Measurements of Lifetime and Attenuation Properties of Ultrasound/Magnetic resonance Multimodality Molecular Probe

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Abstract

It has been shown that microbubble (MB)-enhanced focused ultrasound (FUS) temporally and locally disrupts the blood-brain barrier (BBB), thereby enhancing drug delivery into brain tumors. Imaging tumor angiogenesis with contrast-enhanced ultrasound (US) has been explored with targeted MBs. The BBB opening threshold and BBB opening volume were found to be bubble-size-dependent. However, the relationships between the various components of bubble shells and BBB opening are still unclear. According to a previous study, 1-2 μm bubbles have the most pronounced acoustic activity at frequencies above 10 MHz. The present study developed targeted US/magnetic resonance (MR) multimodality MBs, whose acoustic properties were compared with two commercial MBs, namely Sonovue and Targestar SA. The acoustic activities of these 1.15-2.78 μm MBs with different shells at 10 MHz were investigated. The lifetime 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 MB shells and 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), whereas 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 were 87.96% and 8.74%, respectively, and those of albumin, avidin-albumin, albumin-(Gd-DTPA), 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 their lifetime.

Keywords: Multimodality molecular probe, Albumin-(Gd-DTPA) microbubbles, Avidin-albumin-(Gd-DTPA) microbubbles, Attenuation, Lifetime

1. 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 developed noninvasive technique that shows great promise for local and reversible enhancement of the permeability of the BBB to chemotherapeutic agents [3,4]. 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 [5]. Ultrasound (US) molecular imaging has been conducted with lipid-shelled MBs targeted to vascular
endothelial growth factor receptor 2 (VEGFR2), α,β1 integrin, and endoglin on the tumor vessel [6-8]. Our previous study evaluated albumin-shelled gadolinium (Gd)-diethylene-triaminepentaacetate (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 [9]. In a previous study [10], acoustic activity was found to be most pronounced at lower diagnostic frequencies (<10 MHz), as may be expected with a mean bubble size of 6.8 μm. A strong influence of bubble size on high-frequency attenuation curves was also found, 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 MR imaging contrast enhancement, was 0.45 MPa for 1.2 μm bubbles and 0.30 MPa for both 4-8 μm bubbles [11]. However, the relationships between the various components of bubble shells and BBB opening are still unclear.

In the present study, the acoustic activities of MBs with diameters ranging from 1 to 2.8 μm were measured by a widebandwidth 10 MHz ultrasound probe. The properties of the targeted US/MR multimodality MBs were investigated and compared with those of commercial lipid MBs and targeted lipid MBs.

Gas-filled echogenic MBs are used clinically as the contrast agent for US. Lipid MBs are currently the most commonly used form of contrast agent in ultrasonography [12]. Lipid-coated MBs with Gd(III)-bound shells have recently been used as cavitation probes for MR-imaging-guided FUS therapy applications [13]. Gd (III) can be loaded into the liposomal aqueous core and/or conjugated to the lipid polar head groups in the bilayer [14-16]. Surface conjugation resulted in greater T1-weighted MR relaxation enhancement than that obtained using encapsulation, owing to the greater access of bulk water protons to the Gd(III) ions [13,15]. In addition to Gd(III)-bound lipid MBs, lipid-shelled targeted MBs have also been conjugated with N-succinimidyl-4-[19F]fluorobenzoate-radiolabeled antibodies for micro-position-emission tomography multimodality molecular imaging [8]. The US imaging of tumor angiogenesis with lipid MBs targeted to VEGFR2 has also been explored [6]. Polymer-shelled MBs conjugated with iron oxide (Fe₃O₄) or Gd were recently demonstrated to be an effective multimodality contrast agent for both US and MR imaging [17-19]. It was shown that polymer MBs with embedded Fe₃O₄ could be controlled the release from shells of MBs into cells by sonoporation [20].

Albumin-shelled MBs such as Optison are presented as a suspension of perfluoropropane-filled albumin microspheres with a mean concentration of 5.0-8.0 x 10⁸ microspheres/ml and a mean diameter of 2.0-4.5 μm [21]. MBs of up to 32 μm in diameter are present when preparing Optison, which might be too large to reach peripheral tissues [22]. However, albumin is an important proteinaceous microcapsule that may be modified for biomedical imaging purposes. In addition, the effects of US and albumin-shelled MBs on transfection in vitro for various culture medium heights and the related dynamic behaviors of cavitation bubbles have been investigated [23]. Some drugs have been encapsulated within albumin microspheres [24].

The preparation and properties of sonochemically produced proteinaceous microspheres have been investigated [25]. Some studies have found that local inflammation and angiogenesis can be detected by incorporating the targeting ligands in albumin-shelled MBs [7]. Albumin-shelled MBs can be used as US contrast agents in the clinical setting. A paramagnetic-labeled macromolecule, albumin-(Gd-DTPA), can be used as an MR imaging contrast agent. Our previous study incorporated Gd(III) into albumin-shelled MBs and explored the potential of using these albumin-(Gd-DTPA) MBs for inducing BBB opening and for distinguishing between FUS-induced BBB opening and intracerebral hemorrhage in MR contrast imaging [9].

The present study evaluates the attenuation and lifetime (survival of MBs in the liquid suspension) properties of the targeted US/MR multimodality contrast agent avidin-conjugated albumin (avidin-albumin)-(Gd-DTPA) MBs. The characteristics of the US/MR multimodality molecular imaging probe were investigated and compared with those of commercial US molecular imaging probes. An albumin-shelled MB/US multimodality molecular imaging probe was first produced, the characteristics of which have already been demonstrated [26]. Albumin-shelled MBs, multimodality albumin-shelled MBs, and avidin-incorporated multimodality albumin-shelled MBs (avidin-albumin MBs) with mean diameters of 1.15-2.78 μm were evaluated. The characteristics and acoustic properties of the albumin MBs were measured and compared with those of commercial lipid MBs (SonoVue, Bracco, Milan, Italy) and streptavidin-conjugated lipid MBs (Targestar SA, Targeson, San Diego, CA, USA).

2. Methods

2.1 Production of avidin-albumin-(Gd-DTPA) MBs

Perfluorocarbon-filled albumin, avidin-albumin, and albumin-(Gd-DTPA) MBs were prepared according to previously described methods (Figs. 1(a)-(c)) [9,27,28].

![Figure 1. Schematic diagrams of (a) albumin MBs, (b) avidin-albumin MBs, (c) albumin-(Gd-DTPA) MBs, and (d) avidin-albumin-(Gd-DTPA) MBs.](image-url)
Briefly, for avidin-albumin-(Gd-DTPA) MBs, 2 ml of albumin-DTPA solution (albumin, 70 mg/ml) was treated with 1 ml of GdCl₃ 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 a 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 the free Gd³⁺ ions and avidin (Fig. 1(d)). 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 limits 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 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.

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

The measurement protocol is shown in Fig. 2. 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 avidin-albumin-(Gd-DTPA) MBs were irradiated with US energy with the 1-MHz transducer of a sonoporation gene transfection system (ST 2000V, NepaGene, Chiba, Japan) at an acoustic pressure of 3 W/cm² for 1 min to destroy the MBs (Fig. 2(a)). 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 96-well plates and left at 4°C overnight (Fig. 2(b)). 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 (Fig. 2(c)). 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 (Fig. 2(d)). 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 (H₂SO₄, Sigma-Aldrich, St. Louis, MO, USA) was added (Fig. 2(e)). Colorimetric measurements of avidin were performed at 450 nm using a microplate spectrophotometer (Model 680, Bio-Rad, Hercules, CA, USA; Fig. 2(f)). 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.

![Figure 2](https://example.com/figure2.png)

2.3 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 [10], as shown in Fig. 3. The MBs were held in a

![Figure 3](https://example.com/figure3.png)
sample chamber (50 × 15 × 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 a -6-dB fractional bandwidth of 54.94%, has a frequency range of 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 × 10^11/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 the 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 were normalized to the background noise. The signals were then normalized to the amplitude of each sample after MB destruction. The MBs were destroyed with the sonoporation gene-transfection system (ST 2000V, NepaGene) at an acoustic intensity of 3 W/cm² for 1 min for amplitude normalization because the amplitudes of lipid shells and albumin shells were different.

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

<table>
<thead>
<tr>
<th>Agent</th>
<th>Diameter (μm)</th>
<th>Concentration (per ml)</th>
<th>Avidin concentration (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SonoVue</td>
<td>2.50</td>
<td>2 − 5 × 10^11</td>
<td>N/A</td>
</tr>
<tr>
<td>Targestar SA</td>
<td>2.50</td>
<td>1.00 × 10^10</td>
<td>N/A</td>
</tr>
<tr>
<td>MBs</td>
<td>2.50 ± 0.30</td>
<td>1.15 ± 0.15 × 10^9</td>
<td>N/A</td>
</tr>
<tr>
<td>Av-MBs</td>
<td>2.29 ± 0.86</td>
<td>2.64 ± 0.05 × 10^9</td>
<td>3700</td>
</tr>
<tr>
<td>Gd-MBs</td>
<td>2.78 ± 0.67</td>
<td>2.51 ± 0.07 × 10^9</td>
<td>N/A</td>
</tr>
<tr>
<td>Av-Gd-MBs</td>
<td>1.90 ± 0.53</td>
<td>1.25 ± 0.05 × 10^9</td>
<td>2100</td>
</tr>
</tbody>
</table>

MBs: albumin MBs; Av-MBs: avidin-albumin MBs; Gd-MBs: albumin-(Gd-DTPA) MBs; Av-Gd-MBs: avidin-albumin-(Gd-DTPA) MBs; N/A: not available

2.4 Attenuation in solution

The US attenuation of the solutions relative to water was calculated using [29,30]:

\[
\alpha(f) = \frac{-20}{k} \log_{10}\left(\frac{A_r(f)}{A_i(f)}\right) \text{ dB mm}^{-1}
\]

where \(A_r(f)\) and \(A_i(f)\) 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 length of the propagation path was the focus depth of the transducer (2.54 cm). \(A_r(f)\) and \(A_i(f)\) were calculated using MATLAB (R2010b, MathWorks, Natick, MA, USA).

2.5. 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.

3. Results and discussion

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. 4 and 5 (\(n = 5\)). 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. 4(b) and 5(b)), and their mean diameters were 1.15, 2.29, 2.78, and 1.90 μm, respectively (Table 1; Figs. 4(b) and 5(b)). 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 MB shells may influence the measurement of the ELISA-determined amount of avidin present. Figure 6 shows example histograms of the fluorescence intensity (FI) with and without avidin conjugation for albumin MBs (as the control group;
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 activity at high frequencies. In the present study, although the sizes of all of the samples were 1.0-2.5 μm, nonlinear activity was more significant for avidin-albumin–shelled MBs at 4-18 MHz ($p = 0.025$) and more significant for the other MBs at 6-24 MHz ($p = 0.003-0.044$). Moreover, the attenuation coefficients were higher for MBs that incorporated paramagnetic metal ions ($p < 0.05$). The shell properties thus influenced the attenuation measurements.

Soetanto et al. (2000) reported that MBs coated with surfactants are less soluble and have a longer lifetime [26]. Borrelli et al. (2012) demonstrated that albumin MBs can be stored from 2 weeks to one year depending on their formulation [31]. In the present study, the lifetime of MBs was found to be 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. 8 ($n = 3$).

Figure 6. Typical flow cytometry fluorescence intensity histograms of (a) albumin MBs and (b) albumin-(Gd-DTPA) MBs with and without avidin conjugation.

Figure 7. 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 various frequencies. Data are presented as mean and standard deviation values.

Figure 8. 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 presented as mean and standard deviation values.
The signal changes are listed in Table 2. Initially, the signal intensities of Targetar SA, albumin-(Gd-DTPA) MBs, and avidin-albumin-(Gd-DTPA) MBs were stronger. Then, except for the lipid molecular probe (Targetar SA) ($p = 0.05$), the signal amplitudes of the albumin molecular probes (avidin-albumin MBs and avidin-albumin-(Gd-DTPA) MBs) significantly decreased ($p < 0.001$, $p = 0.0012$) (Table 2). For lifetime measurements, the decay of a signal at 180 min 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. The attenuations of Targetar 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 lifetime of albumin MBs was also decreased by the incorporation of paramagnetic metal ions into their shells (41.38%).

Table 2. US signal changes of various MBs from Fig. 8.

<table>
<thead>
<tr>
<th>Agent</th>
<th>Initial intensity (dB) (0 min)</th>
<th>Final intensity (dB) (180 min)</th>
<th>Decay (%)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SonoVue</td>
<td>1.91 ± 0.01</td>
<td>0.23 ± 0.24</td>
<td>87.96</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Targetar SA</td>
<td>4.46 ± 1.21</td>
<td>4.07 ± 0.68</td>
<td>8.74</td>
<td>0.05</td>
</tr>
<tr>
<td>MBs</td>
<td>3.15 ± 0.74</td>
<td>1.59 ± 0.88</td>
<td>49.52</td>
<td>0.04</td>
</tr>
<tr>
<td>Gd-MBs</td>
<td>4.93 ± 0.75</td>
<td>2.89 ± 1.10</td>
<td>41.38</td>
<td>0.028</td>
</tr>
<tr>
<td>Av-MBs</td>
<td>2.41 ± 0.054</td>
<td>0.61 ± 0.21</td>
<td>74.69</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Av-Gd-MBs</td>
<td>3.54 ± 0.95</td>
<td>0 ± 0.14</td>
<td>100</td>
<td>0.0012</td>
</tr>
</tbody>
</table>

4. 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 were evaluated. The activity of avidin-albumin-shelled MBs was most significant at 4-18 MHz. The attenuation coefficients of the MBs were increased by the incorporation of paramagnetic metal ions. For lifetime measurements, the decay of albumin MBs at 180 min was less than that of SonoVue. Avidin conjugation reduced the lifetime of albumin MBs but not that of Targetar SA. Incorporating paramagnetic metal ions into the shells of albumin MBs influenced their lifetime.

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