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The Lifetime and Attenuation Properties Measurements of a US/MR Multimodality Molecular Probe*

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Abstract—**In our previous studies we explored the potential of using a combined US/magnetic resonance (MR) multimodality contrast agent, albumin-gadolinium-diethylenetriamine-pentacetate (Gd-DTPA) MBs, to induce BBB opening and for distinguishing between FUS-induced BBB opening and intracerebral hemorrhage in MR T1-weighted contrast imaging. According to the previous study in the literature, 1–2 μm bubbles have more pronounced acoustic activity at frequencies above 10 MHz. The present study developed a new targeted US/MR multimodality MB and the acoustic properties were compared with two commercial MBs, SonoVue and Targestar SA. The acoustic activities of these 1.15-2.78 μm MBs with different shells at 10 MHz were investigated. The feasibility of designing a new targeted US/MR multimodality MB was investigated. The lifetime (survival of MBs in the liquid suspension) and attenuation properties of lipid MBs (SonoVue and Targestar SA), albumin-(Gd-DTPA) MBs, and avidin-conjugated albumin (avidin-albumin)-(Gd-DTPA) MBs at 10 MHz were investigated with the pulse-echo substitution method. It was found that incorporating avidin into the albumin MBs and avidin-albumin-(Gd-DTPA) MBs affects the size distribution but does not affect the concentration of MBs produced. The avidin-albumin-shelled MBs had more significant nonlinear activity at 4-18 MHz (*p=0.025*), while the nonlinear activity of the other MBs peaked at 6-24 MHz (*p=0.003-0.044*). Moreover, the incorporation of paramagnetic metal ions into the MB shells increased their attenuation coefficients. With regard to the lifetime of these agents, the attenuations of the SonoVue and Targestar SA lipid MBs were 87.96% and 8.74%, respectively, while those of albumin MBs, avidin-albumin MBs, albumin-(Gd-DTPA) MBs, and avidin-albumin-(Gd-DTPA) MBs were 49.52%, 41.38%, 74.69%, and 100%, respectively. Avidin conjugation decreased the lifetime of the albumin MBs, but not that of the lipid MBs. The incorporation of paramagnetic metal ions into the shells of albumin MBs did not decrease the lifetime.**

I. Introduction

Focused ultrasound (FUS)-enhanced local brain drug delivery relies on the intravenous administration of microbubbles (MBs) and interaction with ultrasonic energy so that the blood–brain barrier (BBB) can be temporarily disrupted. In most previous studies, BBB opening was confirmed with contrast-enhanced T1-weighted magnetic resonance (MR) imaging at targeted locations [1,2]. For malignant glioma, FUS is a recently discovered noninvasive technique that shows great promise for local and reversible enhancement of the permeability of the BBB to chemotherapeutic agents [3]. Molecular imaging for tumor angiogenesis (the growth of new blood vessels) is very important because angiogenesis is promoted in the early stage of tumor growth and plays an important role in tumor growth, invasiveness, and metastatic potential [4]. Ultrasound (US) molecular imaging has been conducted with lipid-shelled MBs targeted to vascular endothelial growth factor receptor 2 (VEGFR2), $\alpha_v\beta_3$ integrin, and endoglin on the tumor vessel [5-7]. In our previous study we evaluated albumin-shelled gadolinium(Gd)-diethylenetriaminepentacetate (Gd-DTPA) MBs that can concurrently serve as a dual-modality contrast agent for US and MR imaging to assist BBB opening and detect intracerebral hemorrhage during brain FUS drug delivery [8]. In previous study in the literature [9], the acoustic activity is most pronounced at lower diagnostic frequencies (<10 MHz) with a mean bubble size of 6.8 μm. It also demonstrated a strong influence of bubble size on high frequency attenuation curves, with bubble diameters of 1–2 μm and below having more pronounced acoustic activity at frequencies above 10 MHz. For BBB opening, the pressure threshold of lipid bubbles, determined by the MRI contrast enhancement, was 0.45 MPa for the 1–2 μm and 0.30 MPa for both the 4–5 and 6–8 μm bubbles [10]. However, the connections between different components of bubble shells and BBB opening are still unclear. The acoustic activities of MBs with the diameters ranged from 1 to 2.8 μm were measured by a wide bandwidth 10 MHz ultrasound probe. The properties of the new targeted US/MR multimodality MBs were investigated and compared with commercial lipid MBs and targeted lipid MBs.

II. Methods

A. Production of avidin-albumin-(Gd-DTPA) MBs

For avidin-albumin-(Gd-DTPA) MBs, 2 ml of albumin-DTPA solution (albumin, 70 mg/ml) was treated with 1 ml of $GdCl_3$ solution (100 mg/ml) and then stirred for 24 hours at 4°C. Perfluorocarbon-filled albumin-(Gd-DTPA) MBs were generated by sonicating 5 ml of the solution containing albumin-(Gd-DTPA), 4 mg of avidin (Pierce Biotechnology, Rockford, IL, USA), and perfluorocarbon gas in phosphate-buffered saline (PBS) using a US sonicator (Branson, Danbury, CT, USA) for 2 min. The avidin-albumin-(Gd-DTPA) MBs were centrifuged (1200 rpm, 128.6×*g*) and then washed three times to eliminate

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the free Gd^{3+} ions and avidin. After the washing process, the number of perfluorocarbon-filled albumin-(Gd-DTPA) MBs in the solution was measured with the MultiSizer III device (Beckman Coulter, Fullerton, CA, USA) using a 30-µm-aperture probe with measurement boundaries of 0.6–20 µm. The size distribution in the suspension was measured by dynamic light scattering (Nanoparticle Analyzer, Horiba, Kyoto, Japan). Surface bioconjugation extends the contrast agents to molecular probes. Flow cytometry was used to analyze the binding of the fluorescein isothiocyanate (FITC)-biotin to the albumin-(Gd-DTPA) MBs and avidin-albumin-(Gd-DTPA) MBs. The biotinylated FITC was then incubated with avidin-albumin-(Gd-DTPA) MBs for 30 min to produce FITC-labeled albumin-(Gd-DTPA) MBs. The produced contrast agents were washed three times to ensure that any unbound biotinylated FITC was removed.

B. Measurements of avidin bound to albumin MBs or albumin-(Gd-DTPA) MBs

The enzyme-linked immunosorbent assay (ELISA) provides an efficient means of screening the amounts of avidin incorporated into the albumin shell of MBs. Before performing the ELISAs, the avidin-conjugated MBs and the avidin-albumin-(Gd-DTPA) MBs were irradiated with US energy with the 1-MHz transducer of the sonoporation gene-transfection system (ST 2000V, NepaGene, Chiba, Japan) at an acoustic pressure of 3 W/cm^2 for 1 min to destroy the MBs. The concentrations before and after destruction were measured using the MultiSizer III device (Beckman Coulter); this process destroyed 95% of the MBs. Avidin, avidin-MB fragments, or the avidin-albumin-(Gd-DTPA) MB fragments at various concentrations in 0.1 M carbonate buffer (pH 9.6) were coated onto the 96-well plates and left at 4°C overnight. The plates were washed three times with PBS containing 0.05% Tween-20, removing the excess liquid as described above. Blocking buffer (200 µl) was added and incubated for 1 hour at room temperature. The plate was then washed three times, 100 µl of primary antibody (mouse antiavidin monoclonal antibody; A3G7, GeneTex, Irvine, CA, USA) was added to each well, and the plate was then incubated for 2 hours at room temperature. The plate was washed three times, 100 µl of secondary antibody (peroxidase-conjugated antimouse IgG; NA931, GE Healthcare, New York, NY, USA) was added to each well, and the plate was then incubated for 1 hour at room temperature. The appropriate enzyme substrate (100 µl; NeA-Bule, Clinical Science Products, Mansfield, MA, USA) solution was then added, and the plate was incubated at room temperature for 30 min or until sufficient color developed. After further washing, 50 µl of the appropriate stop solution (H_2SO_4, Sigma-Aldrich, St. Louis, MO, USA) was added. Colorimetric measurements of avidin were performed at 450 nm using a microplate spectrophotometer (Model 680, Bio-Rad, Hercules, CA, USA). A standard avidin calibration curve was constructed to obtain the corresponding concentration of avidin in avidin-albumin MBs or avidin-albumin-(Gd-DTPA) MBs from the measured absorption peaks. Triplicate measurements were performed for each concentration of avidin.

C. Overview of the measurement system

The lifetime of MBs is defined as their survival time within the liquid suspension. Lifetime and attenuation measurements were made using the pulse-echo substitution method [9], as shown in Fig. 1. The MBs were held in a sample chamber (50 x 15 x 25 mm) located within the beam of a 10-MHz broadband transducer (V311, Panametrics, Waltham, MA, USA) that entered the chamber and was then reflected off an aluminum plate located at the focus. The transducer with −6-dB fractional bandwidth of 54.94% is in the frequency range from 7.1-12.47 MHz. The excitation pulses have a pulse length of 0.498-µs. According to the concentration measurements listed in Table 1, all of the samples were adjusted to the same concentration (2×10^7/ml). Diluted solutions (0.1%) of lipid MBs (SonoVue), 0.02% streptavidin-conjugated lipid MBs (Targestar SA), 0.0067% albumin MBs, 0.0077% avidin-albumin MBs, 0.008% albumin-(Gd-DTPA) MBs, and 0.016% avidin-albumin-(Gd-DTPA) MBs were mixed with a magnetic stirring bar and then placed into the sample chamber. An arbitrary-waveform generator (WW2571, Tabor Electronics, Tel Hanan, Israel) was used to excite the transducer, and a pulser/receiver (5073PR, Olympus, Tokyo, Japan) amplified the received signal. The attenuation and lifetime properties of different MB solutions were measured at 50-ms intervals until 5 min and at 1.08-s intervals until 180 min, respectively. The US pressure amplitude was set at 0.28 MPa. All amplitudes shown in the figure of lifetime are normalized to the background noise. And then, the signals were normalized to the amplitude of each sample after MB destruction. The MBs were destroyed with the sonoporation gene-transfection system (ST 2000V, NepaGene, Chiba, Japan) at an acoustic intensity of 3 W/cm^2 for 1 min for amplitudes normalization because the amplitudes of lipid shells and albumin shells were different.

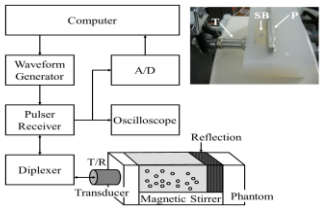

Figure 1. (a) Schematic overview of the hardware employed. (b) Photograph of one of the stirring bars (SB) in the sample chamber; the transducer (T) and reflective steel plates (P) are indicated.

Table 1. Diameters, concentrations, and avidin concentrations of the various MBs used in this study. Data are mean and SD values.

Agent	Diameter (µm)	Concentration (per ml)	Avidin concentration (ng/ml)
SonoVue	2.50	$2-5\times10^8$	N/A
Targestar SA	2.50	1.00×10^9	N/A
MBs	1.15±0.30	$3.18\pm0.15\times10^9$	N/A
Av-MBs	2.29±0.86	$2.64\pm0.05\times10^9$	3700
Gd-MBs	2.78±0.67	$2.51\pm0.07\times10^9$	N/A
Av-Gd-MBs	1.90±0.53	$1.25\pm0.05\times10^9$	2100

III. MATH

A. Attenuation in Solution

The US attenuation of the solutions relative to water was calculated using the following equation:

$$a(f) = \frac{-20}{2L}\log_{10}\frac{|A_s(f)|}{|A_w(f)|}\,dB\ mm^{-1}, \quad (1)$$

where $A_s(f)$ and $A_w(f)$ are the magnitude spectra of the reflected signal from the quartz in the presence and absence, respectively, of a sample of thickness L of the MB solution in the propagation path of the US pulse. In this study the propagation path was the focus depth of the transducer (2.54 cm). $A_w(f)$ and $A_s(f)$ were calculated using MATLAB (R2010b, MathWorks, Natick, MA, USA).

B. Statistical analysis

The obtained data were analyzed statistically using Student's *t*-test. A probability value of $P<0.05$ was considered indicative of a significant difference.

IV. RESULTS AND DISCUSSIONS

The size distributions and concentrations of the albumin MBs, avidin-albumin MBs, albumin-(Gd-DTPA) MBs, and avidin-albumin-(Gd-DTPA) MBs in a suspension are shown in Figs. 2 and 3 (n=5). The mean concentrations of albumin MBs, avidin-albumin MBs, albumin-(Gd-DTPA) MBs, and avidin-albumin-(Gd-DTPA) MBs were 3.18×10^9/ml, 2.64×10^9/ml, 2.51×10^9/ml, and 1.25×10^9/ml, respectively (Table 1). They ranged in size from 0.8 to 18 μm (as measured using the electrical sensing zone system; Figs. 2b and 3b), and their mean diameters were 1.15, 2.29, 2.78, and 1.90 μm, respectively (Figs. 2b and 3b). ELISA revealed that the avidin-albumin MBs and avidin-albumin-(Gd-DTPA) MBs prepared in this study contained approximately 12620 and 15080 avidin molecules per bubble shell area, respectively. As given in Table 1, the loading efficiencies of avidin in avidin-albumin MBs and avidin-albumin-(Gd-DTPA) MBs were 9.25% and 6.25%, respectively. The coating efficiency of MBs shells may influence the measurement of the ELISA-determined amount of avidin present.

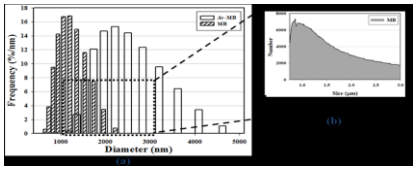

Figure 2. Size (a) and concentration (b) distributions of the albumin MBs (MB) and avidin-albumin MBs (Av-MB) in suspensions measured by dynamic light scattering (DLS) and electrical sensing zone (ESZ), respectively.

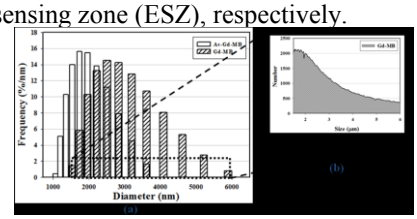

Figure 3. Size (a) and concentration (b) distributions of the albumin-(Gd-DTPA) MBs (Gd-MB) and avidin-albumin-(Gd-DTPA) MBs (Av-Gd-MB) in suspensions as measured by DLS and ESZ.

Fig. 4 shows example histograms of the fluorescence intensity (FI) with and without avidin conjugation for albumin MBs (as the control group; Fig.4a) and albumin-(Gd-DTPA) MBs (Fig. 4b). An increased mean FI, as indicated by a shift of the distribution to the right, confirms more ligand binding of biotin-FITC on the MB surface. The increase in FI with FITC was greater for albumin MBs and albumin-(Gd-DTPA) MBs conjugated with avidin. These results indicate that avidin can be incorporated into the shell of albumin MBs and albumin-(Gd-DTPA) MBs during sonication, and can serve as a direct ligand for biotinylated antibodies. Quantitative analysis revealed that the geometric mean FI was more than twofold higher for avidin-albumin MBs (521.31±57.13) than for nontargeted albumin MBs (258.02±22.03). The geometric mean FI was more than 2.5-fold higher for avidin-albumin-(Gd-DTPA) MBs (414.47±13.21) than for nontargeted albumin-(Gd-DTPA) MBs (161.53±27.12).

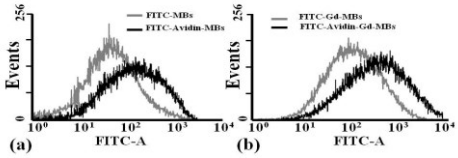

Figure 4. Typical flow-cytometry fluorescence intensity histograms of (a) albumin MBs and (b) albumin-(Gd-DTPA) MBs with and without avidin conjugation.

Figure 5 shows the attenuation coefficients of SonoVue, Targestar SA, albumin MBs, avidin-albumin MBs, albumin-(Gd-DTPA) MBs, and avidin-albumin-(Gd-DTPA) MBs at different frequencies. Although the 10MHz transducer with −6-dB fractional bandwidth of 54.94% is in the frequency range from 7.1-12.47 MHz. Fig. 5 was arranged from 0-30 MHz to help the observations of the changes in the spectrums among different type MBs. The attenuations of albumin MBs, albumin-(Gd-DTPA) MBs and avidin-albumin-(Gd-DTPA) MBs increased steeply from 1 to 6 MHz, decreased gradually from 6 to 18 MHz. The attenuations of avidin-albumin MBs increased steeply from 1 to 4 MHz, decreased gradually from 4 to 24 MHz. The attenuations of SonoVue and Targestar SA increased steeply from 1 to 6 MHz, decreased gradually from 6 to 24 MHz. The attenuation peaks of SonoVue, Targestar SA, albumin MBs, albumin-(Gd-DTPA) MBs and avidin-albumin-(Gd-DTPA) MBs occurred at around 5–10 MHz. The attenuation for avidin-albumin MBs exhibited a diffuse peak in the range of 4–8 MHz, remaining high until 16 MHz. Goertz et al. (2006) found that small bubble populations improved the activity at high frequencies. In the present study, although the sizes of all of the samples were 1.0–2.5 μm, the nonlinear activity was more significant for avidin-albumin-shelled MBs at 4–18 MHz ($p=0.025$) and for the other MBs at 6–24 MHz ($p=0.003$-0.044). Moreover, the attenuation coefficients were higher for MBs incorporated with paramagnetic metal ions than for those without paramagnetic metal ions ($p<0.05$). The shell properties thus influenced the attenuation measurements.

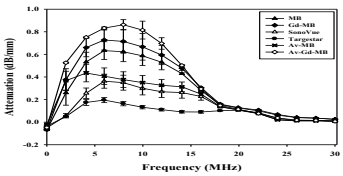

Figure 5. Attenuation coefficients of SonoVue, Targestar SA, albumin MBs (MB), avidin-albumin MBs (Av-MB), albumin-(Gd-DTPA) MBs (Gd-MB), and avidin-albumin-(Gd-DTPA) MBs (Av-Gd-MB) at different frequencies. Data are mean and SD values.

Soetanto et al. (2000) reported that MBs coated with surfactants are less soluble and have longer a lifetime. Borrelli et al. (2012) demonstrated that albumin MBs can be stored from 2 weeks to one year depending on their formulation. In the present study, the lifetime of MBs was influenced by the composition of the shell. The results for SonoVue, Targestar SA, albumin MBs, avidin-albumin MBs, albumin-(Gd-DTPA) MBs, and avidin-albumin-(Gd-DTPA) MBs within a 3-hour period are summarized in Fig. 6 (n=3). Initially the signal intensities of Targestar SA, albumin-(Gd-DTPA) MBs, and avidin-albumin-(Gd-DTPA) MBs were stronger. Except the lipid molecular probe (Targestar SA) ($p=0.05$), the signal amplitudes of the albumin molecular probes (avidin-albumin MBs and avidin-albumin-(Gd-DTPA) MBs) were significant decrease ($p<0.001$, $p=0.0012$) (Table 2). For lifetime measurements, the decay of signal at 180 mins was much lower for albumin MBs (49.52%) than for the lipid MBs (SonoVue, 87.96%). Hence, the lifetime was longer for the laboratory-made albumin MBs than for the commercially available lipid MBs. However, the attenuations of Targestar SA, avidin-albumin MBs and avidin-albumin-(Gd-DTPA) MBs were 8.74%, 74.69%, and 100%, respectively. Avidin conjugation clearly influenced the lifetime of the albumin MBs. The incorporation of paramagnetic metal ions into the shells of albumin MBs also decreased the lifetime of albumin MBs (41.38%).

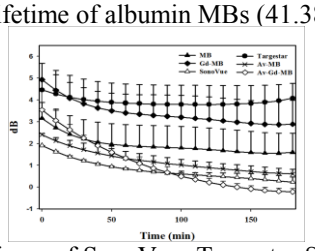

Figure 6. Lifetimes of SonoVue, Targestar SA, albumin MBs (MB), avidin-albumin MBs (Av-MB), albumin-(Gd-DTPA) MBs (Gd-MB), and avidin-albumin-(Gd-DTPA) MBs (Av-Gd-MB) within a 3-hour period. Data are mean and SD values.

Table 2. US signal changes of various MBs from Fig. 6.

Agent	Initial intensity (dB) (0 min)	Final intensity (dB) (180 mins)	Decay (%)	P value
SonoVue	1.91±0.01	0.23±0.24	87.96	<0.001
Targestar SA	4.46±1.21	4.07±0.68	8.74	0.05
MBs	3.15±0.74	1.59±0.88	49.52	0.04
Gd-MBs	4.93±0.75	2.89±1.10	41.38	0.028
Av-MBs	2.41±0.054	0.61±0.21	74.69	<0.001
Av-Gd-MBs	3.54±0.95	0±0.14	100	0.0012

V. Conclusion

The attenuation and lifetime properties of lipid MBs, lipid US molecular probes, paramagnetic-metal-ion-incorporated albumin MBs, albumin molecular MBs, and paramagnetic-metal-ion-incorporated albumin US molecular probes are reported herein. The activity of the avidin-albumin-shelled MBs was most significant at 4–18 MHz. The attenuation coefficients of the MBs were increased by incorporation with paramagnetic metal ions. For lifetime measurements, the decay of albumin MBs at 180 mins was less than SonoVue. Avidin conjugation reduced the lifetime of albumin MBs but not of Targestar SA. Incorporating paramagnetic metal ions into the shells of albumin MBs did not influence their lifetime.

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