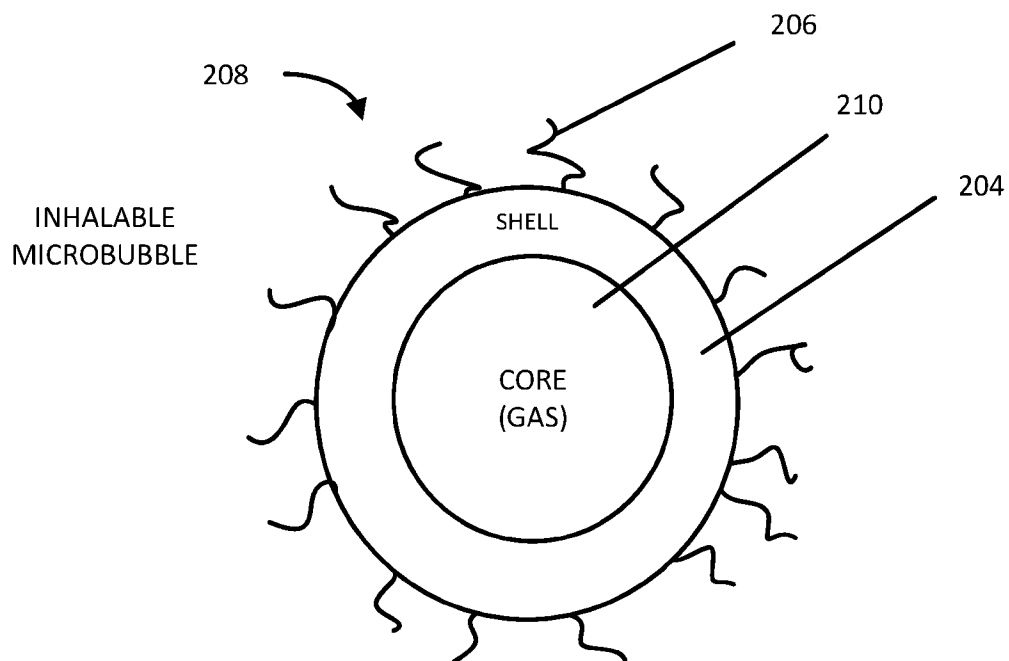


FIG. 2B

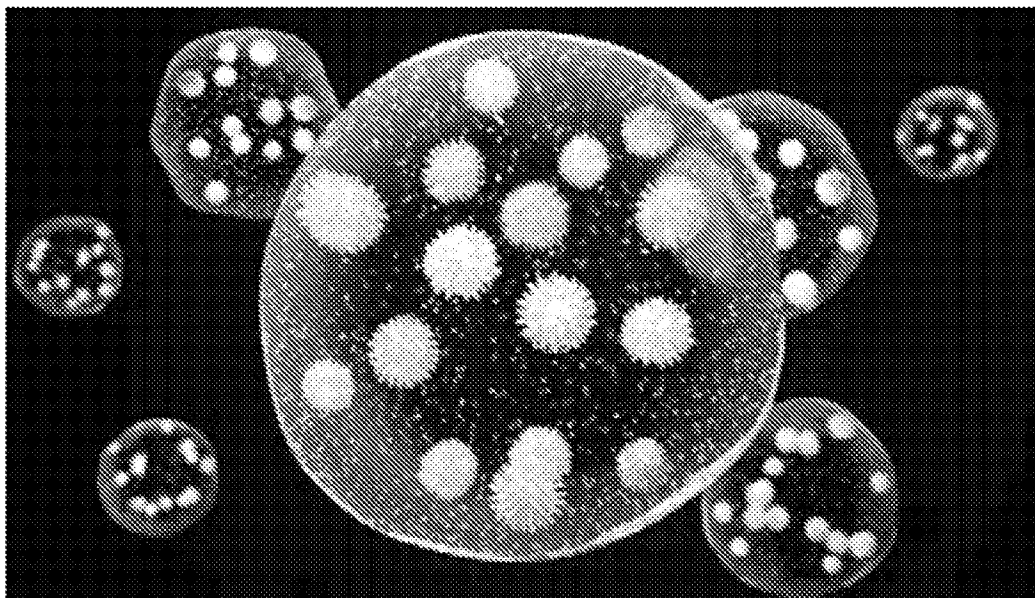


FIG. 3

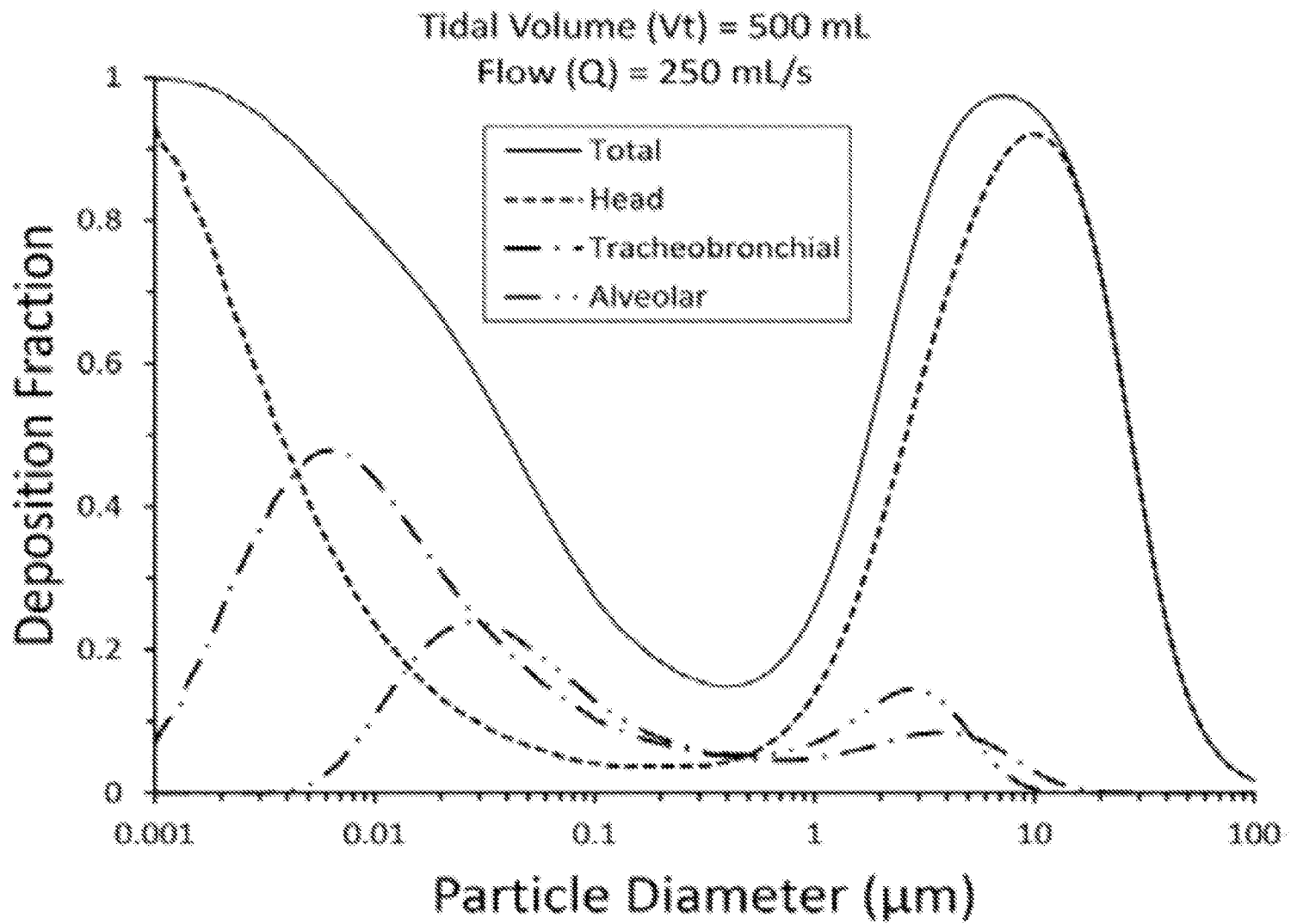


FIG. 4

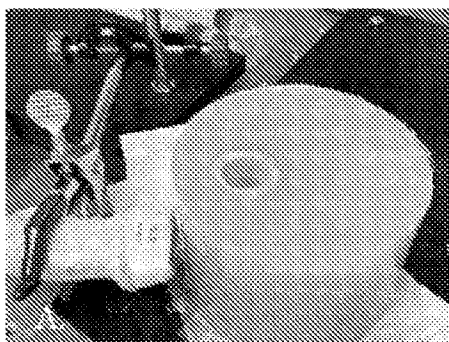


FIG. 5A

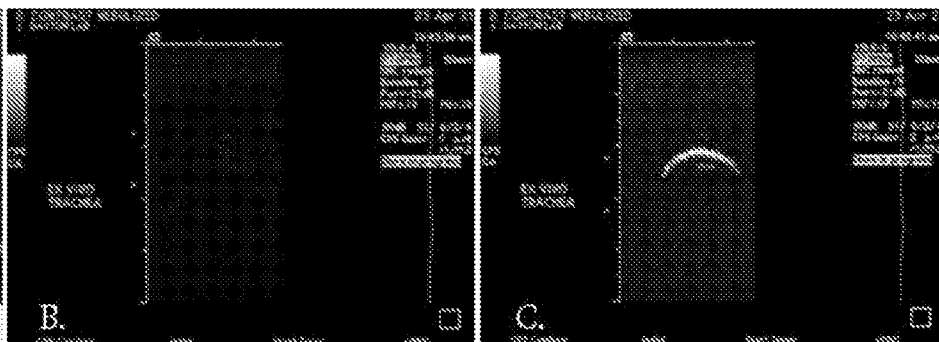


FIG. 5B

FIG. 5C

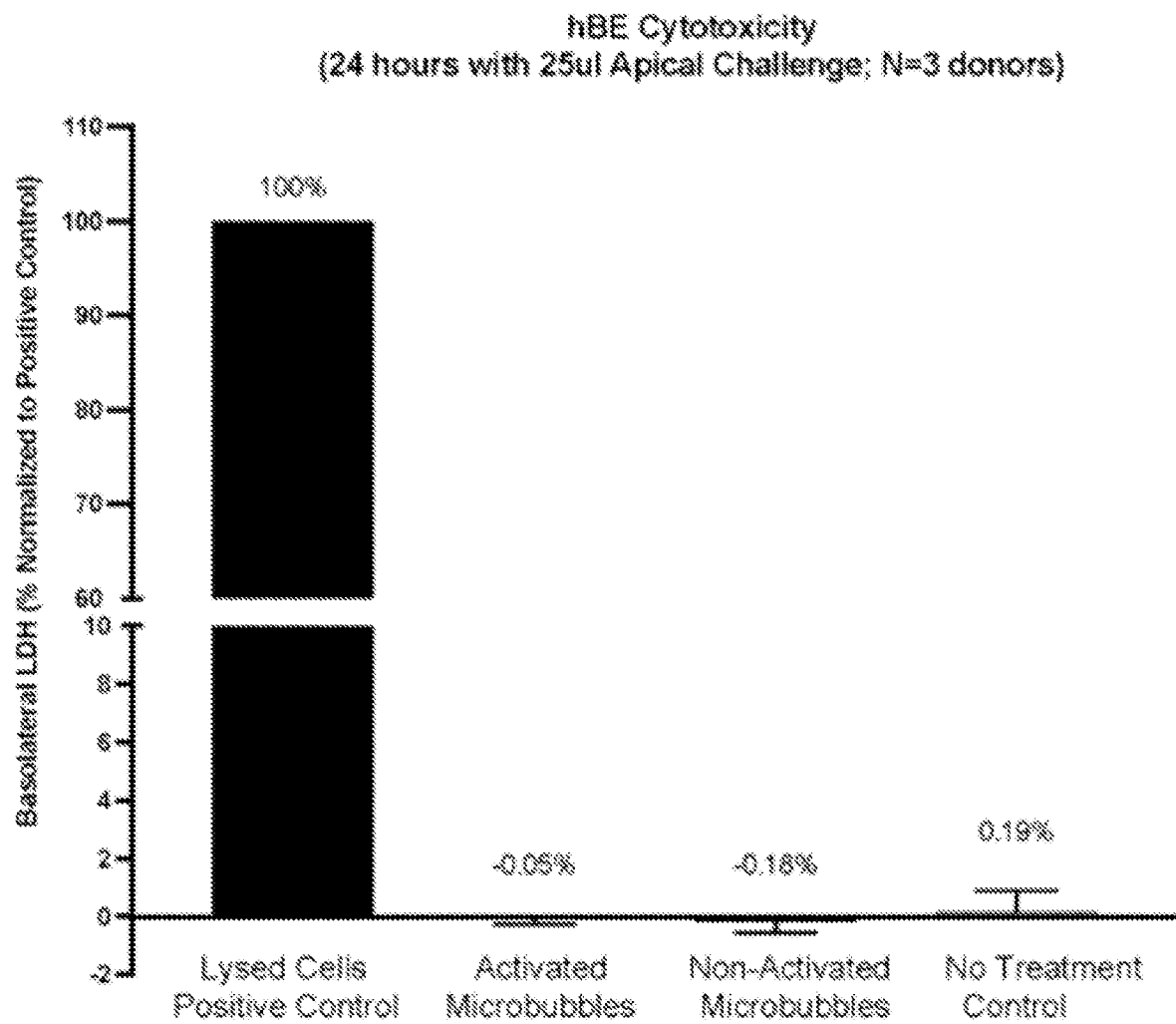


FIG. 6

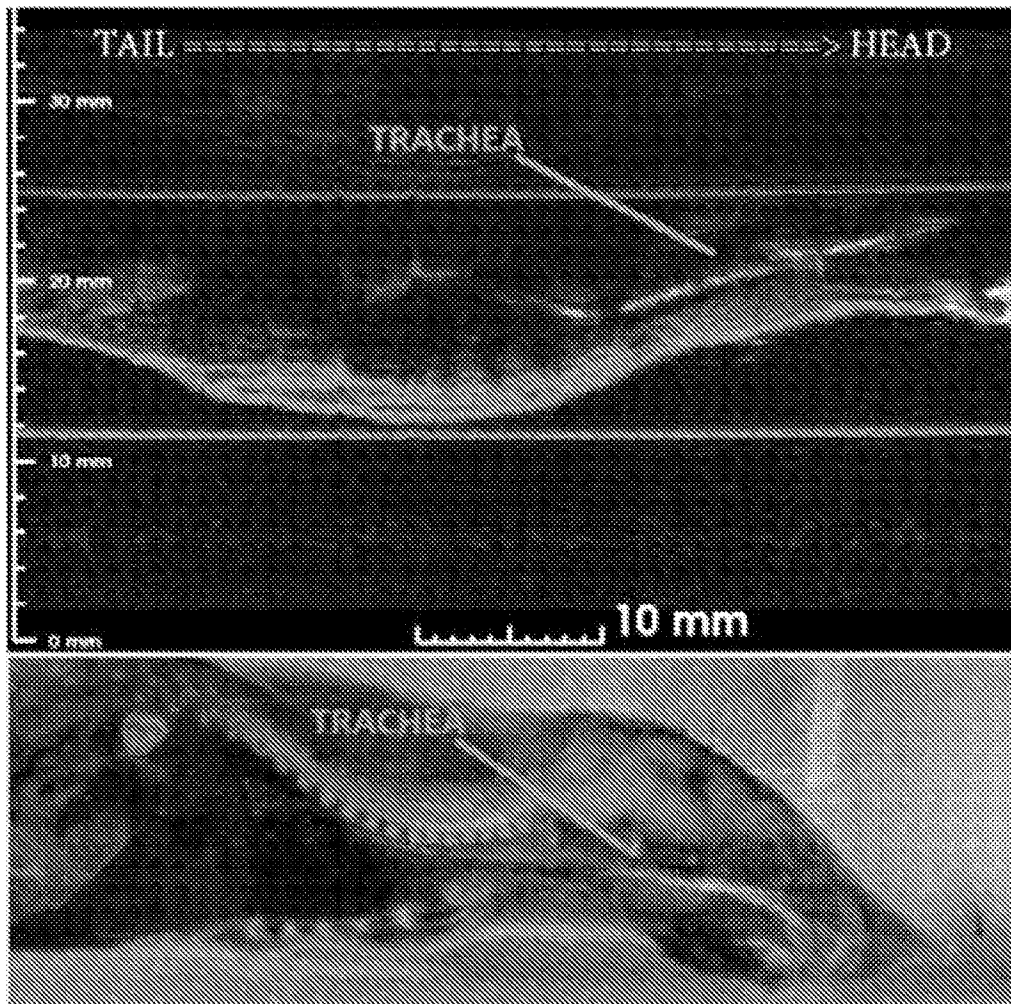


FIG. 7



FIG. 8A

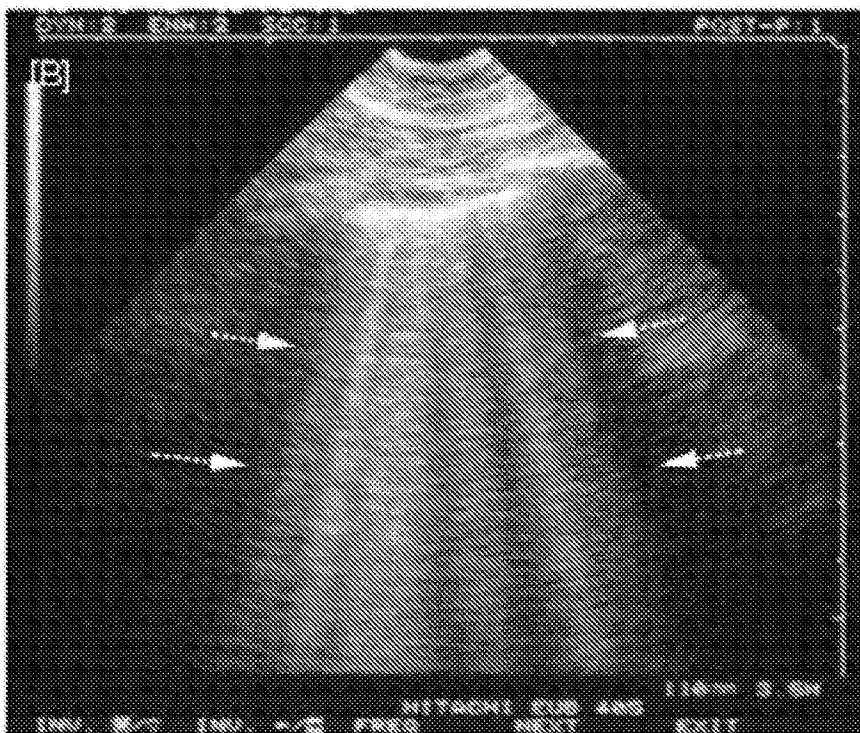


FIG. 8B

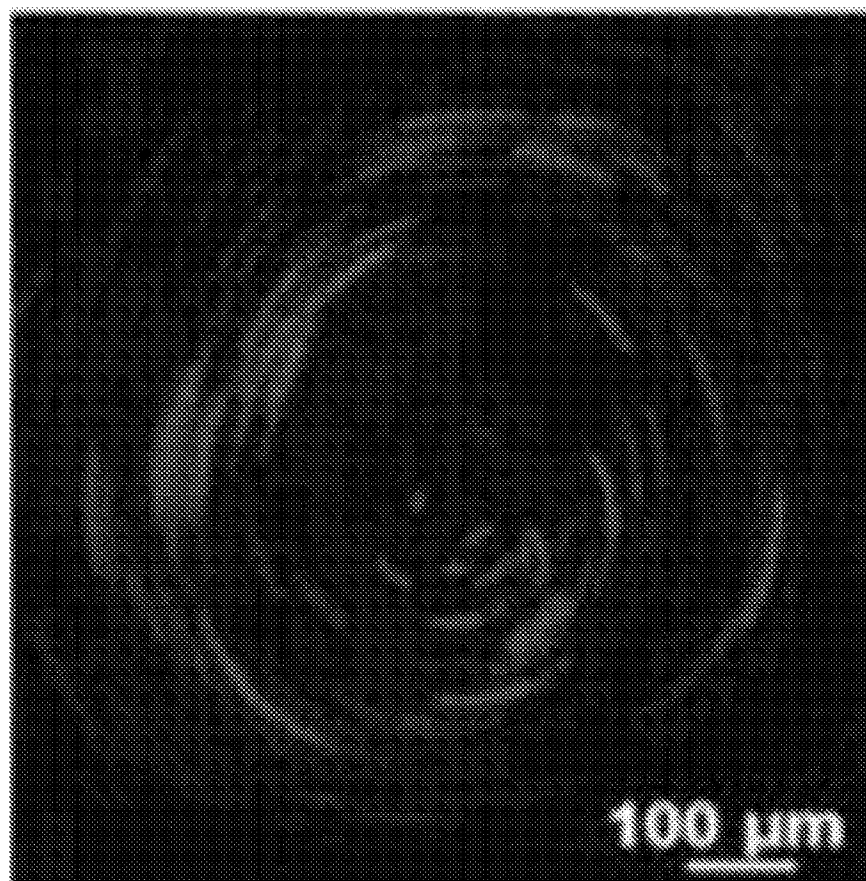


FIG. 9

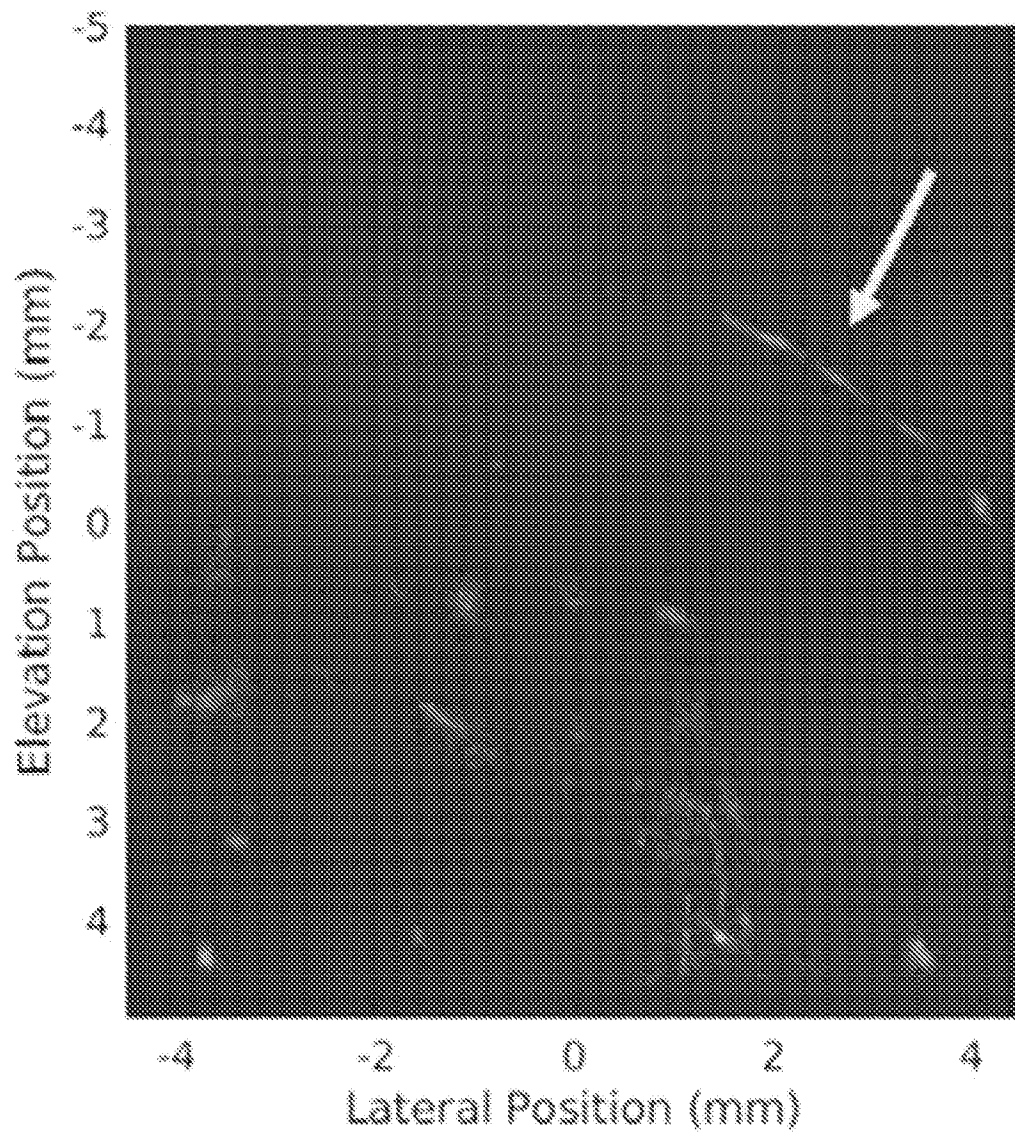


FIG. 10

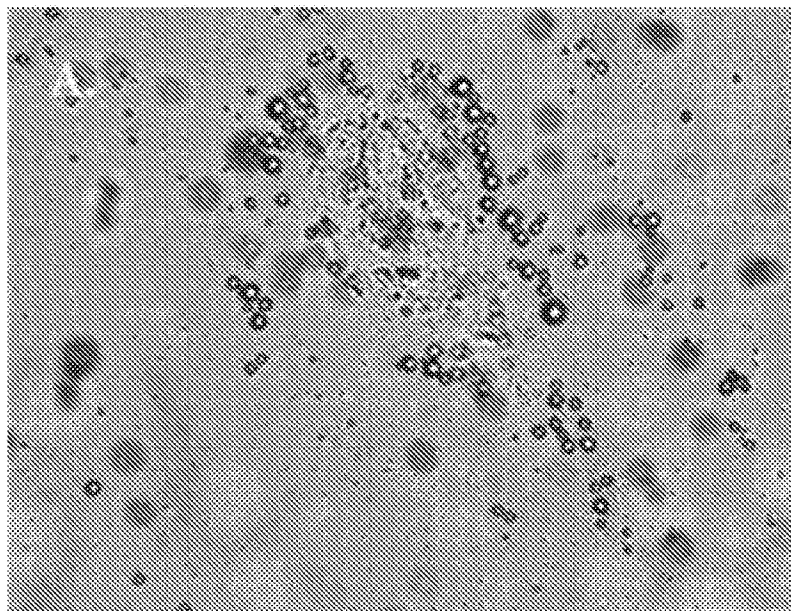


FIG. 11A

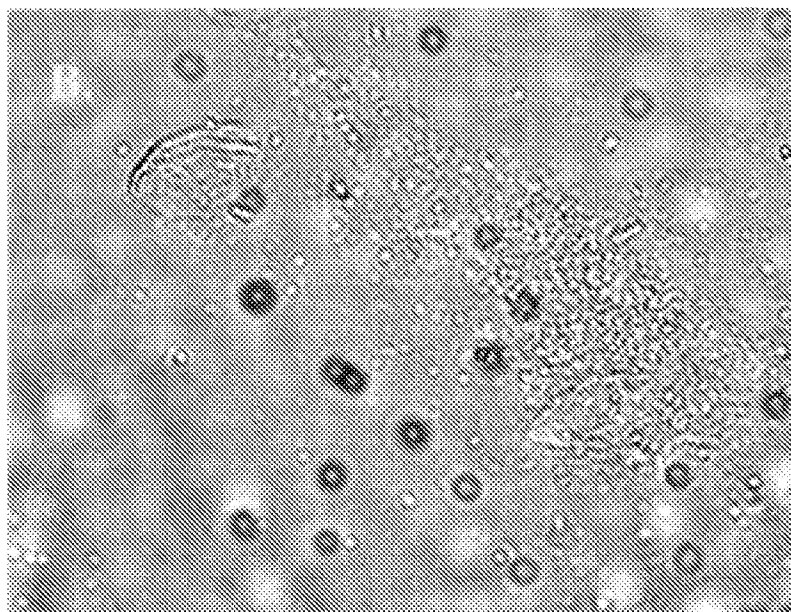


FIG. 11B

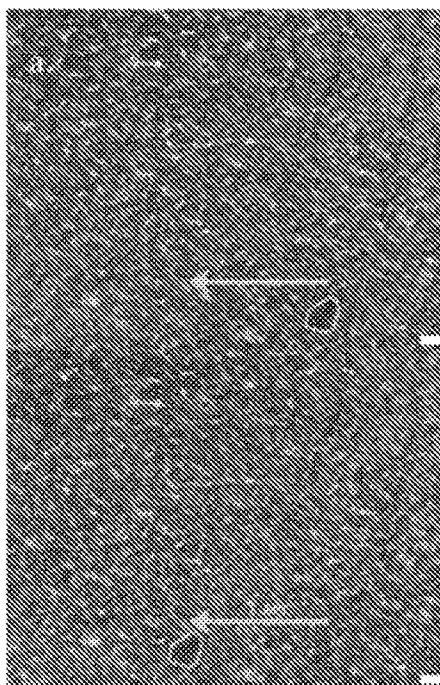


FIG. 12A

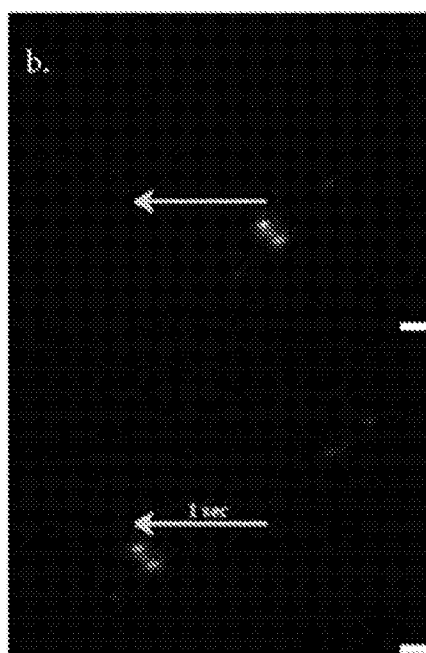


FIG. 12B

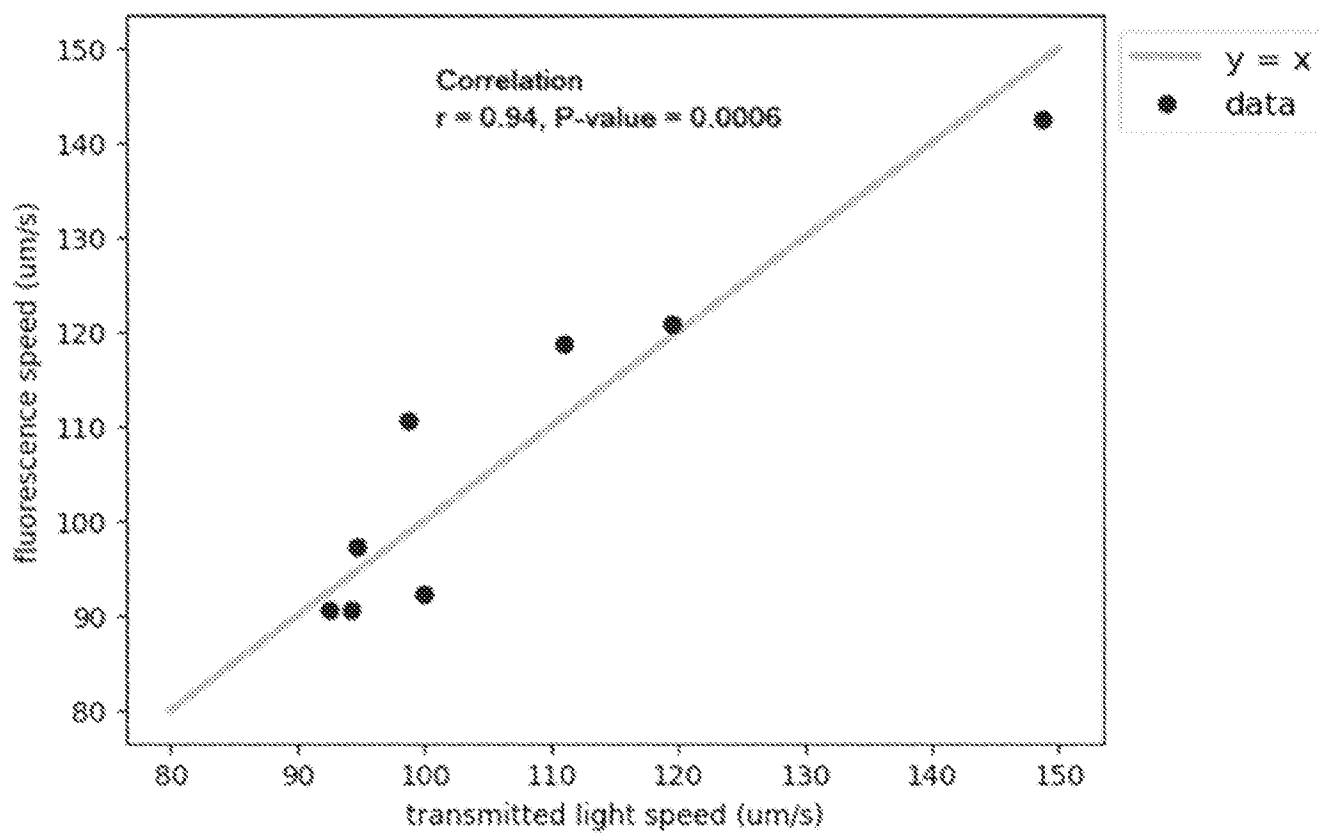


FIG. 13

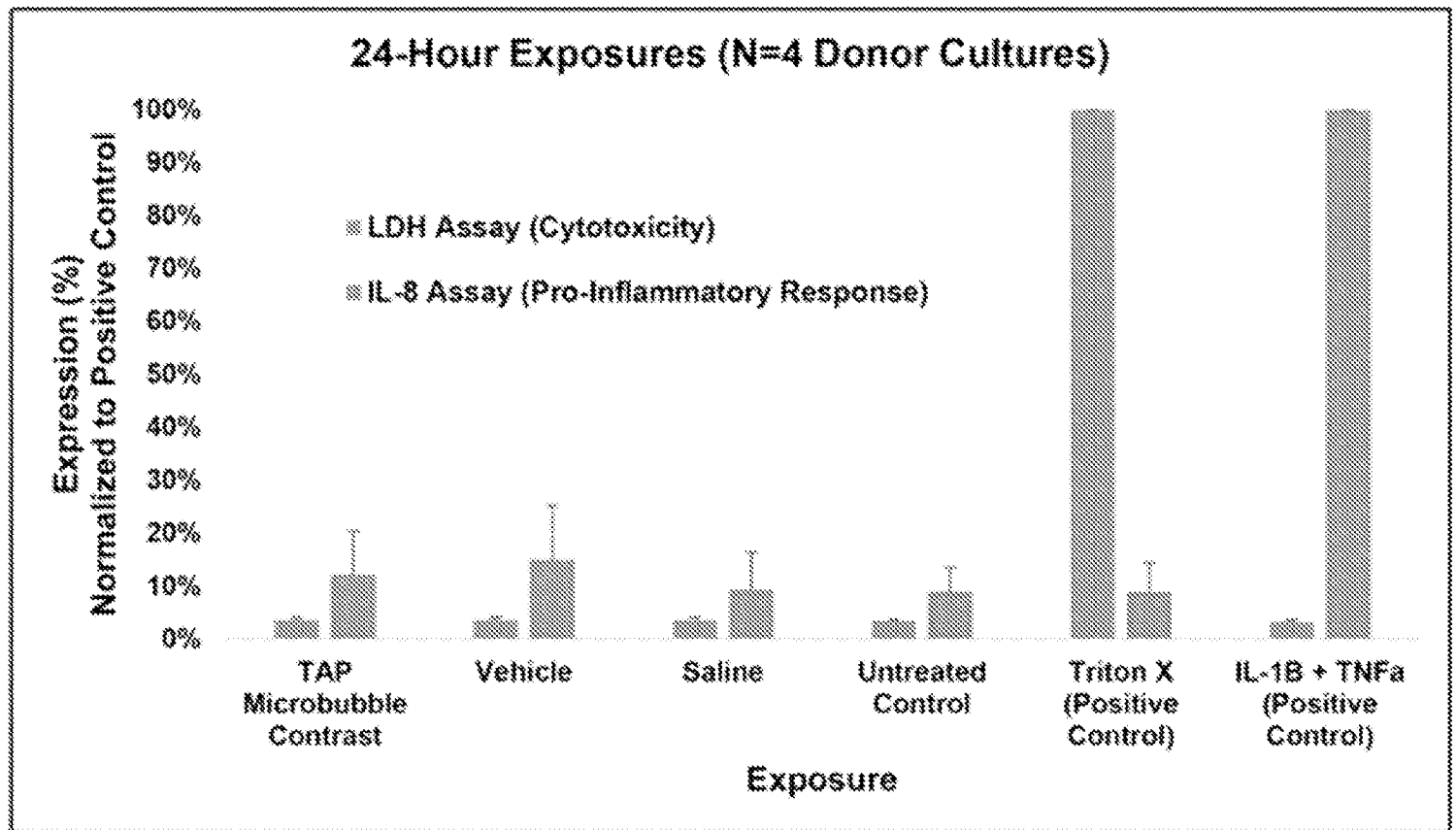


FIG. 14

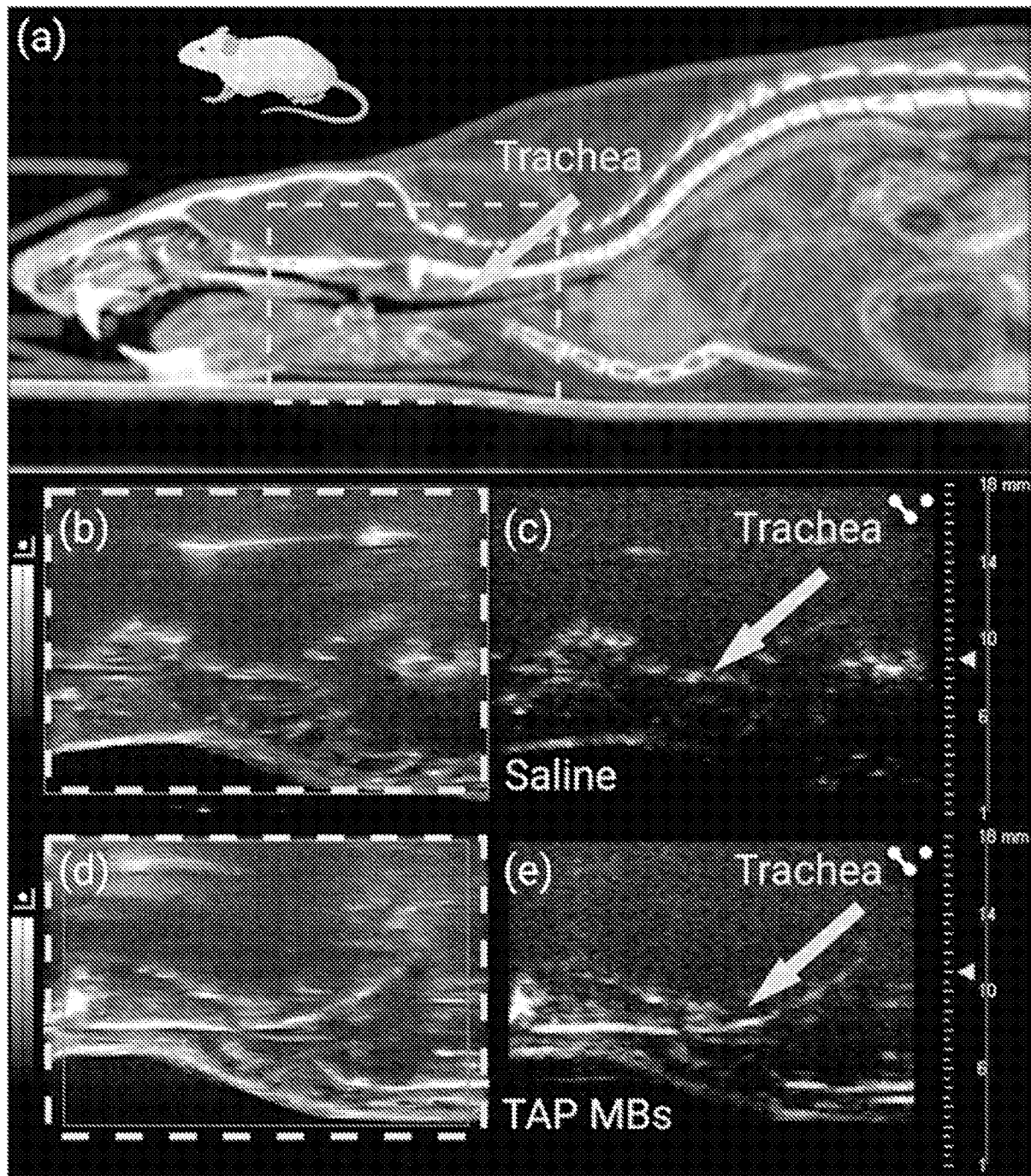


FIG. 15

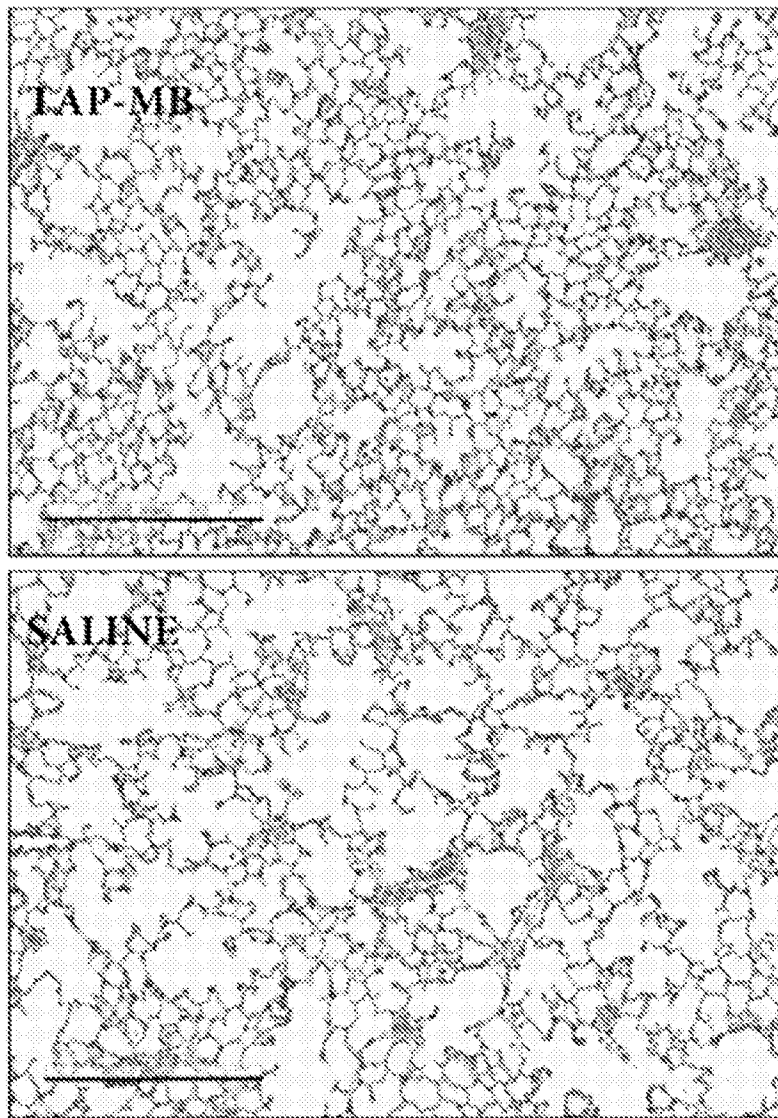


FIG. 16

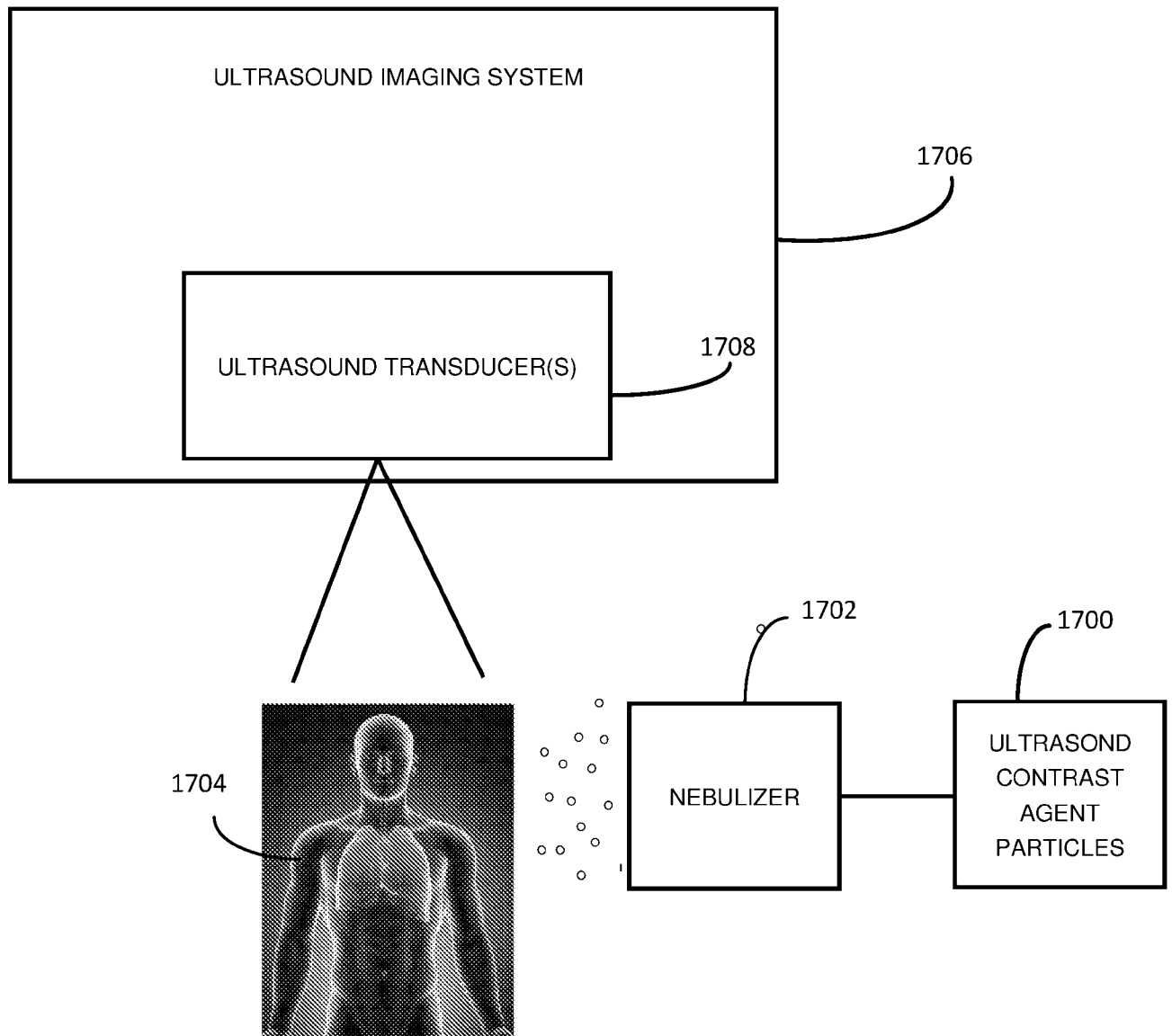


FIG. 17

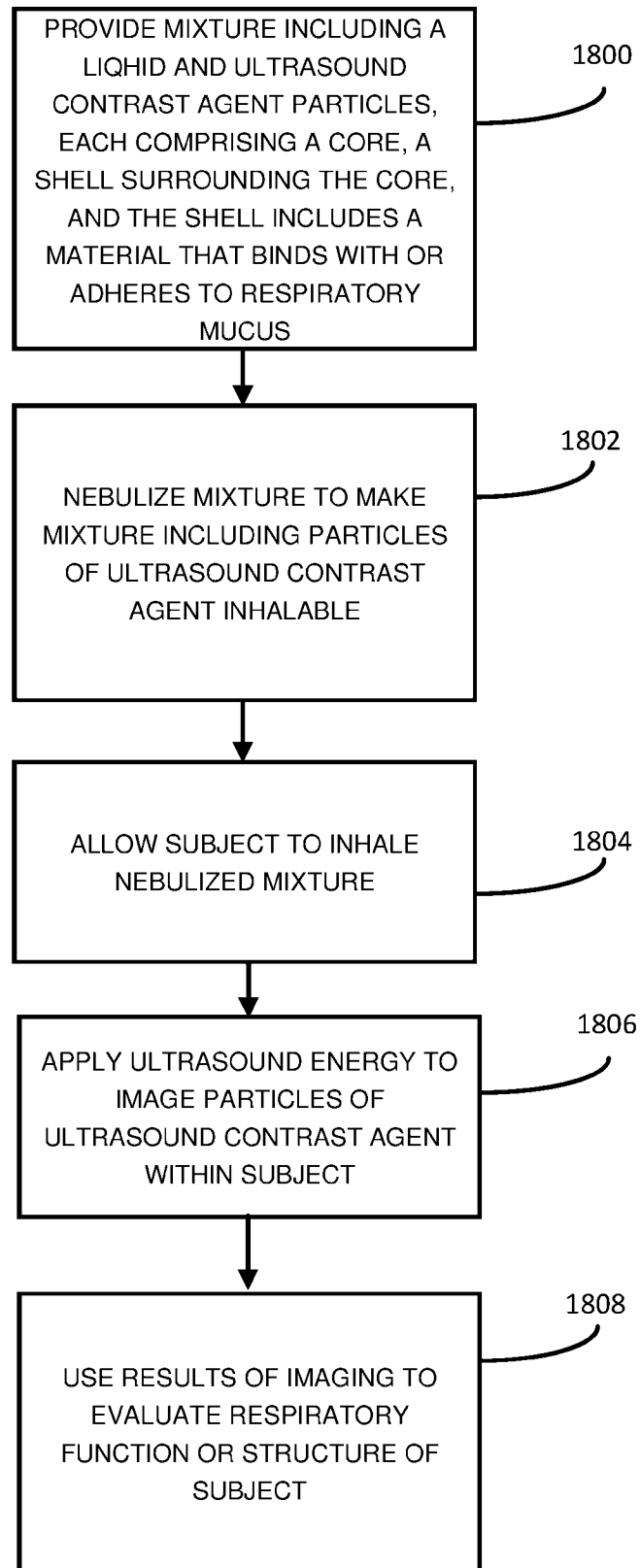


FIG. 18

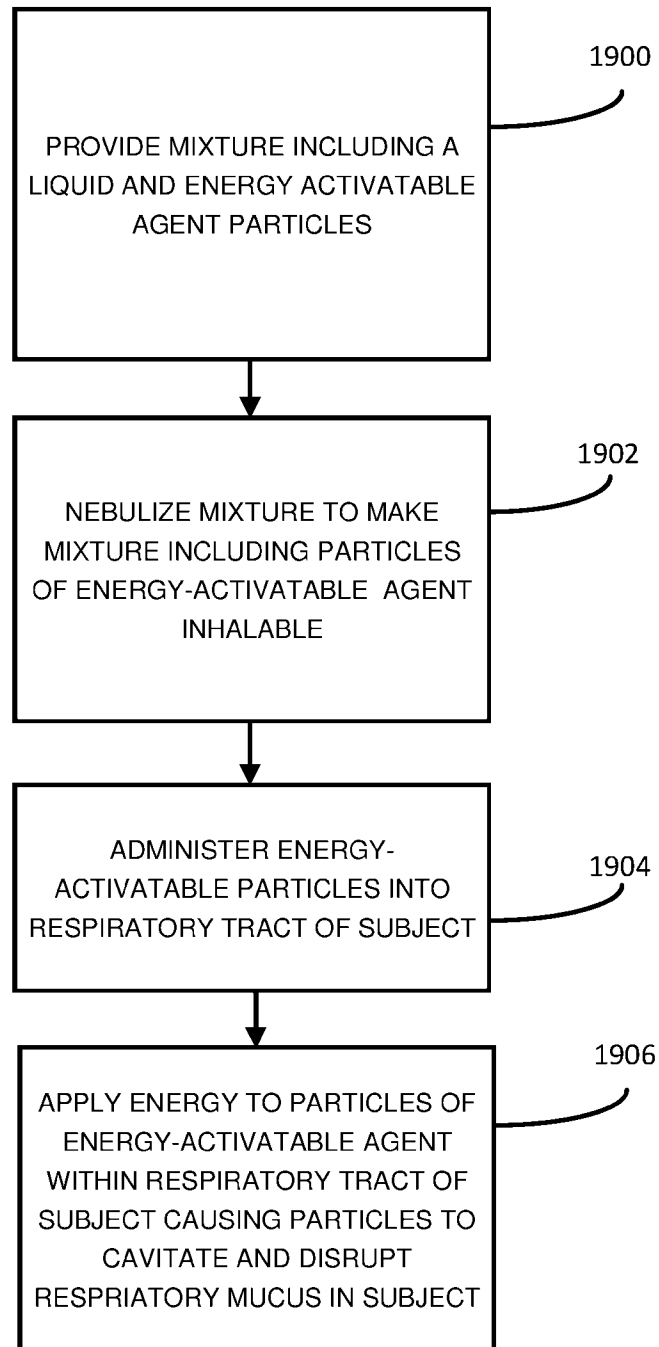


FIG. 19

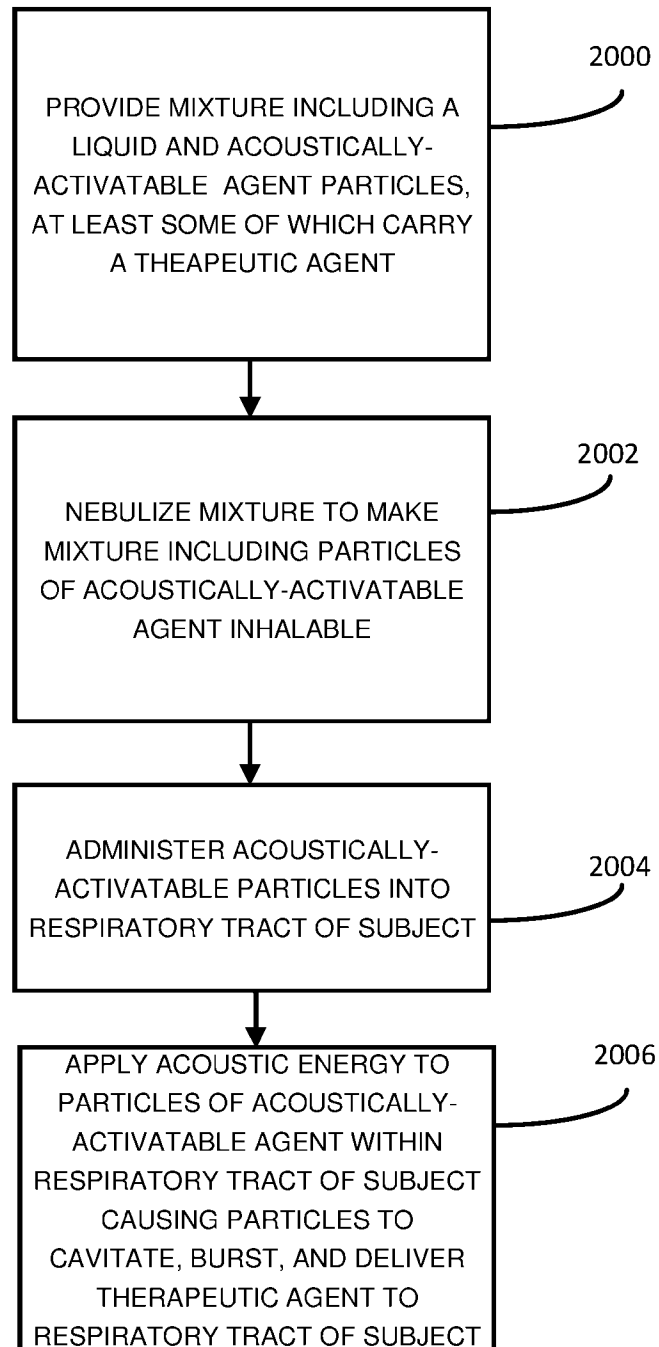


FIG. 20