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TITLE:

Preparation and Characterization of Targeted Microbubbles

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SUMMARY:

The goal of this protocol is to prepare, purify and characterize gas-filled microbubbles (targeted contrast agents for ultrasound molecular imaging). Two targeting systems are described: biotinylated bubbles adherent to streptavidin, and cyclic RGD peptide microbubbles that bind to $\alpha_v\beta_3$, a known tumor neovasculature biomarker.

ABSTRACT:

Targeting of microbubbles (ultrasound contrast agents for molecular imaging) has been researched for more than two decades. However, methods of microbubble preparation and targeting ligand attachment are cumbersome, complicated, and lengthy. Therefore, there is a need to simplify the targeted microbubble preparation procedure to bring it closer to clinical translation. The purpose of this publication is to provide a detailed description and explanation of the steps necessary for targeted microbubble preparation, functional characterization and testing. A sequence of the optimized and simplified procedures is presented for two systems: a biotin-streptavidin targeting pair model, and a cyclic RGD peptide targeting the recombinant $\alpha_v\beta_3$ protein, which is overexpressed on the endothelial lining of the tumor neovasculature.

Here, we show the following: covalent coupling of the targeting ligand to a lipid anchor, assessment of the reagent quality, and tests that confirm the successful completion of the reaction; preparation of the aqueous precursor medium containing microbubble shell components, followed by microbubble preparation via amalgamation; assessment of the efficacy of lipid transfer onto the microbubble stabilizer shell; adjustment of microbubble size distribution by flotation at normal gravity to remove larger microbubbles that might be

detrimental for in vivo use; assessment of microbubble size distribution by electrozone sensing; evaluation of targeted binding of the microbubbles to receptor-coated surface in a static binding assay test (in an inverted dish); and evaluation of targeted binding of the microbubbles to receptor-coated surface in a parallel plate flow chamber test.

INTRODUCTION:

Molecular imaging with targeted microbubbles has been in research and testing for more than two decades. The general concept is straightforward: gas-filled microbubbles that possess selective affinity to the molecular biomarker specific to vascular endothelium in the area of disease are injected intravenously. These particles circulate and accumulate in the target (e.g., tumor neovasculature or the area of ischemic inflammatory injury). Adherent microbubbles are then detected by contrast ultrasound imaging. Early concept research efforts from last century[1,2] are now gradually progressing towards clinical adoption: they have reached medium-scale clinical trial stage just several years ago[3,4]. The purpose of this manuscript is to provide the detailed explanation on the preparation and characterization of such targeted microbubbles, based on two published examples[1,5].

The procedure for the preparation of peptide-PEG-phospholipid, a crucial component for the formulation of these targeted microbubbles, is supplemented with the description of reagent quality control, as required for the successful completion of the reaction. Unfortunately, some active ester lipid reagent suppliers provide material that is hydrolyzed on arrival and therefore is unable to participate in the formation of amide bond. The information on how much of the lipid material is transferred onto the microbubble shell from the aqueous medium during microbubble preparation is provided, as well as the technique to obtain this information.

It is important to prepare microbubbles with a relatively narrow particle size distribution: the co-presence of large microbubbles in the injectable media for intravascular in vivo testing may lead to clogging of microvasculature; nonspecific accumulation of microbubbles that bypass lung shunts might cause nonspecific false-positive tissue enhancement[6], which is avoided by removing microbubbles of larger sizes. Therefore, a simple procedure to achieve particle size selection is presented, supplemented by the description of a method to assess particle concentration and size distribution with a particle counter.

The first test protocol for microbubble targeting assessment, as presented below, describes a purely model system, with biotinylated microbubbles targeted to streptavidin-coated surface[1]. The second protocol is based on a manuscript describing simplified preparation of peptide-targeted microbubbles, decorated with a cyclic RGD peptide that possesses specific affinity towards $\alpha_v\beta_3$, a molecular biomarker of tumor neovasculature[5]. Microbubbles decorated with this cyclo[Arg-Gly-Asp-D-Phe-Lys], i.e., c(RGDfK) peptide by the presented technique have been shown to target tumor neovasculature and achieve ultrasound molecular imaging in a murine tumor model.

PROTOCOL:

1. Covalent coupling of the peptide to NHS-PEG-DSPE

1.1. Dissolve the c(RGDfK) peptide with the primary ε-aminogroup of lysine unprotected and available for coupling, in dimethyl sulfoxide (DMSO, 10 mg/mL). Prepare a methanol or chloroform solution of N-hydroxysuccinimide ester of poly(ethyleneglycol)-3400-distearoyl phosphatidylethanolamine (NHS-PEG-DSPE, 200 mg/mL), and add to 1 mg of peptide in DMSO. Add 3 μL of N,N-diisopropyl ethylamine (DIPEA).

1.1.1. Maintain a peptide-to-NHS-PEG-DSPE molar ratio at least 1:1.2, so that all the primary amino groups will be able to react. Maintain a DIPEA:peptide molar ratio at least 2:1 to assure basic media conditions.

CAUTION: Chloroform, methanol and DIPEA are hazardous materials. Use proper protection, such as gloves, labcoat, goggles and fume hood.

NOTE: Any other peptide or mimetic with a primary amino group can be used instead of c(RGDfK), with unprotected N-terminus or a lysine located outside of the binding site. For the reaction to proceed, all the components (i.e., targeting ligand and lipid) must be soluble in DMSO-chloroform mixture. Alternative solvents, such as dimethylformamide, or its mixtures, may also be tested. Reaction in aqueous media is also possible, but coupling yield will be much lower due to rapid hydrolysis of active ester.

1.2. Following overnight incubation at room temperature, remove the volatile organic material by evaporation (use stream of nitrogen gas or a rotary evaporator, followed by overnight evaporation under a high vacuum pump, to remove DMSO). Redissolve the nonvolatile residue in chloroform at 1 mg/mL for controlled sampling.

1.3. Confirm reaction completion by thin layer chromatography (develop TLC plates in chloroform:methanol solvent media, 2:1 v/v). Confirm presence or absence of the primary amino group with ninhydrin spray upon heating the plate on a 150 °C heating block.

CAUTION: Ninhydrin is hazardous material. Use proper protection, as described above. Heating block treatment of TLC plates must be performed in a fume hood. The heating block is a potential fire hazard.

1.4. Prior to the microbubble preparation, aliquot a sample of peptide-PEG-DSPE from chloroform (e.g., 1 mL of 1 mg/mL solution), evaporate chloroform to dryness in a stream of nitrogen gas, with subsequent high vacuum pump incubation, and add saline to 1 mg/mL, to achieve transparent micellar solution.

1.4.1. If further purification of the reaction mixture is desirable, redissolve in normal saline. Then subject the resulting micellar mixture to dialysis (6-8 kDa molecular weight cut-off, or similar), first against normal saline, and then against several changes of deionized water.

1.4.2. Confirm completion of dialysis by a conductivity check of dialysate. Remove dialyzed material from the dialysis bag, place in a vial with known mass, and lyophilize until complete dryness.

NOTE: An alternative covalent reaction to attach the ligand to the terminus of PEG-lipid is available (e.g., a reaction of maleimide-PEG-DSPE with thiol-ligand). The main advantage of this approach is oriented coupling, if a single thiol is available on the ligand molecule [7]. The main concern is long-term stability: the resulting link between maleimide and thiol may be prone to degradation by a retro-Michael reaction, depending on storage conditions [8].

NOTE: Quality of active ester reagents greatly varies between the manufacturers: it may depend on the transfer and storage conditions (if the material is not stored or transported properly, the active ester will hydrolyze and will not be able to couple with the peptide). Consequently, there is a feeling of obligation to describe a procedure (see below) for the determination of the degree of degradation of NHS-PEG-DSPE, a reagent for covalent attachment of targeting ligands to microbubbles. NHS-PEG-DSPE, supplied as dry powder under argon, is stored in deep freeze. A vial is brought to room temperature (to avoid moisture condensation), a sample weighed, using analytical balance, and dissolved in methanol or DMSO. A vial with bulk dry reagent should be closed under argon and returned to deep freeze, in a sealed container with desiccant, until further use.

1.4.3. Prepare an aqueous micellar solution of peptide-PEG-DSPE (Step 1.4), as well as biotin-PEG-DSPE, by simple addition of dry reagent to aqueous saline, and incubation. Small spherical micelles are formed. To accelerate the transfer the reagent from bulk state to the micellar form, sonication and hot water bath can be applied.

1.5. Assess the reagent quality of NHS-PEG-DSPE active ester.

1.5.1. Confirm that NHS present in the reagent is in the form of active ester, to be able to perform the coupling reaction with amide bond formation.

1.5.2. Place 0.99 mL of 0.1 M sodium tetraborate buffer, pH 9.2, in a quartz or ultraviolet-transparent plastic cuvette (not glass) in a spectrophotometer, and zero at 260 nm wavelength.

1.5.3. Add 10 μL of the freshly prepared 200 mg/mL solution of NHS-PEG-DSPE in methanol to the cuvette. At the same time, start the stopwatch. Rapidly and vigorously mix the cuvette content to achieve uniformity. Place the cuvette in the photometer, close the lid, and start the measurement.

1.5.4. At the start of the spectrophotometer measurement, mark the time of the beginning of the recording, and continuously perform photometric absorbance measurements at 260 nm every 10 s for ~10 min, or until the stabilization of absorbance value.

1.5.5. Plot the kinetic curve, check initial A_{260} time points, and extrapolate the curve to the

reaction start time (i.e., find out A_{260} at the point where reagent was added to aqueous buffer and hydrolysis reaction has started). Assume that prior to the addition of NHS-PEG-DSPE to the buffer in the cuvette no hydrolysis had taken place in organic solvent due to lack of water.

1.5.6. Compute the ratio between A_{260} at the start of the reaction and at the completion of the hydrolysis; it represents the fraction of the degraded active ester. Use this information to select the proper amount of NHS-PEG-DSPE for the complete modification of the primary amino group of the peptide.

2. Preparation of microbubbles by amalgamation

2.1. Preparation of biotinylated microbubbles

2.1.1. Co-dissolve distearoyl phosphatidylcholine (DSPC) and PEG stearate in propylene glycol (PG, 10 mg/mL concentration for each in neat solvent). Use a hot water bath to solubilize the materials.

2.1.2. Add 0.1 mL of this hot PG solution to a vial containing 0.85 mL of hot normal saline, rapidly mix, and add micellar biotin-PEG-DSPE (50 μL, 1 mg/mL in saline) at a 1:20 mass ratio to DSPC.

NOTE: Solubility of lipid components in PG is temperature-dependent, so a hot water bath is necessary. It is helpful to heat the glass vial containing saline in a hot water bath prior to lipid addition, to create a uniform medium without visible particles.

2.1.3. Bring the vial to room temperature, place a rubber stopper on the vial, inserted halfway, and insert PTFE capillary tubing in the vial. Use the flow of decafluorobutane gas to fill the vial, and then close the stopper, while removing the capillary.

NOTE: The temperature of the vial during amalgamation may have a significant effect on the size distribution of the resulting microbubbles [9].

2.1.4. Crimp the stoppered vial, at room temperature, and place in an amalgamator apparatus. Start the amalgamator. The clinical unit used is preset to operate at 4300 rpm for 45 seconds.

2.1.5. When amalgamation is completed, remove the vial with the resulting microbubbles from the amalgamator. Preferably, characterize the microbubble size distribution and composition and use them within several hours of preparation.

2.2. Preparation of peptide-decorated microbubbles

2.2.1. Co-dissolve DSPC and PEG stearate in neat PG (10 mg/mL concentration for each material). Use a hot water bath to solubilize. Add 0.1 mL of this hot solution in PG to a vial containing 0.85 mL of hot normal saline, rapidly mix, and add micellar peptide-PEG-DSPE (50 μL,

1 mg/mL in saline) added at a 1:20 mass ratio to DSPC.

2.2.2. Bring the vial to room temperature, place a rubber stopper on top of the vial, inserted halfway, and insert polytetrafluoroethylene (PTFE) capillary tubing in the vial. Use the flow of decafluorobutane gas to fill the vial, and then close the stopper, while removing the capillary.

2.2.3. Crimp the stoppered vial and place in an amalgamator for mixing. The vial with microbubble dispersion is ready in 45 s.

2.3. Preparation of dye lipid microbubbles for assessment of lipid transfer to microbubble shell

2.3.1. Co-dissolve DSPC and PEG stearate in neat PG, as described above (use a hot water bath) (10 mg/mL concentration for each material).

2.3.2. Add 0.1 mL of this hot PG solution to a vial with 0.89 mL of hot normal saline, rapidly mix, add 10 μL of DiI dye solution (1 mg/mL in neat PG) and mix. Bring the vial to room temperature.

2.3.3. Fill the vial headspace with decafluorobutane gas as above, stopper the vial, crimp and amalgamate as described above.

3. Test DiI lipid transfer from the micellar aqueous medium to the bubble shell

3.1. For the microscopy confirmation that fluorescent lipid material has transferred from the aqueous medium to the microbubble shell, sample an aliquot of microbubbles from the vial through the septum with an insulin syringe.

3.1.1. Then add a drop to a glass slide and cover it with a standard coverslip. First dilute it with a droplet of degassed saline.

3.1.2. Perform microscopy with a video microscope, equipped with a fluorescence epi-illumination, 100x oil objective and high sensitivity video camera.

3.2. To determine the efficacy of transfer of lipid material from the aqueous saline/PG media to the bubble shell during amalgamation, take a sealed vial of microbubbles, invert it and place it in a conical 50 mL tube.

3.2.1. Perform centrifugation in a bucket rotor (10 min, 200 x g). Remove the vial from the centrifuge and keep it inverted.

3.2.2. Insert a needle of an insulin syringe into the septum of the inverted vial. Aspirate a small volume (~50 μL) of clear infranatant slowly.

NOTE: The bevel tip of the needle for infranatant aspiration from the inverted vial after centrifugation must be located as close to the septum as possible, to minimize chances of microbubble intake.

3.3. Place the samples of infranatant (2 μL), as well as the samples of the original lipid-PG-saline medium that was used to generate microbubbles (2 μL) in a 96-well plate with 0.1 mL of phosphate-buffered saline (PBS):ethanol 1:1 medium with 1% Triton X-100.

3.4. Measure red fluorescence of DiI dye (555 nm excitation, 620 nm emission) with a fluorescence microplate reader.

3.4.1. Use black plates with non-transparent bottom for fluorescence spectroscopy due to lower background and lack of inter-well signal transfer that may become an issue for clear plates.

3.4.2. Ensure that the fluorescence signal is within the linear calibration range: excessive dye concentration in the wells may lead to light attenuation and under-reported signal.

NOTE: It may sometimes be desirable to remove the residual shell material that remains in the aqueous medium following amalgamation. To do this, after microbubble flotation centrifugation, take all of the infranatant volume out by slow aspiration from the inverted vial, and replace it by degassed normal saline. To avoid excessive change of ambient pressure in the vial due to removal or addition of liquid, insert an additional long needle in the septum to reach the gas phase inside the vial. To maintain the gas phase inside the vial, first, connect the needle to a syringe filled with fluorocarbon gas, to prevent air contact with the microbubble preparation.

4. Microbubble size distribution adjustment

4.1. Use normal gravity flotation in a static inverted vial to adjust the size distribution of microbubbles, i.e., to remove largest microbubbles quickly and efficiently [5,10].

4.2. Take a vial of microbubbles immediately following preparation as described in Section 2. Invert and place upside down on a stable non-vibrating surface for 15-20 min.

4.3. Following this incubation, insert an insulin syringe needle in the septum of the vial, while it is still inverted, and gently collect 300-500 μL of microbubbles from the lower layer.

NOTE: The needle of the syringe must stay close to septum inner surface to avoid collection of larger bubbles present closer to the top of the liquid in the vial.

4.3.1. Pull back the syringe plunger slowly, to avoid turbulence and hydrostatic expansion of the bubbles in the syringe body. When the collected sample is taken out of the syringe, avoid overpressure to prevent bubble collapse.

4.4. Transfer microbubble dispersion from the syringe to a small volume vial. Then fill with perfluorinated gas, stopper and crimp, for short-term storage, particle counting (see below) and further use.

4.4.1. If sampling is performed directly from the syringe, hold horizontally and rotate to avoid bubble flotation and achieve sample uniformity.

5. Microbubble size distribution assessment

5.1. Use a particle counter (based on electrozone sensing or light obscuration principle) to assess particle size distribution and concentration. Alternatively, use a hemocytometer and a microscope.

5.1.1. Briefly, add a microbubble sample aliquot into normal saline or isotone in a counter chamber (typically, 60-100 mL volume) and count. Compare the microbubble size distributions before and after flotation purification.

NOTE: Subject the vial to gentle mixing (not vortexing) immediately prior to sampling, to achieve representative and reproducible measurements. Sample from the center of the tested volume.

5.1.2. Fill a 100 mL beaker with at least 60 mL of 0.9% saline solution diluent, and place it on the stage of the electrozone sending instrument. Lift the stage so that the tube, outer electrode plate, and stirrer, are all completely immersed in the diluent, and the stirrer can rotate.

NOTE: Diluent volume in the beaker can be precisely determined using its mass, with a scale (saline density is close to 1). Alternatively, a metering pump can be used. The electrodes during electrozone sensing study should be fully immersed.

5.1.3. Record the diluent electrolyte volume in the unit control software. Electrozone sensing counter is equipped with 50 µm orifice, which allows measurement of particles between 1 and 30 µm in diameter. Perform a background run first; it is expected to have less than 5000 counts in 0.5 mL of diluent as blank. The best background levels can be under 100 counts.

5.1.4. Set the sample volume in the unit control software, to account for the dilution factor. Add 10-20 µL of the microbubble mixture to the beaker, and perform the second run.

5.1.5. Use the background size distribution data to account for by the use of the “subtract background run” function within the software. Obtain the concentration of the particles in the original microbubble sample as particle number/mL; it includes the dilution factor.

5.1.5.1. The outer surface of the pipet tip might be also covered with bubbles during sampling; make sure to wipe it off prior to inserting the tip into counting medium. Do not touch

the pipet tip orifice with a wipe.

5.1.5.2. Avoid insufficient dilutions of sample particles during counting, to minimize the issues with coincidence correction, where more than one particle at a time is within the sensor orifice: the system may then underestimate particle concentration.

NOTE: Orifice diameter for particle counting may vary. Selection of the 50 μm orifice allows detection of microbubbles with the diameter down to 1 μm, yet it is not as prone to clogging as smaller diameter options.

5.1.5.3. Calibrate the electrozone sensing counter for the same diluent test media (e.g., sterile-filtered normal saline irrigation solution) as is used in actual tests. A laser obscuration alternative to electrozone sensing has the advantage of not needing filtered saline with controlled salt concentration and electrical conductivity.

6. **Test microbubble targeting in vitro in an adhesion/retention assay**

6.1. Prepare the biomarker receptor surface for microbubble targeting to Petri dishes.

6.1.1. Take polystyrene 35 mm diameter dishes to be used as the target surface. Take advantage of the nonspecific adhesion of proteins to the polystyrene dish surface from normal saline. Use a model protein, streptavidin, for testing of the targeting of biotinylated bubbles that contain biotin-PEG-DSPE as the part of the shell.

6.1.2. Place a droplet of streptavidin solution (0.2 mL, 10 μg/mL in PBS) in the center of each Petri dish and cover it with a 22 mm x 22 mm plastic coverslip to allow for even coating of the dish surface. After overnight incubation at 4 °C in an enclosed moist environment to prevent the dish from drying out, remove the coverslips.

6.1.3. Immediately and exhaustively wash the plates with water, PBS, and block them by incubation with 1.5% bovine serum albumin (BSA) in PBS for at least 4 h to minimize nonspecific adhesion of microbubbles to uncoated surface. Use the clean culture dishes blocked with 1.5% BSA as controls.

6.1.4. Alternatively, use recombinant $\alpha_v\beta_3$ receptor solution (e.g., at 4 μg/mL in PBS)[5] instead of streptavidin. Target microbubbles to this biomarker via cyclic RGD peptide ligand attached to the microbubble shell.

6.1.5. Keep the dish with deposited target receptor wet: receptor protein may be inactivated if it dries out.

NOTE: It is important to use carrier-free receptor proteins that do not contain any carrier proteins or surfactants in the medium – their presence will inactivate adhesion.

6.2. Adhesion of microbubbles in a small droplet: quickly check targeted adhesion in static conditions.

6.2.1. Deposit a droplet of microbubbles (5-20 μL) from underneath onto the target (or control BSA-only) surface in a Petri dish that is inverted upside down. This will bring the bubbles to the receptor-coated surface by flotation.

6.2.2. After 5-10 min incubation of the inverted dish in a moist environment, invert the dish back to a normal position, fill it with PBS and gently rinse to remove free microbubbles. Perform brightfield microscopy to assess targeted adhesion.

6.3. Adhesion of microbubbles on Petri dish surface: perform targeting in a full dish[1].

6.3.1. Take the dish, which has receptor surface coating, and fill it completely with degassed PBS-BSA buffer (over 10 mL for the 35 mm dish), so that the meniscus of buffer extends over the top of the dish and is held by capillary force. Inject the bubbles (50 μL) into the bulk of the buffer and rapidly mix to achieve homogeneity. Avoid formation of air bubbles during mixing.

6.3.2. Place a segment of transparent packaging tape or a culture plate sealer tape, backed with a flat piece of plastic over the dish quickly. Pressure-seal the film to the dish, invert the "assembly" and place it upside down for 30 min to allow the microbubbles float upwards, touch the target surface and adhere.

6.3.3. Invert the sealed dish "assembly" back to "down-side-down" configuration, remove the seal, and wash away the non-adherent microbubbles by rinsing with degassed buffer solution. Observe targeted microbubbles by microscopy or by ultrasound imaging.

NOTE: When the inverted sealed dish completely filled with the microbubble dispersion is incubated, it can be placed at a slight angle, so if any large bubbles are present, they will float to the edge of the dish, outside of the center region of interest. Forced rinsing with fast flow coming from a micropipette tip during microscopy can be used to assess how firmly bubbles are adherent to the target (bubbles completely detach from control surface even in slow flow) [11]. Degassed buffer is preferable for bubble dilution, because excess of dissolved air will lead to uncontrolled growth of microbubbles.

7. Test microbubble targeting in vitro: assess dynamic adhesion/retention assays in a parallel plate flow chamber

NOTE: We test the adhesion of biotinylated bubbles to streptavidin layer with ultrasound imaging.

7.1. Use a commercially available parallel plate flow chamber with a custom-built inverter holder to observe targeted adhesion to a 35 mm Petri dish from flowing medium. After adsorption of target protein to the dish (see 6.1.1), insert the chamber body with preinstalled

gasket in the dish and seal it in the holder. Use gaskets with channel height (i.e., gasket thickness) 0.127 mm and channel width 2.5 mm.

NOTE: During the assembly of parallel plate flow chamber, the amount of silicon grease used on the gasket should be minimal. Do not allow grease to get in the channel area: avoid covering the biomarker protein-coated surface with grease. To achieve a proper seal, select 35 mm dishes carefully (see **Table of Materials** for the chamber and dish information).

7.2. For the flow chamber assembly, do not screw the cap and frame that holds the dish-flow chamber combination too tightly, to avoid leakage.

7.3. Connect the flow tubing to a syringe pump operated in a withdrawal mode, and on the feeder side, connect a thin polyethylene tubing (PE50) to the vial with a dilute microbubble dispersion, subjected to constant mixing with a stir bar via a magnetic stirrer[12]. Control the wall shear rate (WSR) parameter of the chamber by adjusting the pump volumetric flow rate based on the formula $6Q/bh^2$, where Q is the flow rate, 'b' is width of the channel, and 'h' is height of the channel.

NOTE: Remove the air bubbles from the entire system prior to perfusion of the microbubble-containing medium or PBS, as any air bubble passing through the channel will dislodge adherent microbubbles from the target surface, and invalidate the experiment. Connection between the flow chamber tubing and the syringe must be properly sealed.

7.4. Prepare a microbubble dispersion by adding a calculated volume of concentrated bubbles to the microbubble reservoir (a 20 mL scintillation vial) to achieve concentration of 10^6 microbubbles/mL, in PBS buffer with 0.1% BSA. Place the feeder reservoir on a magnetic plate stirrer. Insert a 1 cm x 2 cm magnetic stir bar and stir at ~400 RPM to maintain homogeneity during the course of the study.

7.5. Perform ultrasound imaging of the flow chamber in a water tank[13].

7.5.1. Submerge the flow chamber assembly in degassed water and hold it in place by a weight to prevent movement during experiments.

7.5.2. Place the imaging probe clamped directly above the channel, and tilt it at a 15° angle backwards, as well as a 5° angle clockwise to minimize specular reflection from the dish surface. Position the flow chamber channel within the imaging plane.

7.5.3. Use the following imaging conditions: 15L8 transducer, contrast-specific imaging mode, dynamic range 50 dB, 7 MHz, Mechanical Index (MI) = 0.18, CPS Gain = 0. Keep the time gain compensation uniform across the entire image.

7.5.4. Place the transducer face so it is touching the water surface, not deeply immersed. Alternatively, use a rubber protective sleeve filled with ultrasound gel.

NOTE: To warrant reproducible positioning of the flow chamber system for imaging, the markers should be placed on the water basin, or on the ultrasound system screen.

7.6. Draw the microbubble dispersion from the reservoir through the chamber and into the pump for 2 min.

7.6.1. Switch the flow to PBS, to remove non-adherent bubbles from the channel and assess acoustic backscatter of the remaining adherent (targeted) bubbles. To obtain a background image, increase the MI to 1.9 to destroy the adherent bubbles in the ultrasound imaging field of view.

NOTE: Microbubble dispersion should be replaced for repeated runs: at high dilution, microbubbles gradually degrade with time.

7.7. Export individual images from the screen recording video stream: before PBS flushing, after flushing, and after destruction, to be imported into ImageJ for offline analysis. Select the region-of-interest (ROI) to exclude the inlet and outlet portions of the chamber. After subtraction of the background ROI signal, quantify the echo intensity as the mean pixel intensity within ROI.

NOTE: As an alternative to ultrasound imaging, the adhesion of microbubbles to the target surface can be observed by video microscopy, when the parallel plate flow chamber assembly in the inverted holder is placed on a stage of a compound microscope, with video recording [5,11,12,14]. This is especially useful for imaging targeted adhesion of microbubbles to cell surface biomarker receptors in cell culture, when receptor-expressing cells are grown on the tissue culture dish, and the number of microbubbles bound to each target cell can be counted directly.

REPRESENTATIVE RESULTS:

Covalent coupling of peptide and lipid

Reaction completion and desired product formation was confirmed by TLC. A separate unreacted peptide control did not move up during TLC: it was retained at the start, and its spot was positive for the primary amino group, as observed after ninhydrin spray, upon heating. This ninhydrin-positive free peptide spot was no longer observed in the mixture following reaction completion, following TLC of the reaction mixture sample, after the removal of DIPEA, DMSO, and re-dissolution in chloroform. As for the crucial issue of NHS ester reagent quality, **Figure 1** presents spectrophotometric track of hydrolysis kinetics, with the zero time point at the beginning of the reaction being when the NHS ester in an organic solvent was added to the cuvette. This confirms the functionality of NHS active ester of carboxy-PEG-DSPE (see Methods Section 1). At the zero time point, the extrapolated A_{260}=0.33 represents the material that was already hydrolyzed prior to testing. At the completion of the hydrolysis reaction, in excess of 10-15 min, A_{260}=1.54 (when absorbance does not increase considerably anymore). This confirms the presence of active ester. It also provides quantitative data, that over 78% of the

material is not pre-hydrolyzed NHS, and can be thus successfully used for peptide coupling, with the proper adjustment of the reagent amount.

Preparation and transfer of lipid material from the aqueous media onto the bubble shell: fluorescence lipid

The microbubbles for this study were prepared to contain a trace amount (under 1%) of the fluorescent dye DiI, with characteristic red fluorescence, that was added as a solution in PG to the saline-PG solution of DSPC and PEG stearate. Resulting microbubbles clearly demonstrate shell fluorescence when green light excitation and red emission filters are used in the microscope (see **Figure 2**, left). Brightfield microscopy of microbubble gas phase (**Figure 2**, right) can be compared with microbubble shell fluorescence. For the quantitative assessment of lipid material transfer from the aqueous phase to the bubble shell, microbubbles were floated using centrifugation, and the fluorescence signal of the clear infranatant phase was compared with the fluorescence of the initial solution, prior to microbubble amalgamation. Almost an order of magnitude signal reduction was observed (**Figure 3**), i.e., over 85% of the lipid material has transferred to the microbubble shell by amalgamation.

Preparation and size distribution correction of microbubbles

Microbubbles generated by amalgamation demonstrated a typical size distribution, with high concentration (e.g., ~4.8 x 10^9 particles per mL for biotinylated bubbles). The size distribution was wide, with particles present within the measured range (between 1 and 30 μm); ~6.3% microbubbles exceed 5 μm in diameter (**Figure 4, green curve**). Intravascular administration of large microbubbles may lead to their nonspecific accumulation in the blood capillaries and should be avoided. A short (15-17 min) flotation of the inverted vial in normal gravity, with the subsequent collection of 0.3 mL close to the septum surface, allows removal of larger microbubbles completely, with minor loss in the total particle number concentration, down to ~4.6 x 10^9: following flotation, only 0.01% of the particles in the purified sample have diameters over 5 μm (**Figure 4, red curve**).

Adhesion of microbubbles to receptor-coated surface: static assay

This procedure has been first described in the previous century[1], and is being used as a quick test that confirms functionality of targeted microbubbles. Microbubbles are allowed to contact the receptor-carrying dish surface. If the ligand-receptor interaction takes place, bubbles may be retained on the surface despite the vigorous wash. An example of such a quick test of the functional adhesion of c(RGDfK)-microbubbles onto the surface coated with recombinant $\alpha_v\beta_3$ is presented. **Figure 5** is a representative brightfield microscopy image of adherent microbubbles on the receptor surface in a Petri dish, following a wash with PBS, to remove unbound bubbles. Bubbles in this type of microscopy present as dark circular patterns. In similar condition, if the surface is only coated with albumin (to block nonspecific adhesion), microbubbles will not adhere and will be easily washed away even by the gentle rinse.

Binding of microbubbles from the flowing medium: parallel plate flow chamber

This procedure has been initially proposed as a tool for study of cell adhesion in a controlled flow setting[15] and adapted for study of microbubble targeting decades later[11]. Testing in a flow-

through system, unlike a static assay, is much more realistic for a clinical imaging scenario, where circulating bubbles in a flow of blood briefly touch the vessel wall and may adhere to it if the target receptor is present. Two examples of such studies are presented. The first example is a more traditional approach, where the adhesion of peptide-decorated microbubbles to the receptor-coated surface is monitored by video microscopy. Microscopy allows distinguishing adherent microbubbles from the flowing ones. It also allows to quantify those adherent microbubbles in the microscope imaging frame: many more c(RGDfK) microbubbles (left column) adhere to the surface, when compared with control, where scrambled c(RADfK) peptide is used, or if the surface is only coated with BSA (**Figure 6**).

The second example is contrast ultrasound imaging of the Petri dish coated with streptavidin (**Figure 7**, right side) to which biotinylated microbubbles adsorb successfully from the flowing medium, and can be detected by contrast ultrasound imaging following a flush with PBS. The control dish surface does not retain any adherent microbubbles from the flow, so essentially all ultrasound contrast signal is removed with PBS flow. Ultrasound contrast signal quantification shows strong statistical significance of the difference observed; the ratio of target and control signals exceeded an order of magnitude.

FIGURE LEGENDS:

Figure 1. Kinetics of hydrolysis of NHS-PEG-DSPE active ester, observed by NHS release in alkaline medium by spectrophotometric testing at 260 nm wavelength. Zero time point is the time of addition of NHS-PEG-DSPE in organic solvent to the 0.1 M borate buffer, pH 9.2.

Figure 2. Microscopy of gas-filled microbubbles following amalgamation. Left, fluorescence microscopy (green excitation, red emission, DiI lipid shell dye). Right, brightfield microscopy (gas phase observation), same magnification. Frame width, 85 um (10 μm stage micrometer embedded on bottom right of each image).

Figure 3. Fluorescence spectroscopy of DiI lipid dye sample from the microbubble preparation medium before amalgamation (right) and following amalgamation and removal of microbubbles by centrifugal flotation (left). Fluorescence excitation - 555 nm, emission - 620 nm. Data presented as Mean ± Standard Deviation.

Figure 4. Particle size distribution of the number concentration of microbubbles following amalgamation preparation (green), with subsequent normal gravity flotation for the removal of large microbubbles (red) and diluent-only background count (blue). Electrozone sensing particle counting in normal saline, 50 μm orifice.

Figure 5. Brightfield microscopy of c(RGDfK)-microbubbles on a dish coated with $\alpha_v\beta_3$. Image frame width is 106 μm; bar is 10 μm.

Figure 6. *In vitro* parallel plate flow chamber targeting of peptide-decorated microbubbles to the surface coated with recombinant $\alpha_v\beta_3$. cRGDfK-decorated microbubbles efficiently adhered to the dish (left), attachment of control non-targeted cRADfK (scrambled, center)

617 microbubbles was minimal ($p<0.00005$), as was microbubble retention at the albumin-only
618 control surface (right, $p<0.0025$). Chamber flow wall shear stress at 1 dyn/cm^2. Microbubble
619 adhesion monitored by video microscopy; the number of particles in the field of view is
620 presented. Accumulation time is 4 min. Data presented as Mean ± Standard Deviation.
621 Reprinted with permission from[5]. Copyright, 2018, American Chemical Society.
622
623 **Figure 7. Contrast ultrasound imaging of a parallel plate flow chamber following targeted**
624 **adhesion and buffer flush of biotinylated microbubbles on the dish coated with streptavidin**
625 **(middle, adherent targeted microbubbles, right, same dish, following high-MI ultrasound**
626 **burst), and control dish coated only with albumin (left).** Two minutes of perfusion of
627 microbubble dispersion (PBS/BSA, 10^6 particles/mL) at 450 s^{-1} shear rate, followed by buffer
628 flush. Quantification of ultrasound signal is performed from the regions of interest in the video
629 frames after background subtraction.
630
631 **DISCUSSION:**
632 The importance of a simple technique for the preparation of ligand-decorated microbubbles is
633 evident. The use of amalgamation technique for microbubble preparation, as pioneered by
634 Unger et al.,[16] may serve this purpose for a number of reasons. Manufacturing of microbubbles
635 by amalgamator is easy to perform. A small-footprint desktop single-phase 120 V unit is
636 available and inexpensive. The procedure is quick (45 seconds) and efficient: 1 mL of
637 microbubble dispersion in an aqueous medium is prepared at once. It contains billions of
638 particles per mL, more than sufficient for research studies. Manufacturing occurs in a sealed
639 vial with perfluorinated gas headspace. If necessary, vial contents will remain sterile from the
640 time of aseptic filling, during manufacturing (amalgamation), and until use. This makes the
641 approach relevant for clinical use, as it does not require elaborate preparations in a dedicated
642 sterile environment in clinic.
643
644 The procedure is based on self-assembly: during mixing, as high shear is applied to gas-water
645 interface within the moving vial, small gas fragments are formed, which assume spherical shape
646 due to action of surface tension. PG, as a cosurfactant, present in the medium at high
647 concentration, reduces surface tension and energy required to generate gas-water interface
648 during shear. Next, more “classical” surfactants, such as PEG-lipids and phospholipids, which
649 are present at much lower concentrations, get to the interface, most likely displacing PG and
650 establishing a monomolecular layer at the bubble surface. This shell is reasonably stable; it is
651 likely due to a combination of a “solid” lipid (DSPC phase transition temperature is 56 °C, so it is
652 not prone to inter-membrane fusion) and an extended PEG brush coat that surrounds the
653 microbubbles and inhibits direct monolayer contact of neighboring bubbles. One can speculate
654 that the presence of a high concentration of PG in the media may lower the microbubble shell
655 stability. In its absence, microbubbles are stable in the sealed vials under fluorocarbon
656 atmosphere for many months, with only moderate fusion between the bubbles. For clinical use,
657 with a small amalgamator device at the bedside, the interval between microbubble preparation
658 and use can be short, minutes or hours. With PG present in the media, microbubble
659 concentration does not show a significant drop, at least for several hours of refrigerated
660 storage.

An added advantage of the described procedure (assisted by the use of PG cosurfactant in the bubble preparation medium) is high efficacy (>85%) of the lipid transfer to the shell, whereas the traditional sonication provides ~20% efficacy[5] and modern microfluidic methods even lower[17]. High level of transfer efficiency is important not just because the waste of lipid material and expensive ligand is reduced, but because the amount of bubble-free ligand co-present in the media is minimized as well. Then the free ligand may not have an opportunity to block the biomarker target receptor to which the microbubbles are expected to bind via ligand on their surface. The general amount of the biomarker receptor on the target vasculature is often quite high, so this might not be of utmost importance. From the available patent literature[18] one might suggest that at least 50% of lipid shell material and targeting ligand in the microbubble formulations in clinical testing may be associated with the bubble shell. This can be generally compared with radiolabeled antibodies or peptides that are widely used in nuclear medicine receptor imaging studies: most of those targeting ligand molecules actually do not carry "hot" radioisotope even for the highest specific activity reported[19], whereas for targeted microbubbles, the shell material in this study (including ligand-lipid) is mostly attached to microbubbles.

Selective adhesion of targeted microbubbles prepared by this technique in vitro was demonstrated, in two sets of targeting models: static adhesion, and a flow chamber targeting experiment. In a static assay, targeted microbubbles adhered to the target receptor layer tightly and were not dislodged with buffer rinse, unlike in a control setting, where microbubbles were removed from the surface even with a gentle rinse. Likewise, in a flow-through test, performed in a parallel plate flow chamber, biotinylated bubbles demonstrated statistically significant and superb adhesion to streptavidin layer on a polystyrene dish, when compared with the control albumin-only surface. Peptide c(RGDfK)-decorated microbubbles selectively adhered to $\alpha_v\beta_3$-coated surface, both in the static adhesion assay, and in a parallel plate flow chamber.

The following issues can be considered as the limitations of the described protocol. First, the procedure does not account for the submicron particles. The instrument that was used in the study was not set up to detect nanobubbles (i.e., particles under 1 μm in diameter). These particles might have been present in the formulation. Although their acoustic backscatter signal is generally known to be low, and they were not observed in this study by microscopy, the presence of nanobubbles should still be considered. The second significant issue is the size heterogeneity of the microbubbles. Despite the removal of larger particles, the size of the resulting bubbles is far from uniform. This should be a consideration and justification for further research in the area of microbubble formulation.

In conclusion, the narrative given in this manuscript should provide sufficient level of technical detail to manufacture targeted microbubbles quickly and easily. The steps to perform additional purification (if desirable), adjusting the size and/or assessing the small amount of the shell material that remains in the aqueous medium are provided. The detailed analytical tools for the assessment of the microbubble parameters, such as size distribution and concentration, and in vitro ability of ligand-decorated microbubbles to adhere to target receptors are described.

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DISCLOSURES:

A. Klibanov is a co-founder and minority shareholder of Targeson Inc, a startup in the area of preclinical targeted microbubbles, now dissolved. His UVA laboratory has a subcontract via NIH R44 HL139241 from SoundPipe Therapeutics.

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Figure 1

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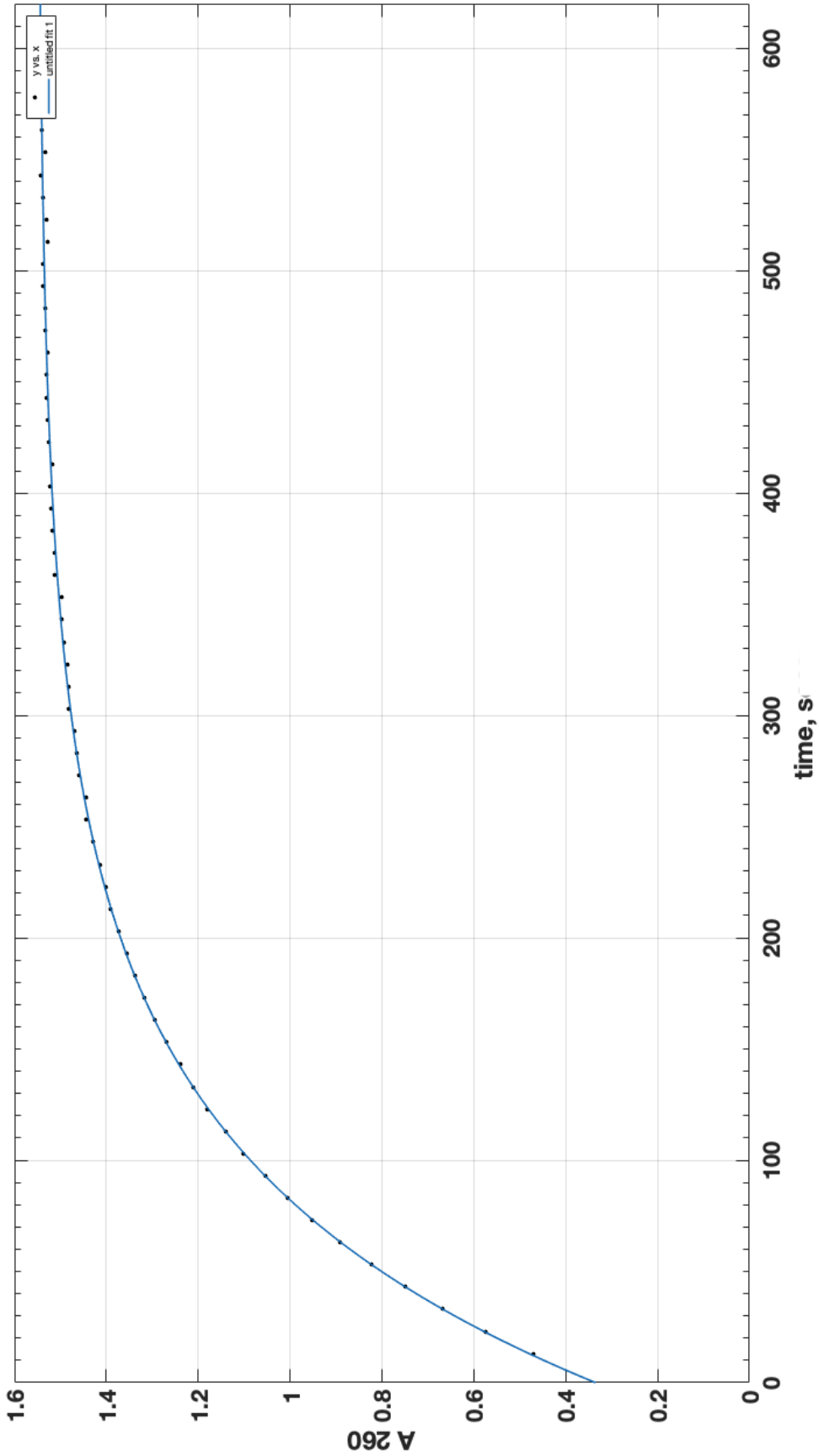

Figure 2

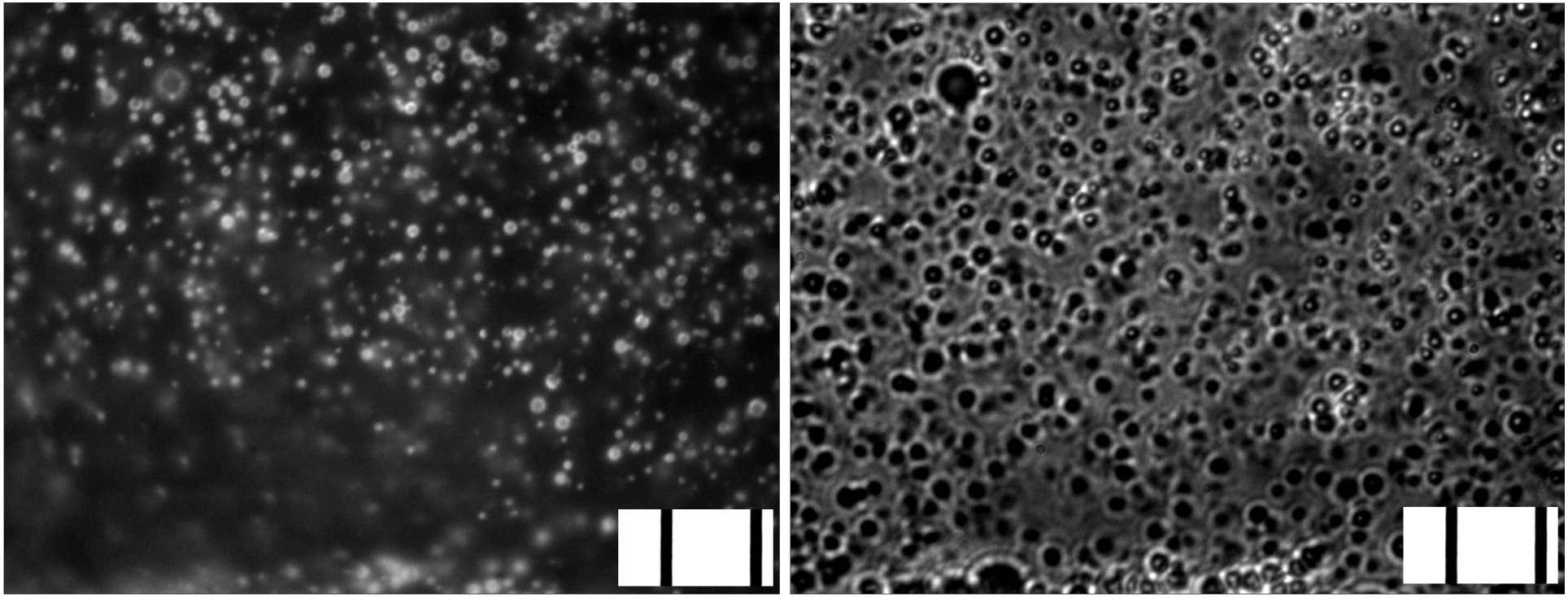

Figure 3

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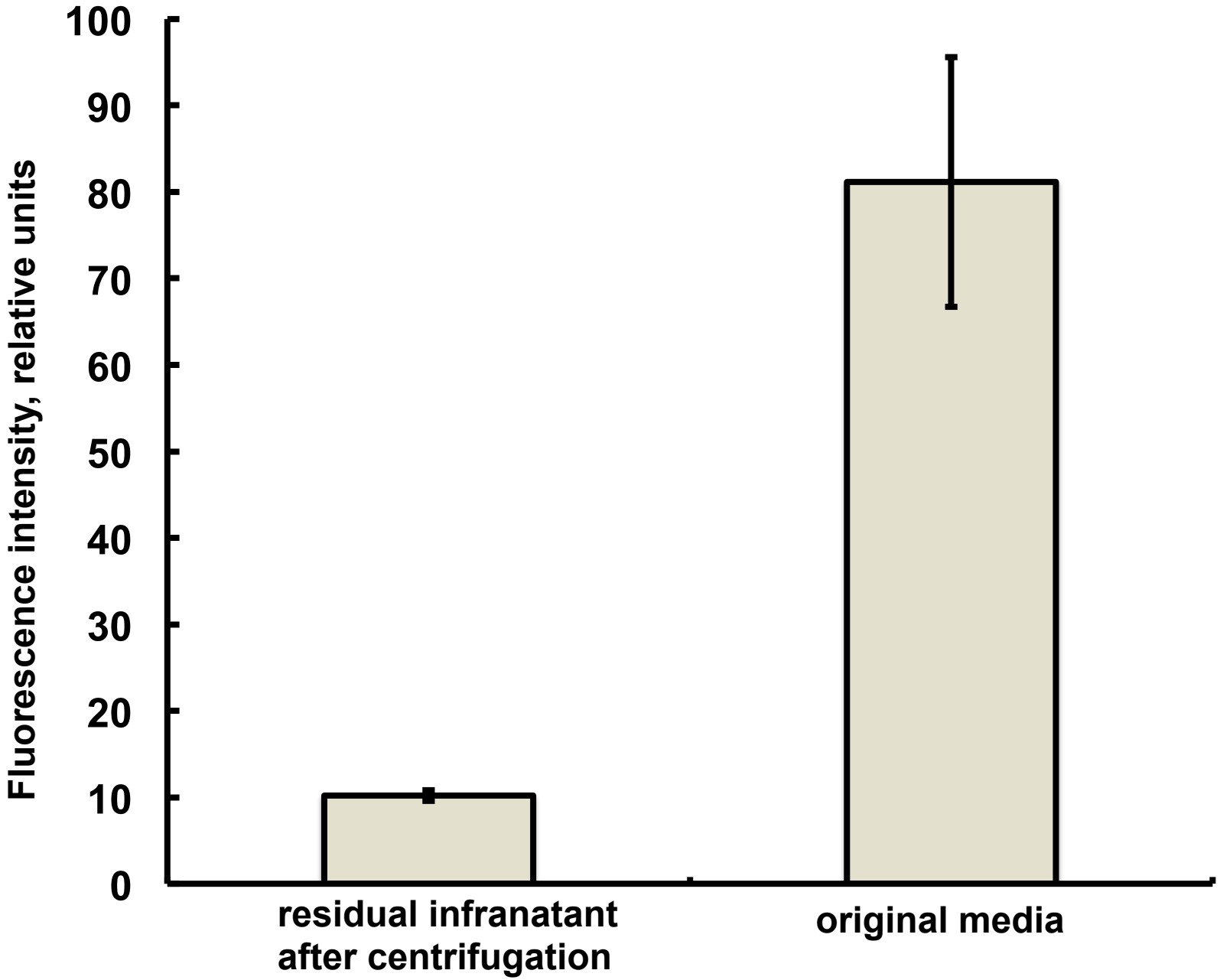

Figure 4

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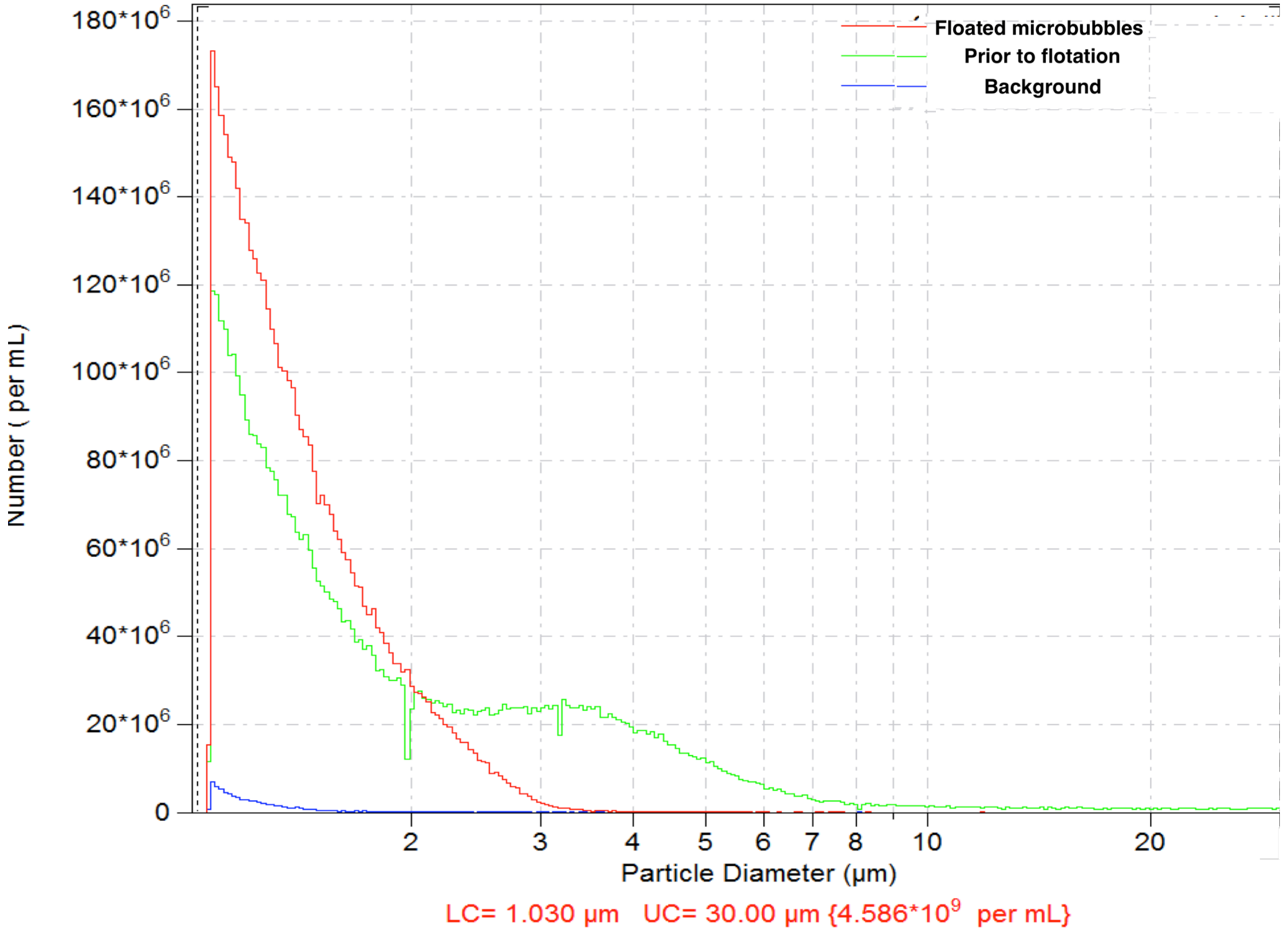

Figure 5

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Figure 6

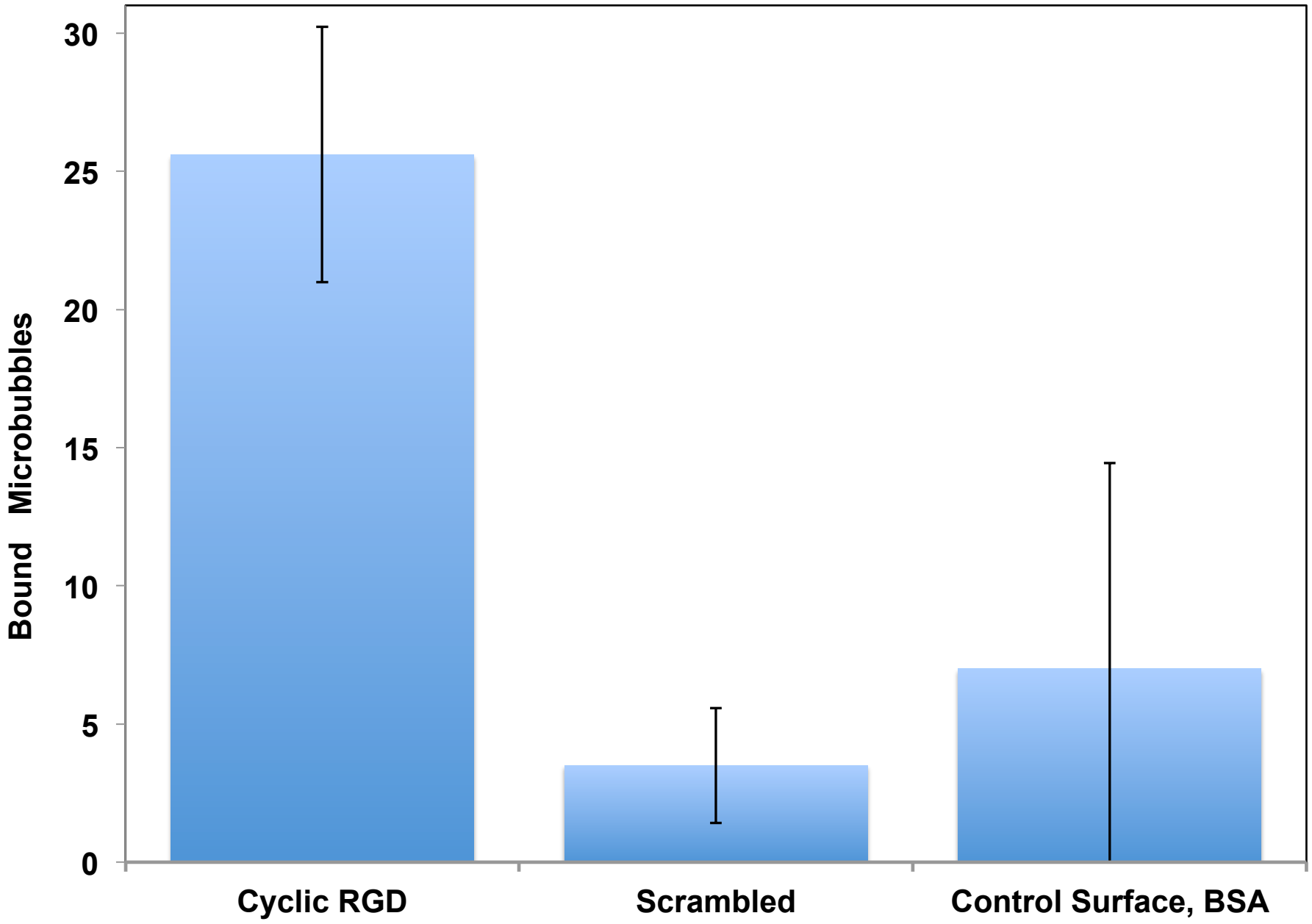

Figure 7

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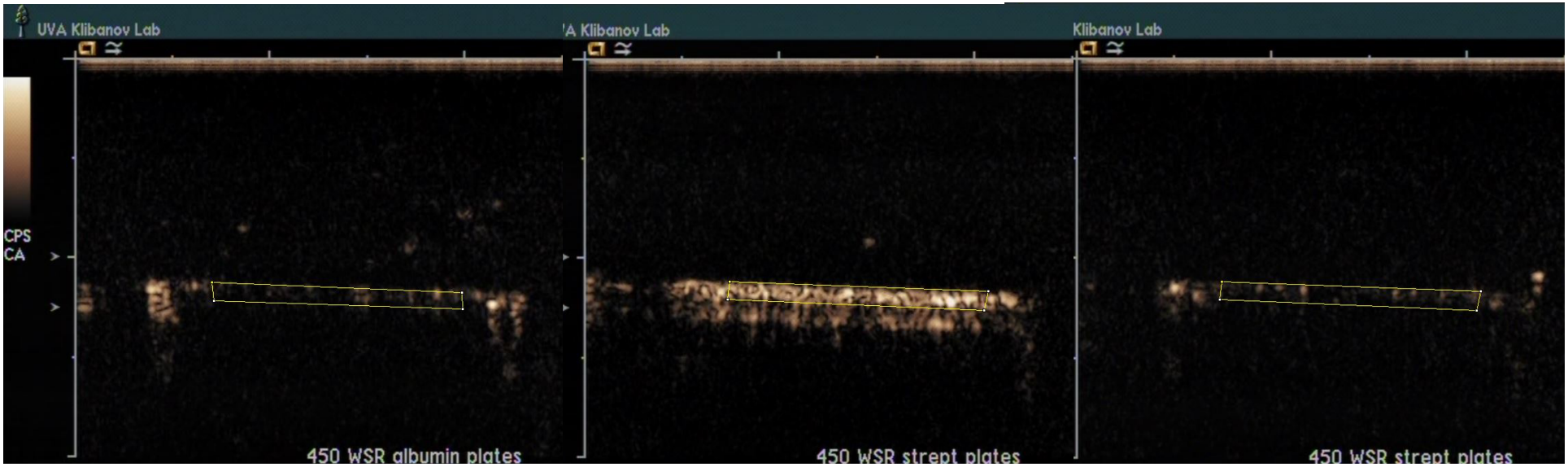

Control Petri dish: BSA coat only

Microbubble targeting: streptavidin coat

Microbubble targeting: streptavidin coat, post-burst

Name of Material/Equipment	Company	Catalog Number	Comments/Description
Amalgamator	Lantheus, Billerica, MA.	Vialmix	ESPE Capmix, Wig-L-Bug or another a
biotin-PEG3400-DSPE	Laysan Bio, Arab, AL.	Biotin-PEG-DSPE-3400	
Bovine Serum Albumin (BSA)	Fisher Scientific, Waltham, MA	BP1600-100 or similar	
Ca-Mg-free PBS	Fisher Scientific, Waltham, MA	14190-144	
Centrifuge with a bucket rotor	IEC/Thermo, Fisher Scientific, Waltham, MA.	NH-SII	Any centrifuge with a bucket rotor
chloroform	Fisher Scientific, Waltham, MA	C297-4	
cyclic (RGDfK) peptide	AnaSpec, Fremont, CA	AS-61111	
Decafluorobutane	F2 Chemicals, Preston UK	CAS 355-25-9	
DiI	Sigma-Aldrich, St. Louis, MO.	468495-100MG	
DIPEA	Sigma-Aldrich, St. Louis, MO.	387649	
Disposable UV cuvette, 1.5 ml	BrandTech, Essex, CT.	759150	
DMSO	Sigma-Aldrich, St. Louis, MO.	276855	
Dry block heater, high temperature	Techne Cole Palmer, Staffordshire UK	DB-3A	
DSPC	Lipoid, Ludwigshafen, Germany	LIPOID PC 18:0/18:0	
Fluorescence microplate reader	Molecular Devices, San Jose, CA.	Spectramax Gemini XS	No longer available, superceded by G
Microscope with fluorescence epi-illumination.	Leica	Laborlux 11	No longer available; any fluorescence
NHS-PEG3400-DSPE	NOF-America, White Plains, NY.	SUNBRIGHT DSPE-034GS	Some of the alternative manufacture
Ninhydrin spray for TLC plates	BVDA, Haarlem, The Netherlands	AS-72177	
Normal saline irrigation solution (0.9% NaCl)	Baxter, Deerfield IL.	2F7124	

Parallel plate flow chamber, for 35mm Corning Petri Dish	Glycotech, Gaithersburg, MD.	31-001	May only work with Corning Petri dis
Particle sizing system	Beckman Coulter, Hialeah, FL	Multisizer 3	No longer available, superceded by M
PEG 6000 monostearate	Kessco Stepan, Joliet, IL.	CAS 9004-99-3	
Petri Dishes, 35 mm diameter, 10 mm tall	Corning, Corning, NY.	430165	
Plastic coverslips, 22x22mm	Cardinal Health, McGaw Park, IL.	M6100	
Propylene glycol	Sigma-Aldrich, St. Louis, MO.	P4347	
Recombinant murine alphavbeta3, carrier-free	R&D Systems, Minneaposis, MN.	7889-AV-050	
Rubber stoppers, 13mm	Kimble-Chase, Vineland, NJ	W017900	
Serum vials, 2 ml, 13mm	Kimble-Chase, Vineland, NJ	223683	
Silica TLC Plates, F254	Analtech, Newark, DE	P02521	
Sodium tetraborate	Sigma-Aldrich, St. Louis, MO.	S9640	
Streptavidin	AnaSpec, Fremont, CA	AS-72177	
Syringe pump, infuse/withdraw option	Harvard Apparatus, Holliston, MA	PHD2000, 70-2001	
Ultrasound imaging system with contrast-specific mode.	Siemens/Acuson, Mountain View CA	Sequoia c512, 15L8 probe	Old generation Sequoia is out of proc
UV Spectrophotometer	Beckman, Brea, CA.	DU640	No longer available, may be replaced

malgamator capable of 4300 rpm can be used.

emini XPS; any fluorescence plate reader with red dye detection capability will work

microscope is sufficient; high-sensitivity video camera is required for image stream collection

rs provide material that is mostly, or completely, hydrolyzed on arrival

hes, but not necessarily with other makers, due to different dimensions
Multisizer 4, with similar electrozone sensing principle. Alternatively, optical methods, e.g., Accusizer, can be used.

duction for more than a decade. Available as used equipment. CPS mode has to be unlocked for the 15L8 transducer.
with any 260 nm ultraviolet-capable unit

Dear Editor,
Thank you very much for your message and for the suggestion to move the notes closer to the respective protocol steps; in the amended manuscript this was accomplished as suggested. Just in case, I did not remove the old rebuttal letter for the submission file set. The video file will be finalized and uploaded shortly, hope it is of acceptable quality.
Thank you very much for your help, advice and consideration with this manuscript submission. If there are any questions or suggestions, I will be happy to address those ASAP,
Sincerely,
Sasha Klibanov

Dear Editor,

Thank you very much for considering this manuscript for publication. Please find enclosed below the response to the comments and suggestions by the reviewers. We apologize for slower than expected video processing – this is due to continued coronavirus limitations and should be completed and uploaded in a couple of days.

Thank you very much for your help and consideration,

Sincerely,

A. Klibanov

Editorial and production comments:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Thank you for the suggestion, an additional check was performed.
2. Please provide an institutional email address for each author.

Institutional address for all authors was provided during original submission; they all receive the email from the JOVE office.

3. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.
For example: speedvac, Vialmix, ESPE Capmix, Wig-L-Bug, Hamamatsu Orca video camera, Gemini XS, Molecular Devices, Accusizer, Beckman Coulter, Multisizer, Coulter counter, Corning, Cadence, etc.

Done, except CPS Gain – that parameter is required to describe exact imaging conditions.

5. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

Done.

6. Line 91-94: Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. How is the evaporation performed? How much chloroform is used for redissolving the nonvolatile residue?

Information added.

7. Please move the notes compiled at the end of a particular section to the appropriate steps of

the protocol. In the JoVE Protocol format, "Notes" should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step. Please consider moving some of the notes about the protocol to the discussion section (Lines: 107-134, 197-202, 225-242, 256-263, 287-309, 358-373, 417-442)

We would prefer to disagree with this suggestion; the technical details as we describe in the notes are not of the "general discussion" type, but specifically address the reasons, potential issues and pitfalls of the experimental steps as well. Reviewers also pointed at these details as the positive feature of the manuscript.

8. Line 249/350: Please use standard abbreviations for time units preceded by a numeral. Do not abbreviate day, week, month, and year. Examples: 5 h, 10 min, 100 s, 8 days, 10 weeks

Corrected as suggested.

9. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

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10. Please include the limitations of the Protocol in the Discussion section.

Limitation paragraph added to Discussion as suggested.

11. Figure 1: Please use standard abbreviations for time units in the X- axis. Please revise "time, seconds " to "time (s)"

X-axis adjusted to "time, s", as suggested.

12. Figure 2/5: Please include scalebars in all the images of the panel and define them in the Figure legends. Consider adding the details of the magnification to make the image more informative.

Figure 2 has the fragment of the stage micrometer image embedded in the lower right corner of each image (distance between vertical bars of the pictured stage micrometer is 10 μm). Frame width is 85 um, which is also marked in the figure legend.

Figure 5 has the scale bar added; frame size and scale bar information added to the figure legend.

13. Please sort the Table of Materials in alphabetical order.

Chemicals had been sorted in alphabetical order. We now, separately, sort equipment in alphabetical order, separately from chemicals.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The goal of this protocol is to prepare, purify and characterize gas-filled microbubbles, targeted contrast agents for ultrasound molecular imaging. The authors describe two targeting systems: biotinylated bubbles adherent to streptavidin and cyclic RGD peptide microbubbles that bind to αvβ3, a known tumor neovasculature biomarker. Very detailed description and explanation of the steps necessary for targeted microbubble preparation. In addition, methods to test bubble preparation and transfer of lipid materials.

Overall: This protocol will be very helpful with the microbubble and ultrasound community to have a well-documented protocol to making bubbles in the research lab. There are several bubble preparation or sizing bubble protocols previously published, but hard to replicate, if at all. The authors did a great job at detailing the steps throughout the steps. It is very detailed but sometimes hard to follow steps. I would encourage to go through the document one more time and make it a bit clearer in certain steps. If more sentences are needed to make it detailed, please add them. There are some parts not in the video footage. For a researcher getting started with the microbubble process for the first time, it would be great to have more portions shown in the video footage, if possible. Or even include footage you submit that could be added as supplementary. The notes documentation is great for the microbubble preparation throughout the document and for alternatives. This protocol could serve as a big stepping stone for a detailed microbubble fabrication document for many researchers.

We thank the reviewer for the positive assessment of our effort. In the revision we have tried to address all of the suggestions to the best of our ability.

Major Concerns:
Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol.

Thank you for noting these issues; numerous amendments were made as required.

Any text that cannot be written in the imperative tense may be added as a "Note." There are many sections throughout the document that just make statements. Please elaborate these areas. Line 136 -139: this is a little hard to read, please rewrite for clear message

Text edited to achieve better clarity and state the intended goal.

Line 372-373: This needs to be cited and does normal saline irrigation solution cause the bubbles to expand when being used to characterize the bubbles in the Counter?

The phenomena that take place following introduction of fluorocarbon bubbles into air-saturated medium have been discussed in detail since last century, starting with Van Liew (PMID:8828702) and Kabalnov (PMID: 9695277) - here we just suggest to minimize this potential source of a significant variability, because dependence on the level of dissolved air and temperature history of the medium on the bubble size can be drastic. Indeed, as suggested by the reviewer, degassing Coulter diluent saline and saturating it with perfluorocarbon gas will provide a somewhat different size distribution pattern (the number and volume of largest bubbles will drop). A separate systematic study may be considered separately from this manuscript, which is already close to the allowed page limit. Normally, for particle counting, regular air-saturated saline, equilibrated at room temperature, is used: some say that it would serve as a better mimic for in vivo following intravenous administration, when compared with degassed medium. Ideally, all bubbles should be of uniform size, which is not the current real use scenario. That is why we present here a simple flotation tool to remove largest microbubbles, so they will not grow further in any medium.

Line 418: cover the biomarker protein surface with what?

Thank you for suggesting the clarification. We want to warn the user to avoid covering the target receptor surface with silicon grease. Text amended to point at that.

Line 437-442: This portion is hard to understand of what the reader is to do as an alternative approach.

Thank you for the suggestion; text reworded to clarify.

Line 583-584: citation needed for bubbles lasting years in a sealed vial.

There is no formal citation, sorry, this is our own personal experience; we have adjusted the text to remove “years”. May need to perform a separate formal study to investigate long-term stability of microbubbles, if we obtain funding.

Figures
Figure 2: Both images have white boxes in bottom right. Was the scale not properly added or was corrupted in uploading? Lines 525 - 528 state fluorescence, upload the fluorescence image to differentiate between the fluorescence and brightfield

These white boxes with black marks in them are fragments of the microscopy image of the stage micrometer where vertical black lines represent the “ruler” with the ten-micrometer “step”. Figure legend expanded to clarify. The frame on the left is a fluorescence-only image. The frame on the right is brightfield-only image (same frame, taken several seconds later).

Figure 5: It is very blurry, unless the upload disrupted the quality. Please upload a clear image of the bubbles on the petri dish and add a scale.

Dynamic range of the camera resulted in high background level observed, following conversion to pdf format, and resulting fuzziness, now corrected by background adjustment. The width of the image is 106 um (information has been added to the figure legend), size bar added.

Figure 7: In the notes, the author described that increasing the MI to 1.9 would remove the bubbles. Please add an additional image that shows another panel for removing bubbles with high MI to compare with the streptavidin coated bubbles panel.

Third imaging frame added (now on the right). Figure legend amended to include.
Lines 53-55: cite the two published examples

Amended as suggested.

Minor Concerns:
Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Thank you for making this suggestion – we did an additional check.

Please sort the Materials Table alphabetically by the name of the material.

First part of the table is Equipment (now in alphabetical order). Then we have reagents – also in alphabetical order.

There are certain portions of the document that has common acronyms without spelling it out. Please look throughout the document to catch these.

Done.

The words coulter counter and Multisizer is used interchangeably but this can be confusing for the reader.

Both words have been removed and replaced with “electrozone sensing”, which is an established generic term for coulter counting.

In certain steps, it mentions notes. Please make sure to remove the notes or commentary into note sections. Line 254: Regarding the coulter counter, notes on using the coulter counter, add that it will be discussed in section 5 Microbubble size distribution assessment.

Text added as suggested.

Line 265 - 285: For the Microbubble counter steps. Use the term Particle sizing system as mentioned in your materials document. Multisizer and coulter counter are used interchangeably.

Amended as suggested.

List all centrifugation speeds in terms of centrifugal g-force instead of rpm. Avoid the use of commercial language, including ™/®/© symbols or company brand names before/after an instrument or reagent. Cite these in the Table of Materials instead.
Two mentions of rpm are not related to centrifugation: one is the rotation of the magnetic stirrer, and another is the movement of the amalgamator. Centrifugation description (yet another procedure) does report g-force value.

We have not used ™/®/© in the initial version of the manuscript. We have removed company and brand names from the text into the Materials spreadsheet.
Spell out acronyms and use proper symbols, here are a few examples found:
Lines 56: Spell out PEG

This has been spelled out in the original version of the manuscript, line 83-84

Lines 59: Spell out NHS-PEG-DSPE

This has been spelled out in the original version of the manuscript, line 83-84

Line 75: spell out c(RDGfK) -> cyclo[Arg-Gly-Asp-D-Phe-Lys]

Done.

Line 81: write epsilon as ε

Done.

Reviewer #2:
Manuscript Summary:
As discussed in the paper, microbubbles targeted to upregulated vascular proteins has been

pursued for multiple disease states over many years. This manuscript is of interest particularly as regards targeting using peptide ligands and the attachment of these to microbubbles. The description of microbubble manufacture and purifications of these as well as the in vitro assessment is generally well written, although there are a few typos etc that need correcting.

We thank the reviewer for the positive assessment of our effort. In the revision we have tried to address all of the suggestions to the best of our ability.

Major Concerns:
None

Minor Concerns:
1.4 could the authors add a brief description of creating the micellar solution of peptide-PEG-DSPE in saline (or biotin version)?

PEG-DSPE molecules turn into small spherical micelles upon addition of aqueous medium, by themselves, with sufficient time. Note N1.5 added for clarification.

1.5.2 needs to change to 260nm in last sentence.
Thank you for noticing this misprint! It is now corrected.

2.1.1, states that DSPC and PEG Stearate were co-dissolved in propylene glycol (PG, 10mg/ml for both). Could the authors clarify that the PG used at a neat concentration and that the 10mg/ml refers to the DSPC and PEG stearate?

It was indeed each of these components at 10mg/ml, in neat PG. The text has been amended to clarify.

Reviewer #3:
Manuscript Summary:
The authors have described the process of formulating targeted microbubbles with enough detail to make the procedure accessible to those without extensive experience in microbubble fabrication. This will be a valuable resource for anyone interested in formulating targeted or non-targeted microbubbles. The authors have taken care to point out procedural precautions that may not have been performed if they were not called to the reader's attention. I wish all protocols were written with this much attention to detail and I look forward to viewing the video protocol.

We thank the reviewer for the kind assessment of the manuscript and our effort.

Major Concerns:
The authors used the uptake of DiI into the microbubble shell to monitor the efficacy of lipid transfer. They cited the loss of 85% of DiI fluorescence of the unshaken lipid solution compared to the infranatant recovered after centrifuging microbubble suspensions as evidence that 85% of the available lipid in the solution was incorporated into the microbubble shell. The authors' prior publication (#6, doi:10.1021/acs.langmuir.8b03551) used fluorescently tagged lipids to assess the efficacy of lipid transfer and saw about 70% transfer to microbubble shells. Interpreting the result from the fluorescently tagged lipid experiments is straightforward but much more expensive than using DiI dye. Interpreting the DiI dye experiment is a little more complicated since fluorescence of this dye is enhanced when it is incorporated into a lipid environment. Do the authors have additional data to validate the use of DiI dye for assessing the efficacy of lipid transfer? (My concern is that DiI fluorescence could be enhanced by lipid micelles in the lipid solution or by nano bubbles remaining in the infranatant - complicating interpretation of these results.) If it's simple to explain the validity of using DiI, please add text as appropriate. Otherwise, let the

reader know that 70% lipid transfer was observed in Ref #6 and that the DiI method provided a similar measure.

Thank you for pointing at this difference. DiI (as all of the bis-C18-lipid cyanine dyes in the family, from DiO to DiR) have a moderate size fluorescence molecule, outfitted with two stearyl residues. So from physical chemistry standpoint, such as solubility in water, DiI is similar to diacyl lipid with similar C18 fatty residues. Reference 6 (now Reference 5) study describes transfer of a different molecule, not the basic phospholipid or labeled dye, but a mimic of ligand-PEG-DSPE, namely, fluorescein-PEG5000-DSPE, which has a somewhat higher critical micellar concentration (i.e., solubility of individual molecules in water) than DiI or phospholipids. Therefore, it is not surprising that PEG-lipids will transfer to the shell to a somewhat lower extent. The validity of fluorescence measurement performed with DiI in this study had been achieved by the selection of the fluorescence measurement medium, as outlined in Section 3.3 (lines 219-223 of the first version of the manuscript): PBS:ethanol 1:1, with 1% Triton X-100. In these conditions, with large excess of Triton X-100 and presence of ethanol, all lipid molecules are disassociated, all membrane structures or potential nanobubbles are destroyed, so the dye molecules cannot be subjected to potential concentration quenching anymore. All measurements, prior to or after the separation of bubbles from the preparation medium are made in these conditions.

Minor Concerns:
1) In the description of the parallel-plate flow apparatus, please include the name of the manufacturer (Glycotech) otherwise a reader may expect more technical details to be included so that they can reproduce this apparatus.

Thank you for suggesting this clarification. As the author instructions require, the name of the parallel plate plug manufacturer could not be present in the text. It is present in the Table of Materials.

.
2) In Figure 6, please provide complete units for the Y-axis. Was this microbubbles per mm^2, per video frame, per 35mm plate?

Figure 6 Y-scale represents the number of particles in the field of view following four minutes of accumulation time. Figure legend is now slightly amended to clarify.

Permission for figure from Ref 5

Click here to access/download;Supplemental File (Figures, Permissions, etc.);renamed_20510.pdf

Formation of Microbubbles for Targeted Ultrasound Contrast Imaging: Practical Translation Considerations

Author: Sunil Unnikrishnan, Zhongmin Du, Galina B. Diakova, et al

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