Tissue-mimicking phantoms for photoacoustic and ultrasonic imaging

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Abstract: In both photoacoustic (PA) and ultrasonic (US) imaging, overall image quality is influenced by the optical and acoustical properties of the medium. Consequently, with the increased use of combined PA and US (PAUS) imaging in preclinical and clinical applications, the ability to provide phantoms that are capable of mimicking desired properties of soft tissues is critical. To this end, gelatin-based phantoms were constructed with various additives to provide realistic acoustic and optical properties. Forty-micron, spherical silica particles were used to induce acoustic scattering, Intralipid® 20% IV fat emulsion was employed to enhance optical scattering and ultrasonic attenuation, while India Ink, Direct Red 81, and Evans blue dyes were utilized to achieve optical absorption typical of soft tissues. The following parameters were then measured in each phantom formulation: speed of sound, acoustic attenuation (from 6 to 22 MHz), acoustic backscatter coefficient (from 6 to 22 MHz), optical absorption (from 400 nm to 1300 nm), and optical scattering (from 400 nm to 1300 nm). Results from these measurements were then compared to similar measurements, which are offered by the literature, for various soft tissue types. Based on these comparisons, it was shown that a reasonably accurate tissue-mimicking phantom could be constructed using a gelatin base with the aforementioned additives. Thus, it is possible to construct a phantom that mimics specific tissue acoustical and/or optical properties for the purpose of PAUS imaging studies.

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1. Introduction

1.1. Ultrasonic and photoacoustic imaging

Medical ultrasonic (US) imaging relies on the transmission of a high-frequency (MHz range) acoustic pulse into tissue through a transducer that is capable of both transmitting and receiving high-frequency sound [1]. As the transmitted pulse propagates through tissue, acoustic impedance mismatches in the propagation path cause the sound to be scattered. The backscattered portion of this energy, traveling in a direction opposite to the pulse propagation direction, can be received by the aforementioned transducer. The amplitude of the modulated signal of this received echo is related to specific tissue structure, while the signal’s temporal component denotes spatial position. US transducers can be comprised of a single element, made from material capable of electromechanical transduction, or of multiple elements, with the latter configuration allowing for electronic transmit and receive beamforming.

Photoacoustic (PA) imaging is a technique that combines ultrasonics with photonics [2]. With PA imaging, unlike US imaging, acoustic generation is achieved through the irradiation of tissue with nanosecond, low-energy laser pulses. This laser light is absorbed by tissue and results in thermoelastic expansion, which generates broadband acoustic waves that emanate from the optical absorption volume; the amplitude of this acoustic perturbation is proportional to both local fluence and absorption. These acoustic waves can then be received with conventional ultrasound transducers to form an image that is related to the local optical absorption of tissue. It is important to note that PA imaging does not require ballistic photon propagation, as laser irradiation is merely a mechanism to achieve localized thermoelastic expansion. PA imaging can achieve a significant contrast enhancement for highly absorbing tissue constituents (e.g., blood) or highly absorbing contrast agents (e.g., plasmonic nanoparticles or fluorescent dyes) [3,4]. Unlike US imaging, PA imaging generally does not provide sufficient soft tissue contrast. Given their common use of a conventional ultrasound transducer, it is possible to combine PA and US (PAUS) imaging and obtain co-registered images. Thus, PAUS imaging affords a researcher or clinician reliable soft tissue contrast (provided by US imaging) while simultaneously offering drastically improved sensitivity to blood or photoabsorbing contrast agents (provided by PA imaging). PAUS imaging has been successfully utilized in preclinical small-animal imaging studies, from investigating the dynamics of cancer models [5,6] to assessing blood oxygenation in a mouse model [7]. Furthermore, there has been growing interest in utilizing PAUS imaging in clinical applications, such as brachytherapy seed treatment monitoring [8], in vivo vascular imaging [9], sentinel lymph node assessment [10], and atherosclerotic plaque characterization [11].

1.2. Tissue-mimicking phantoms

In medical imaging, phantoms are imaging specimens of known geometric and material composition and are commonly used in the development and characterization of imaging systems or algorithms. A tissue-mimicking phantom emulates important properties of biological tissue for the purpose of providing a more clinically realistic imaging environment. Depending on the imaging modality, certain physical properties are of critical importance when constructing a tissue-mimicking phantom. In the case of US imaging, important phantom properties are the material’s speed of sound, acoustic attenuation coefficient, and acoustic backscatter coefficient. In soft tissue, the average speed of sound is 1540 m/s [12],

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while attenuation coefficients range from approximately 0.5 to 3.3 dB cm\(^{-1}\) MHz\(^{-1}\); they are as low as 0.18 dB cm\(^{-1}\) MHz\(^{-1}\) for blood [1]. Backscatter coefficients in tissue typically range from 10\(^{-5}\) to 10\(^{-1}\) cm\(^{-1}\) sr\(^{-1}\) [13–17]. Both the acoustic attenuation and backscatter coefficients have been shown to be frequency-dependent in tissue. Inconsistencies in the speed of sound through a phantom can result in beamforming errors that lead to geometric aberrations in the image, while variations in attenuation and backscatter coefficients affect the echogenicity of the image. The primary component of a US phantom tends to be water-based (e.g., gelatin, polyvinyl alcohol, agarose, polyacrylamide) which results in a speed of sound similar to tissue [18]. In addition, a scattering agent is suspended in the medium to produce the backscatter that makes US imaging possible. Scattering agents tend to be particulate matter and have included graphite particles, silica particles, and polystyrene spheres [12,19,20].

In the case of PA imaging, optical absorption and scattering are the two most important parameters when constructing an imaging phantom. As previously indicated, the magnitude of a generated PA signal is related to both the local fluence and optical absorption at the signal-generating source (e.g., blood or plasmonic nanoparticles). Consequently, optical scattering, the predominate optical loss mechanism in soft tissue, becomes important when attempting to achieve biologically realistic local fluences. Scattering also increases the turbidity of a medium, which tends to increase the irradiation volume. Typical optical reduced scattering values in soft tissue range from 3.84 cm\(^{-1}\) in blood [21] to 4000 cm\(^{-1}\) in the epidermis [22]. While optical absorption tends to be a much less significant loss mechanism in tissue (when compared to scattering), it does play a critical role in the generation of the PA signal itself. Typical optical absorption values in soft tissue range from 0.09 cm\(^{-1}\) in fat [21] to 6600 cm\(^{-1}\) in the epidermis [21,23]. The primary endogenous optical absorbers in deep tissues are oxygenated hemoglobin (between 10 cm\(^{-1}\) and 100 cm\(^{-1}\) in the range of 400 nm to 1300 nm) and deoxygenated hemoglobin (between 2 cm\(^{-1}\) and 105 cm\(^{-1}\) in the range of 400 nm to 1300 nm), which both generate an appreciable PA signal at clinically safe fluences [24]. Both scattering and absorption have strong wavelength dependence in tissue. As ballistic photon propagation is not required, PA imaging phantoms need only be translucent. Much like US phantoms, gel-based backgrounds are utilized for PA phantoms as they allow for the suspension of scattering- and absorption-inducing agents. To promote absorption at a specific wavelength, colored dyes are commonly introduced, while Intralipid\(^{®}\) solution or polystyrene beads are frequently incorporated to induce optical scattering [25,26].

1.3. Study and motivation

The purpose of this investigation is to give researchers and clinicians the tools to construct realistic tissue-mimicking phantoms for the continued development of PAUS imaging. In this study, gelatin was utilized as the phantom base because of its reasonable cost, satisfactory temporal stability, and straightforward fabrication protocol [27]. Silica particles were utilized as acoustic scattering agents as they allow for translucency (unlike graphite, for instance). US transducers were chosen to cover both the (lower) clinical and (higher) preclinical frequency range (6 to 22 MHz), while the range of investigated optical spectra was chosen to coincide with what is currently of greatest research interest in the literature (400 to 1300 nm).

2. Materials and methods

Phantoms were prepared by combining type-A, 300-Bloom gelatin derived from acid-cured porcine skin (G2500, Sigma-Aldrich Corp., St. Louis, MO) with ultrapure deionized water. The water was heated to 30°C and combined with formaldehyde (37% by weight, Thermo-Fisher Scientific Inc., Waltham, MA) to enhance cross-linking of the phantom and improve its long-term stability [18]. The mixture was vigorously stirred, and gelatin powder was slowly added to mitigate aggregation of the powder in the water. The solution was continuously stirred, covered to minimize vapor loss, and slowly heated to 45°C over the course of 10 minutes. To remove the entrapped air that resulted from the stirring process, the samples were placed into a vacuum chamber at 21 kPa\(^{\text{absolute}}\) for approximately 10 minutes. The solution
was then transferred into a mold with a syringe to further minimize the reintroduction of air. Finally, the mold was placed into a 4°C refrigerator for one day to allow the solution to fully cross-link.

To enhance ultrasonic backscatter, 40-µm diameter silica particles (MIN-U-SIL-40, U.S. Silica Co., Mill Creek, OK) were added to the gelatin solution before the addition of the formaldehyde. India Ink (Dr. Ph. Martin’s Bombay Black, Salis International Inc., Golden, CO), Direct Red 81 (Sigma-Aldrich Corp., St. Louis, MO), and Evans Blue (Sigma-Aldrich Corp., St. Louis, MO) were added to the water solution to increase the optical absorption properties of the phantom. The optical scattering of the phantom was increased by adding Intralipid® 20% IV fat emulsion (2B6022, Baxter International Inc., Deerfield, IL).

2.1. Characterization of ultrasound properties

2.1.1. Speed of sound and ultrasound attenuation

The phantom molds used to characterize ultrasonic attenuation and speed of sound were constructed from a 7.8-cm inner diameter PVC pipe cut into 1-cm tall cylinders. Taut sections of polyethylene food service film were epoxied to each side of the cylinder to serve as acoustic windows and desiccation prevention layers, while a hole was cut into the side of the pipe section to form an injection port for the gelatin solution. The phantoms were prepared as previously described; the different phantom types are listed in Table 1, where phantom A contains only water in the phantom mold.

![Figure 1. Experimental setup for the speed of sound and ultrasound attenuation measurements.](image)

Two single-element immersion transducers were selected to cover both clinical and preclinical imaging frequencies. The clinical transducer (V320, Olympus NDT Inc., Waltham, MA) had a central frequency of 7.5 MHz, a diameter of 1.27 cm, and a focal length of 5.08 cm. The preclinical transducer (V324, Olympus NDT Inc., Waltham, MA) had a central frequency of 25 MHz, a diameter of 0.64 cm, and a focal length of 2.54 cm. The transducers were interfaced to a pulser/receiver (5073PR, Olympus NDT Inc., Waltham, MA) operating at a pulse repetition rate of 2 kHz. The resulting signal was captured with an oscilloscope (CompuScope 12400, Gage Applied Technologies Inc., Lockport, IL) operating at 400 MHz. The ultrasound focus was positioned at the incident surface of the phantom, as shown in Table 1. Samples for ultrasound characterization

| Phantom | Gelatin Concentration (% m/V) | Formaldehyde Concentration (% m/V) | Silica Concentration (% m/V) | Intralipid® Concentration (% V/V) |
|---------|-------------------------------|-----------------------------------|-----------------------------|----------------------------------|
| A       | -                             | -                                 | -                           | -                                |
| B-F     | 4, 6, 8, 10, 12               | 0.1                               | -                           | -                                |
| G-J     | 8                             | 0.1                               | 0.1, 0.5, 1.0, 2.0           | -                                |
| K-N     | 8                             | 0.1                               | -                           | 5, 10, 15, 20                    |
Fig. 1. At five different positions around the center of the phantom (2 mm apart), 13 A-lines were captured.

To accurately determine the speed of sound through each phantom, the position of the plate ($z_1 + z_2 + z_3$ from Eq. (1)) was measured using the ultrasound system and a tank containing deionized water. A phantom mold filled with deionized water was then placed between the plate and transducer to determine the axial positions of the desiccation layers. The speed of sound ($c_s$) was determined by the change in time of the echo reflected from the metal plate in the experimental setup shown in Fig. 1 as

$$c_s = \frac{z_1 + z_2 + z_3}{z_2} c_w - \frac{z_1 + z_2}{z_2} c_w,$$  

where $c_s$ is the speed of sound of the sample, $c_w$ is the total measured speed of sound, and $c_w = 1482.66$ m/s is the speed of sound in water at 20°C [28].

The frequency-dependent ultrasound attenuation ($\alpha_s(f)$) was estimated using Fourier analysis of the signals obtained during the speed of sound measurements and is expressed as

$$\alpha_s(f) = 10 \log_{10} \left[ \frac{\langle V_s(f,z) \rangle}{\langle V_s(f,z) \rangle} \right] 10^{\frac{\alpha_w(f)}{10}},$$  

where $\alpha_w(f)$ is the frequency-dependent ultrasound attenuation of water in dB/cm, $\alpha_s(f)$ is the frequency-dependent ultrasound attenuation of the sample in dB/cm, $z_2$ is the thickness of the sample in cm, $\langle V_s(f,z) \rangle$ is the average spectral voltage with the sample between the transducer and plate, and $\langle V_s(f,z) \rangle$ is the average spectral voltage without the sample [29].

2.1.2. Ultrasound backscatter coefficient

For US backscatter coefficient (BSC) measurements, an aluminum plate was introduced 1 mm under one of the desiccation layers of the phantom mold. The same acquisition system used for the attenuation and speed of sound measurements was utilized for the BSC measurements. For the reference phantom, the ultrasound focus was placed at the aluminum plate, as shown in Fig. 2, and four A-lines were captured at each point of a 50-by-50 raster scan in 0.5-mm increments. The metal plate in the reference phantom simulated an ideal ultrasound scatterer. The measurement was repeated in phantoms without a metal plate, assuring that the ultrasound focus was at the same depth for the incident surface of the phantom (Fig. 2).

![Fig. 2. Setup for the ultrasound backscatter coefficient measurement. The reference measurement was performed with the focus 1 mm in the phantom background at an aluminum plate (left image). The phantom measurement (right image) was performed without the aluminum plate.](image)

The frequency-dependent backscatter coefficient ($\eta(f)$) was calculated using a short-time Fourier analysis technique as
\[
\eta(f) = \frac{\langle S_s(f, F) \rangle}{\langle S_r(f, F) \rangle} \times \frac{R^2 k^2 a^2}{8 \pi d \left[ 1 + \frac{(ka^2)^2}{4F^2} \right]},
\]

where \( \langle S_s(f, F) \rangle \) is the average signal from the phantom at the focus of the ultrasound transducer, \( \langle S_r(f, F) \rangle \) is the average signal from the metal plate at the focus of the ultrasound transducer, \( R \) is the reflection coefficient (i.e., 0.715) between the water and aluminum, \( k \) is the wavenumber in cm\(^{-1} \), \( a \) is the radius of the transducer in cm, \( d \) is the gate length in cm, and \( F \) is the focal length of the transducer in cm [30–32].

2.2. Characterization of optical properties

2.2.1. Optical absorption

To mimic the optical absorption of tissue, India Ink, Direct Red 81, and Evans Blue were introduced into the phantom. Optical extinction was obtained using a spectrophotometer (UV-3600, Shimadzu Corp., Kyoto, Japan) to measure gelatin samples containing India Ink, Direct Red 81, or Evans Blue (see Table 2). The samples were poured into 1-cm-path-length cuvettes, covered to minimize evaporation, and placed into a 4°C refrigerator for one day to cross-link.

The reduced optical extinction \( (\mu'') \) was then measured with the spectrophotometer (shown in Fig. 3) between wavelengths of 400 nm and 1300 nm (in 2-nm increments) as

\[
\mu'' = \frac{1}{z} \ln \frac{I}{I_0},
\]

where \( z \) is the path length in cm\(^{-1} \), \( I_0 \) is the intensity of light through the cuvette filled with air, and \( I \) is the intensity of light through the sample. Assuming optical scattering \( (\mu_s) \) of the samples is negligible [33], the reduced optical extinction coefficient \( (\mu'') = \mu'' + \mu_a \) reduces to the optical absorption coefficient \( (\mu_a) \). The sample was measured with a 5-nm slit width and in dual-beam mode to minimize system noise.

![Fig. 3. Setup to measure the optical extinction coefficient of samples containing different concentrations of gelatin and India Ink, Evans Blue, or Direct Red 81. The dashed lines indicate scattered photons; S is the light source; and D is the light detector.](image)

### Table 2. Samples for optical absorption characterization

| Phantom       | Gelatin Concentration % (m/V) | Formaldehyde Concentration % (m/V) | Dye Concentration |
|---------------|------------------------------|-----------------------------------|------------------|
| India Ink     | 0, 4, 5, 6, 8, 10, 12        | 0.1                               | 0.01% V/V and 0.02% V/V |
| Evans Blue    | 0, 4, 5, 6, 8, 10, 12        | 0.1                               | 0.01 mM and 0.02 mM |
| Direct Red 81 | 0, 4, 5, 6, 8, 10, 12        | 0.1                               | 0.01 mM and 0.02 mM |
2.2.2. Optical scattering

Optical scattering of tissue was mimicked using dilutions of 20% Intralipid® solution. The inverse adding-doubling (IAD) method was used to find the optical scattering of the turbid samples using total reflection and total transmission measurements. This method is applicable to homogeneous scattering samples with any optical thickness, albedo, or phase function and can be applied to a sample that is bound by glass. Optical scattering was obtained by iterating an adding-doubling solution of the radiative transport equation until the calculated values of the reflection and transmission matched the measured values [34,35].

Intralipid® solution was used as the principle source of optical scattering in all phantoms. Samples (see Table 3) were prepared by heating the Intralipid® dilutions to 30°C. Formaldehyde and gelatin powder were added while vigorously stirring the Intralipid® solution. The mixture was then slowly heated to 45°C over the course of 10 minutes. The mixture was vacuumed to remove trapped air and pipetted between two 75-mm-by-25-mm by-1-mm microscope glass slides (Thermo-Fisher Scientific Inc., Waltham, MA). The slides were spaced 100 µm apart using stainless steel shims and placed in a 4°C refrigerator for 30 minutes.

Reflection and transmission measurements were made between 400 nm and 1300 nm (in 2-nm increments) using an ISR-3100 integrating sphere attachment for the Shimadzu UV-3600. The samples were placed flush with the sample port, and four measurements (two on each side) were made on each sample. The experimental setup for the total reflection and total transmission measurements is shown in Fig. 4. System noise was again minimized using a dual-beam measurement.

The total diffuse reflectance and transmittance measurements were input into the IAD program [36]. Total diffuse reflectance was calculated as

$$r_{\text{sample}} = r_{\text{std}} \frac{P_{\text{sample}} - P_0}{P_{\text{std}} - P_0},$$  \hspace{1cm} (5)

while total diffuse transmission was calculated as

$$t_{\text{sample}} = t_{\text{std}} \frac{P'_{\text{sample}} - P'_0}{P'_{\text{std}} - P'_0},$$  \hspace{1cm} (6)
where $P_{std}$, $P_{sample}$, and $P_0$ are reflectance measurements from the setup in Figs. 4a-c and $P'_{std}$, $P'_{sample}$, and $P'_0$ are the transmittance measurements from the setup in Figs. 4d-e. The test standards, $r_{std}$ and $t_{std}$, were constructed from barium-sulfate with a fractional reflectance of 0.96. Given that optical scattering anisotropy ($g$) is not significantly sensitive to the calculation of the reduced optical scattering coefficient ($\mu'_s = (1-g) \mu_s$) with the IAD method, an anisotropy value of 0.85 was assumed.

Table 3. Samples for optical scattering characterization

| Phantom         | Gelatin Concentration | Formaldehyde Concentration |
|-----------------|-----------------------|-----------------------------|
|                 | % (m/V)               | % (m/V)                     |
| 4.0% Intralipid | 0, 4, 5, 6, 8, 10     | 0.1                         |
| 4.5% Intralipid | 0, 4, 5, 6, 8, 10     | 0.1                         |
| 5.0% Intralipid | 0, 4, 5, 6, 8, 10     | 0.1                         |

Based on a preliminary study, the silica concentrations used to induce ultrasound backscatter do not exhibit enough optical scattering for the IAD method; therefore, a Monte Carlo model was introduced to determine the optical scattering contribution of the silica. Mie calculations of the optical scattering of 40-µm silica spheres in water were performed for volume fractions corresponding to 0.1%, 0.5%, 1.0%, and 2.0% m/V concentrations [37].

3. Results and discussion

3.1. Speed of sound

Speed of sound dependence on gelatin concentration is shown in Fig. 5a; no significant change in the speed of sound was observed with the introduction of silica particles. A slight net decrease in the speed of sound was observed with the addition of Intralipid ® solution, as shown in Fig. 5b.

The speed of sound illustrated in Fig. 5 is similar to intra-vitam fat (~1480 m/s), kidney (~1555 m/s), spleen (~1553 m/s), liver (~1570 m/s), and post-mortem muscle (~1575 m/s) [38]. Note that the fastest speed of sound in Fig. 5a is 1530 m/s. To achieve faster speeds of sound in gelatin phantoms, iso-propyl alcohol may be added to attain speeds over 1600 m/s [18,39]. If using Intralipid ® solution in the phantoms, however, alcohol will interact with the lipid particles and change both the ultrasound and optical properties of the phantom.

3.2. Ultrasound attenuation

Frequency-dependent ultrasound attenuation was determined using Fourier analysis of the signal from the aluminum plate. A −6 dB cutoff was used to determine the bandwidth of each transducer in the aforementioned system. Attenuation in water is proportional to frequency; thus, most of the upper band (22 MHz to 32 MHz) of the 25 MHz transducer fell below the −6 dB cutoff. The contribution of the attenuation of gelatin was removed from phantoms G-N in
Fig. 6. Therefore, the attenuation curves shown in Fig. 6b are of the silica microparticles alone (i.e., with the attenuation due to gelatin removed), and the attenuation curves shown in Fig. 6c are of the of Intralipid® particles alone.

Fig. 6. The dependence of ultrasound attenuation on gelatin concentration (a), silica concentration (b), and Intralipid® concentration (c) for the phantoms listed in Table 1. The contribution of ultrasound attenuation from the gelatin was removed from the graphs for silica and Intralipid® formulations.

The attenuation difference between phantom D and phantom G was not significant, and thus the data for phantom G are not shown in Fig. 6b. Using least-squares regression, the ultrasound attenuation was found to exhibit a frequency-dependent relationship as

$$\alpha(f) = A f^n,$$

where $\alpha(f)$ is the frequency-dependent ultrasound attenuation in dB cm$^{-1}$ MHz$^{-1}$, $f$ is frequency in MHz, $A$ is a constant in dB cm$^{-1}$ MHz$^{-1}$, and $n$ is a constant. The solid black lines in Fig. 6 were generated using Eq. (7) with the coefficients in Table 4.

Typical attenuation values for specific tissue types are as follows: fat (0.63 dB cm$^{-1}$ MHz$^{-1}$), skeletal muscle (3.3 dB cm$^{-1}$ MHz$^{-1}$), cardiac muscle (1.8 dB cm$^{-1}$ MHz$^{-1}$), blood (0.18 dB cm$^{-1}$ MHz$^{-1}$), liver (0.94 dB cm$^{-1}$ MHz$^{-1}$), kidney (1.0 dB cm$^{-1}$ MHz$^{-1}$), brain white matter (1.2 dB cm$^{-1}$ MHz$^{-1}$), and brain gray matter (0.5 - 1.0 dB cm$^{-1}$ MHz$^{-1}$) [1]. Generally, the combination of silica and gelatin alone does not provide attenuation values typical of these tissues. The addition of Intralipid® solution, however, allows for accurate modeling of the ultrasound attenuation of these tissues, with coefficients for Eq. (7) shown in Table 4.

Table 4. Constants for ultrasonic attenuation of phantoms

| Phantom | B | C | D | E | F | H | I | J | K | L | M | N |
|---------|---|---|---|---|---|---|---|---|---|---|---|---|
| A       | 0.015 | 0.021 | 0.032 | 0.040 | 0.053 | 0.017 | 0.062 | 0.102 | 0.233 | 0.392 | 0.495 | 0.687 |
| n       | 1.619 | 1.602 | 1.542 | 1.535 | 1.497 | 1.396 | 1.073 | 1.130 | 0.994 | 1.045 | 1.054 | 1.036 |

3.3. Ultrasound backscatter

The frequency-dependent BSC was found for phantoms B-N. As expected, no significant backscatter was observed in phantoms B-F, which lacked an added scattering agent. The BSC data for phantoms G-J are shown in Fig. 7. The data in Fig. 7 exhibit a proportional change in the backscatter coefficient with silica particle concentration.

The frequency-dependent BSC for samples G-N was regressed as

$$\eta(f) = A f^n,$$

where $\eta(f)$ is the frequency-dependent ultrasound BSC in cm$^{-1}$ sr$^{-1}$ MHz$^{-1}$, $f$ is the frequency in MHz, $A$ is a constant in cm$^{-1}$ sr$^{-1}$ MHz$^{-1}$, and $n$ is a constant. The solid black lines in Fig. 7 were generated using Eq. (8) with the regression coefficients in Table 5. Phantoms K-N exhibited frequency independence and are presented with an exponent of zero in the table.
Fig. 7. The dependence of the ultrasound backscatter coefficient with increasing silica concentration for phantoms listed in Table 1. Error bars were calculated from 2500 positions on 3 samples.

Table 5. Constants for ultrasonic backscatter coefficient of phantoms

| Phantom | G     | H     | I     | J     | K     | L     | M     | N     |
|---------|-------|-------|-------|-------|-------|-------|-------|-------|
| A       | 2.11e-2 | 1.51e-2 | 2.83e-2 | 4.63e-2 | 3.02e-4 | 3.50e-4 | 3.76e-4 | 4.21e-4 |
| n       | 0.4828 | 0.9228 | 0.7797 | 0.7496 | 0     | 0     | 0     | 0     |

The backscatter coefficient of several tissues can be simulated using Intralipid® solution alone: blood (10^{-5}-10^{-3} cm^{-1} sr^{-1}) [16], fatty liver tissue (10^{-4}-10^{-3} cm^{-1} sr^{-1}) [14], breast fat (10^{-4}-10^{-3} cm^{-1} sr^{-1}) [13], breast infiltrating duct carcinoma (10^{-4}-10^{-3} cm^{-1} sr^{-1}) [13], brain white matter (10^{-5}-10^{-4} cm^{-1} sr^{-1}) [15], and spleen (10^{-4}-10^{-3} cm^{-1} sr^{-1}) [15]. Silica microparticles, however, must be included in the phantom when mimicking tissues that possess greater backscatter coefficients. These tissues include normal liver tissue (10^{-3}-10^{-2} cm^{-1} sr^{-1}) [14], breast parenchyma (10^{-3}-10^{-2} cm^{-1} sr^{-1}) [13], and skeletal muscle (10^{-4}-10^{-1} cm^{-1} sr^{-1}) [17].

The increase in attenuation with increasing gelatin concentration (Fig. 6a) was due to an increase in ultrasound absorption as no significant backscatter was observed from any concentration of gelatin. Adding silica microparticles also increases the attenuation by primarily increasing the ultrasound scattering (see, Fig. 6b, Fig. 7, and Table 5). Using the coefficients in Table 5 with Eq. (8) for phantoms H-J validates a monotonic increase in the backscatter coefficient. Although incorporating Intralipid® solution in the phantom greatly increases the ultrasound attenuation by both absorption and scattering mechanisms, the primary contribution of Intralipid® to the ultrasound attenuation (Fig. 6c) was absorption, as evidenced by the relatively small backscatter coefficients in Table 5.

3.4. Optical absorption

As a verification of all optical absorption measurements, the spectrum of ultrapure water, which is well characterized, was determined with the aforementioned system [37,40]. The main assumption in the measurement of the absorption spectrum for gelatin is that optical scattering is negligible (i.e., \( \mu_s = \mu_s + \mu_a = \mu_a \)). As shown in Fig. 8a, the optical absorption spectrum of gelatin (4%-12%) closely follows the optical absorption of an aqueous solution of 0.1% V/V formaldehyde (0% gelatin) between 950 nm and 1300 nm. At wavelengths between 400 nm and 950 nm (see the magnified region in Fig. 8b), an increase in optical absorption is present. The optical absorption increased proportionally to the concentration of gelatin (i.e., the higher the gelatin concentration, the higher the optical absorption between 400 nm and 950 nm).

India Ink, Direct Red 81, and Evans Blue were used to change the absorption spectra of the phantoms. Two concentrations of India Ink (0.01% and 0.02% V/V) were tested with six concentrations of gelatin (4%, 5%, 6%, 8%, 10%, and 12% m/V). The absorption of gelatin was removed from the absorption spectra of the samples by subtracting the results shown in
Fig. 8. The spectra for each India ink concentration were then scaled to 100% concentration and averaged (shown in black in Fig. 9a). A similar procedure—except scaled to 1 mM—was then repeated for Evans Blue and Direct Red 81. The Evans Blue in gelatin spectra is shown in Fig. 9b as the dark-blue spectrum and the Direct Red 81 in gelatin spectrum is shown in Fig. 9c as the red curve. The cyan spectra in Figs. 9a-c are of the respective dyes dispersed in only ultrapure water (i.e., not in gelatin). Results were obtained for dyes in an aqueous solution of 0.1% V/V formaldehyde and were shown to have the same spectra as dyes dispersed in only ultrapure water.

The changes in the absorption peaks of India Ink, Evans Blue, and Direct Red 81 were due to the interaction of the dyes with the gelatin. The nonpolar molecules in gelatin reduce the interaction of water with the dye molecules. The weakened dipole-dipole interaction causes larger absorption cross-sections of the polar dyes, thus red-shifting and broadening the optical absorption peak. Red and blue shifts can be seen in other dye species as well as nanoparticles, depending on their chemistry [41,42].

Fig. 9. Optical absorption spectrum of India Ink (a), Evans Blue (b), and Direct Red 81 (c) with error bars; the solid cyan line is the same molarity of dyes in water (i.e., not gelatin).

3.5. Optical scattering

We verified the IAD method to measure optical scattering by comparing the reduced optical scattering coefficient of Intralipid® solution with results found in the literature [43,44]. An anisotropic scattering value of zero was used for the IAD program. The anisotropic scattering coefficient does not significantly change the calculation of the reduced scattering coefficient in the IAD program [45]. The results in Fig. 10 were scaled, representing reduced scattering in
a concentration of 20% Intralipid® solution. The influence of increased gelatin concentration monotonically decreased the overall scattering of the samples. Therefore, we speculate that increasing the gelatin concentration increases the index of refraction in the gelatin background. This causes the difference in index of refraction between the gelatin background and Intralipid® particles to decrease; thus, this results in a decrease in reduced optical scattering.

![Fig. 10. Optical scattering spectrum of 20% Intralipid® in various concentrations of gelatin.](image)

Mie calculation of the optical scattering of silica in phantoms G-J resulted in reduced scattering of approximately 0.005, 0.025, 0.05, and 0.1 cm$^{-1}$, respectively. The calculated anisotropy coefficient ranged between 0.967 at 400 nm and 0.958 at 1300 nm. These calculations were performed in water with an index of refraction of 1.33. The index of refraction of gelatin is closer to the index of refraction of silica (~1.5); therefore, we can assume that the optical scattering contribution of silica is even less significant.

4. Conclusion

We have demonstrated that an accurate tissue-mimicking phantom for PAUS imaging can be constructed for most soft tissues. US attenuation can be controlled by varying the concentration of gelatin, silica microparticles, and/or Intralipid® solution. The US backscatter coefficient can be tailored for a specific tissue by adjusting the silica microparticle and/or Intralipid® solution concentration. Although silica microparticles produce some optical scattering, Intralipid® solution produces scattering values that are more common to tissue. Lastly, optical absorption, the source of the PA signal, can be accurately established at a specific wavelength by varying the concentration of India Ink, Evans Blue, or Direct Red 81.

One interesting finding is the red-shift in the optical absorption of India Ink, Evans Blue, and Direct Red 81 in gelatin, as opposed to water. This effect illustrates the notion that PA contrast agents, especially molecular agents, may perform differently in gelatin than in other solutions. For this reason, optical absorption measurements for a desired PA imaging contrast agent must be performed in gelatin to accurately predict the PA response. In addition to this limitation, phantoms prepared using the aforementioned dyes only mimic a tissue at a specific wavelength. The development of a tissue-mimicking phantom for spectroscopic PAUS imaging is outside the scope of this paper. Attempting to combine dyes for spectroscopic tissue modeling may change the spectral absorption characteristics of each dye because of molecular interactions.

Phantoms were stable over the duration of the testing (approximately one week). A more comprehensive longitudinal study would have to be performed in future work to assess the stability of phantoms over experimentally relevant storage durations/conditions. The phantoms used in our study were sealed in air-tight molds in a 4°C refrigerator between tests. Other investigations have shown that gelatin phantoms maintain good stability for several months with similar recipes by preventing desiccation of the phantoms [18,27]. Storing the phantom at low temperatures slows bacterial growth within the phantom, further improving temporal stability.
Tissue-mimicking phantoms for PAUS imaging are vital for testing and simulating in vivo conditions in both preclinical and clinical research. We have quantified the dependence of gelatin, silica microparticles, dye, and Intralipid® solution concentration on the speed of sound, US attenuation, US backscatter, optical absorption, and optical reduced scattering. Careful manipulation of the concentrations of each of these ingredients can be used to create an accurate tissue-mimicking phantom for the continued development of PAUS imaging of soft tissues.

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