Multifunctional microbubbles for image-guided antivascular endothelial growth factor therapy

Leilei Zhang, Jeff S. Xu, Virginia M. Sanders, Alan D. Letson, Cynthia J. Roberts, and Ronald X. Xu

Abstract. We synthesize multifunctional microbubbles (MBs) for targeted delivery of antivascular endothelial growth factor (antiVEGF) therapy with multimodal imaging guidance. Poly-lactic-co-glycolic acid (PLGA) MBs encapsulating Texas Red dye are fabricated by a modified double-emulsion process. Simultaneous ultrasound and fluorescence imaging are achieved using Texas Red encapsulated MBs. The MBs are conjugated with Avastin, an antiVEGF antibody for treating neovascular age-related macular degeneration (AMD). The conjugation efficiency is characterized by enzyme-linked immunosorbent assay (ELISA). The efficiency for targeted binding of Avastin-conjugated MBs is characterized by microscopic imaging. Our work demonstrates the technical potential of using multifunctional MBs for targeted delivery of antiVEGF therapy in the treatment of exudative AMD.

Keywords: microbubble; age-related macular degeneration; fluorescence; ultrasound; multimodal imaging; Avastin; bevacizumab; drug delivery.

This work reports our recent effort on synthesizing multifunctional PLGA MBs for targeted pharmacotherapeutics of neovascular AMD. Many drug-delivery devices have been synthesized using biocompatible and biodegradable materials, such as liposome, albumin, polylactide (PLA), and poly-lactic-co-glycolic acid (PLGA). PLGA has been approved by the FDA for implantation applications, and is now widely investigated as a biodegradable carrier for controlled drug release. We have previously encapsulated and characterized fluorescence dyes in PLGA microbubbles (MBs) and nanobubbles (NBs) for multimodal imaging using different modalities such as fluorescence (FL), ultrasound (US), and photoacoustic tomography (PAT). We have also modified and conjugated PLGA MBs and NBs for cancer targeting and imaging. Similar fabrication and conjugation techniques can be applied to synthesize drug-loaded microdevices for image-guided antiVEGF therapy.

Address all correspondence to: Ronald X. Xu, Tel: 614-688-3635; Fax: 614-292-7301; E-Mail: xu.202@osu.edu

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were filled in three centrifuge tubes respectively for FL and US imaging. An additional centrifuge tube filled with water was used as the control. FL images were acquired using a Maestro EX Fluorescence imaging system (CRi, Woburn, Massachusetts) at the excitation wavelength of 590 nm and an emission wavelength of 610 nm, as shown in Fig. 2(a). Immediately after FL imaging, US images were acquired by a Vevo 2100 US system (VisualSonics Incorporated, Toronto, Canada) with a 20-MHz transducer, as shown in Fig. 2(b). FL and US image intensities were estimated at different MB concentrations, as plotted in Fig. 3. Linear correlations were observed between the FL intensity and the MB concentration \( R^2 = 0.960 \) and between the US intensity and the MB concentration \( R^2 = 0.991 \), indicating the technical potential of using FL and US imaging for doximetric assessment and control.

The carboxylic acid terminated PLGA MBs were surface modified and Avastin conjugated for VEGF targeting and drug delivery. Briefly, MBs were dispersed in a 2-(N-morpholino)ethanesulfonic acid (MES) buffer (0.1 M, pH = 5.6) at concentrations of 5, 2.5, and 1.25 mg/mL, respectively. Then, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) was added into MBs suspensions to a final concentration of 2 mM. Immediately after that, N-hydroxy succinimide (NHS) was added to a final concentration of 5 mM. The suspensions were stirred slightly for 15 min and washed by MES buffer three times. The precipitates were then collected and redispersed in phosphate buffered saline (1 \( \times \) PBS, pH = 7.4). To conjugate Avastin with NHS-ester active MBs, Avastin (Genetech, San Francisco, California) was added into the MB suspension to a final concentration of 167 \( \mu \)g/mL. After that, the suspension was stirred slightly at room temperature for 2 h followed by centrifugation at 5000 rpm for 5 min. The supernatant was collected for enzyme-linked immunosorbent assay (ELISA) assessment of the conjugation efficiency, while the precipitate was collected and redispersed in 1 \( \times \) PBS for further binding tests.

The Avastin conjugation efficiency was determined by an indirect ELISA method, where the concentration of supernatant Avastin without reactivation with MBs was measured. The conjugation efficiency was derived by subtracting the supernatant Avastin concentration from the total concentration. Briefly, the 96-well ELISA plate (Fishers) was first coated with 15-\( \mu \)g/mL monoclonal antihuman IgG1 (Fab specific) antibody (Sigma, Saint Louis, Missouri) in 50-mM carbonate buffer (pH = 9.6) at room temperature overnight, and then blocked by 1% bovine serum albumin (BSA) for 1 h. The bound Avastin was detected by using an antihuman IgG antibody (Fc specific) with alkaline phosphatase (Sigma) on a SpectraMax Plus microplate reader (Molecular Devices, Sunnyvale, California). Figure 4 shows the standard ELISA curve fitted by 4-P logistic regression \( R^2 = 0.996 \). The Avastin conjugation efficiencies at MB concentration levels of 1.25, 2.5, and 5 mg/mL are 70.22, 47.20, and 57.35%, respectively, as shown in Fig. 4.

The ELISA plate was also used to test the targeted binding efficiency of Avastin conjugated MBs. The monoclonal antihuman IgG1 (Fab specific) antibody was first incubated in the wells of the ELISA plate overnight and then blocked by 1% BSA. Instead of using the supernatant, the redispersed MBs after conjugation were incubated in the wells at concentration levels of 5, 2.5, and 1.25 mg/mL, respectively. Meanwhile, the control MBs without Avastin conjugation were also incubated at the same concentration levels in different wells of the same plate. After an overnight incubation at room temperature, the plate was washed six times with 0.05% Tween-20 in 1 \( \times \) PBS and then imaged by an inverted microscope. MB distributions for both the samples and the controls were estimated at three concentration levels. The density of distributed MBs was then averaged in six randomly selected regions of interests for each test case. As shown in Fig. 5, the Avastin conjugated MBs (sample) have greater targeted binding effi-

![Fig. 1](image1.png)  
Fig. 1 A typical SEM image of Texas Red encapsulated MBs.

![Fig. 2](image2.png)  
Fig. 2 FL and US images of Texas Red encapsulated MBs stored in different centrifuge tubes. The MB concentrations from left to right are: 1. 5 mg/mL, 2. 2.5 mg/mL, 3. 1.25 mg/mL, and 4. 0 mg/mL. (a) FL images of the centrifuge tubes (front view). (b) The corresponding US images (cross sectional view).

![Fig. 3](image3.png)  
Fig. 3 FL intensities (red dots) and US intensities (blue rectangles) of Texas Red encapsulated MBs at the following MB concentration levels: 0, 1.25, 2.5, and 5 mg/mL. (Color online only.)
The targeted binding efficiency for Avastin conjugated MBs is significantly higher than that for bare MBs at the MB concentration level of 5 mg/mL.

In summary, we synthesize a multifunctional MB agent that can potentially be used for image-guided anti-VEGF therapy in exudative AMD. Our next step is to encapsulate biodegradable dyes such as indocyanine green (ICG) in MBs for in vitro and in vivo validation of this image-guided drug delivery schema. Since microbubbles have strong scattering contrast for optical coherence tomography (OCT), it is also possible to use OCT for image-guided intravitreous delivery of drug-loaded MBs. In addition to drug-loaded MBs, we also fabricate MBs that can be activated by an external energy source. The MB targeting mechanism described in this work may facilitate the sustained release of anti-VEGF agents by sequential ultrasound sessions in the later time.

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