Investigation of silk as a phantom material for ultrasound and photoacoustic imaging

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\section{Introduction}

Tissue mimicking phantoms with well-defined properties play a significant role in characterizing and comparing the performance of both commercial and laboratory imaging devices. It is imperative for the phantoms to demonstrate properties that match biological tissues and have long-term stability when evaluating medical imaging devices. The properties to be expressed by these tissue mimicking phantoms are specific to the type of imaging modality or device under consideration and a wide range of materials have been studied for this purpose. For example, in the case of ultrasound (US) imaging that relies on the pulse-echo sequence of an acoustic wave generated and detected by a single or multi-element transducer, human-tissue mimicking phantoms must have tunable acoustic properties such as the speed of sound and acoustic attenuation. In devices incorporating multiple imaging modalities, the phantom material should mimic all the modality-specific attributes. For example, photoacoustic (PA) imaging may be combined with ultrasound imaging due to their similar receiver electronics, to obtain co-registered structural and functional images\textsuperscript{[1–3]}. PA relies on the generation of acoustic waves following nanosecond pulsed optical excitation and follows similar image formation methods as ultrasound imaging. Devices combining both of these modalities must use phantoms that have both tunable acoustic properties such as those previously mentioned, and optical properties such as absorption and scattering, while also maintaining long-term structural integrity. Additionally, the phantom material should have the flexibility to be molded into various shapes, inclusions, and lesions to mimic different biological structures in the body\textsuperscript{[4]}. Various water and oil-based materials have been utilized as phantom materials for ultrasound and photoacoustic imaging\textsuperscript{[5]}. Most are composed of synthetic polymers, natural polymers, or a combination of both. Water-based materials such as gelatin\textsuperscript{[5–11]}, agarose\textsuperscript{[9,10,12–15]}, and polyvinyl alcohol cryogel (PVAc)\textsuperscript{[9,10,15–17]} are readily available, relatively low cost, and simple to prepare\textsuperscript{[18,19]}. However, the longevity of these phantoms is inconsistent due to their fragility resulting in damage from mishandling or improper storage. Additionally, they will dehydrate if stored in air or swell if stored in liquid. Instead, oil-based materials such as paraffin gel wax\textsuperscript{[9,20,21]},
polyvinyl chloride-plastisol (PVCP) [5,10,22–24], and styrene-ethylene/butylene-styrene copolymer (SEBS) [9,19,25,26] have been used previously and can be stored long-term. However, their commercial counterparts exhibit batch-to-batch variations or their formulations are proprietary. Additionally, these commercial phantoms along with copolymers in oil have a low speed of sound which may restrict utility [4]. These materials display properties such as speed of sound, acoustic attenuation, and Young’s modulus that cover an extensive range of tissue types, however, they are acoustically and optically transparent and need additional dopants such as silica powder or titanium oxide to produce ultrasound and optical contrast. Additional precautions need to be taken during fabrication to ensure homogenous distribution of the scatterers in these tissue phantoms. Furthermore, with the emergence of engineered tissues for various medical applications, it is key to develop phantoms mimicking these bioengineered samples that can change their physical, chemical, and biological properties with relevant external cues. Previously utilized water-based biocompatible materials such as gelatin, while conducive for cell incorporation, undergo rapid contraction or expansion and oil-based materials are not optimal to support long-term cell growth.

The use of silk proteins as an imaging phantom material is proposed here to address some of the aforementioned issues. Silk fibroin, referred to as silk henceforth, is an established material in the field of bio- and tissue engineering for its desirable properties such as low immunogenicity, the formation of porous structures, biocompatibility, and biodegradability [27]. These properties allow for the rapid transition from ex vivo phantom experiments to in vitro cell experiments, as well as in vivo translation studies, while maintaining similar imaging conditions and geometries. This is illustrated by the fact that silk displays intrinsic acoustic scattering, thereby circumventing the need for the addition of exogenous scattering agents such as titanium oxide or silica particles. Furthermore, by tuning the specific cells seeded into the silk scaffold and introducing patterned structures, the resulting scaffolds may better represent human tissue in its native environment [28]. In general, these human mimicking tissue engineered scaffolds are generated in a variety of sizes and shapes and depending on the application, the dimensions can be several millimeters to centimeters or larger. Given that silk displays desirable tissue mimicking phantom properties which may be used to initially characterize the system, and then form tissue engineered constructs for biological studies, we propose the use of silk as a biologically relevant US and PA imaging phantom material.

The goal of this study was to assess basic material properties of the silk material related to phantom performance. Using single element ultrasonic transducers covering both clinical and preclinical frequencies, ultrasound speed and attenuation were measured over a one-month period. The results indicate that silk displays acoustic properties similar to those of human tissues and maintained these properties for the duration of measurement. For use in photoacoustic imaging, the optical absorption and scattering coefficients were obtained in the range of 400–1200 nm to coincide with prevalent wavelengths utilized for in vivo applications. The results show a relatively flat absorption curve throughout the visible spectrum which expectedly peaks upon approaching near-infrared (NIR) wavelengths (~800–2500 nm) due to absorption by water. A moderately negative correlation with increasing wavelength was observed for the reduced scattering coefficient. Confirmation of optical absorption properties of silk phantoms was performed within the range of 690–950 nm by measuring the photoacoustic spectrum of pencil lead and indocyanine green (ICG) dye in the presence and absence of silk phantoms. Within this wavelength range, silk has a relatively low PA response [29]. Lastly, mechanical properties of phantoms fabricated with silk were obtained over a one-month period and compared to traditionally used gelatin and agar phantoms of similar size. Within this time frame, silk was more stable than gelatin in terms of volume, and more stable than gelatin and agar in terms of Young’s modulus. Based on these results, silk was determined to be a suitable phantom material, displaying stable mechanical properties and desirable acoustic and optical properties, while producing innately low photoacoustic signals due to low absorption.

2. Materials and methods

2.1. Preparation of gelatin, agar, and silk phantoms

Gelatin phantoms (8% w/v and 16% w/v) were prepared by combining gelatin powder (G2500, Sigma-Aldrich) with deionized (DI) water. Deionized water was filled into a glass beaker and heated to 40 °C on a hotplate. Gelatin powder was then slowly added and stirred using a magnetic stir rod. The beaker was covered to minimize vapor loss, and slowly heated to 50 °C over 10 min to fully dissolve the powder. The solution was then placed into a vacuum chamber to remove entrapped vapor.

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**Fig. 1.** Schematic demonstrating the silk extraction process. Production time from cocoons to scaffold requires approximately 5 days. Enface photographs of the rectangular silk phantoms used in this study are shown on the bottom left. Concentrations increase from left to right. Scale bar on 8% silk phantom = 5 mm. Created with BioRender.com.
air from the stirring process for approximately 10 min. Once cooled to 30 °C, the solution was poured into a rectangular mold (50 mm × 50 mm x 25 mm) lined with Vaseline and placed into a 4 °C refrigerator for one day to allow the solution to fully cross-link. The sample was then removed from the mold and cut into approximately 15 mm × 5 mm x 5 mm rectangle shaped phantoms.

Agar phantoms (1% w/v and 2% w/v) were prepared by combining agarose powder (A1296, Sigma-Aldrich) with deionized (DI) water. Deionized water was filled into a glass beaker and heated to 50 °C on a hotplate. Agar powder was then slowly added and stirred using a magnetic stir rod for 1 min. The solution was then placed into a microwave for 15 s after removing the stir bar. The solution was returned to the hotplate and stirred for another minute. These two steps were repeated until the temperature of the solution was between 80 and 100 °C. The solution was then placed into a vacuum chamber to remove entrapped air from the stirring process for approximately 5 min. Once cooled to 40 °C, the solution was poured into a rectangular mold (50 mm × 50 mm x 25 mm) lined with Vaseline and placed into a 4 °C refrigerator for one day to allow the solution to fully cross-link. The sample was then removed from the mold and cut into approximately 15 mm × 5 mm x 5 mm rectangle shaped phantoms.

Silk scaffolds were prepared from silk extracted from Bombyx mori silkworm cocoons (Fig. 1). Briefly, each batch utilized 5 g of cocoons which were cut into small pieces and boiled for 30 min in 2 L of a 0.020 M sodium carbonate (Na2CO3) solution. The resulting degummed fibers were thoroughly rinsed three times for 20 min each in deionized (DI) water to remove any residual Na2CO3 solution. After the last rinse, the fibers were stretched out and air dried overnight. Dried silk fibers were dissolved in a 9.3 M lithium bromide (LiBr) solution for 1 h in a 60 °C oven at which the content was stirred at the 30-minute mark. The resulting content was dialyzed for 2 days in which the water was changed 4-5 times on the first day and 3 times on the second day to yield a fibroin water solution. After the last water change, the solution was centrifuged twice at 9000 RPM in 4 °C, switching the tube after each spin to remove any impurities. To determine the resulting concentration of the silk solution, the weight of a dried volume was divided against its wet counterpart. For use as scaffolds, the solution was diluted to a concentration of 4% w/v, 6% w/v, 8% w/v and poured into rectangular PDMS molds (15 mm × 5 mm x 5 mm) which were frozen at −20 °C overnight and then transferred to a lyophilizer for drying. Dried silk scaffolds were then autoclaved to induce a β-sheet conformation and soaked in phosphate-buffered saline (PBS) for 48 h before use. In total, 4 days are required to extract the silk solution which may then be stored for up to one month. Creating scaffolds from the solution requires 1 day and then 2 more days to hydrate the scaffolds before use. Further in-depth explanations of the extraction and preparation process can be found elsewhere [30].

2.2. Measurement of speed of sound and ultrasound attenuation of silk phantoms

Rectangular silk phantoms were placed into a custom 3D-printed mount, immersed in DI water, and measured using two single-element immersion transducers to span both clinical and preclinical acoustic frequencies. The low frequency transducer (V310, Olympus) had a central frequency of 5 MHz, an element diameter of 6.35 mm, and a focal length of 26.0 mm. The high frequency transducer (V324, Olympus) had a central frequency of 25 MHz, an element diameter of 6.35 mm, and a focal length of 26.6 mm. Acoustic pulses were generated using a pulser/receiver (DDPR506, JSR Ultrasons) following triggers from a function generator (SDG1032X, Siglent). The same trigger was immediately passed to a data acquisition card (CSE161G2, GeGe) for digitization of the echoes as previously reported [1]. The data acquisition sampling rate was set to 200 MHz. An aluminum plate (BA2, Thorlabs) was placed below the sample to reflect the ultrasound echo. To move and align the transducer to the top surface of the scaffold before imaging, the transducer was mounted to a 3D axis stage comprised of three stacked linear stages (X-LSM, Zaber). After alignment, 200 A-lines at three different horizontal positions spaced 500 μm apart were captured. This was repeated for each silk concentration. (Fig. 2).

Measurement of the speed of sound relied on determining the shift in the ultrasound echo obtained from the aluminum plate with and without the sample, and the axial distance between the following three components: transducer, phantom, and aluminum plate [6]. The sample speed of sound (c_sample) was calculated following Eq. (1) where c_measured is the total measured speed of sound with the sample, c_water = 1493.5 m/s is the speed of sound in water at 23.7 °C [31], and z_1, z_2, z_3 are the axial distance between the transducer and top surface of the phantom, the thickness of the phantom, and the axial distance between the bottom surface of the phantom and aluminum plate, respectively. To obtain z_1, z_2, z_3, the first distance between the transducer and aluminum plate (z_1 + z_2 + z_3) was measured using regular ultrasound by multiplying the “speed of sound in water” by the “number of data samples collected” divided by the “sampling rate of the data acquisition card”. Similarly, z_1 was obtained using the same method once the phantom was placed in the transducer’s imaging path. Calipers were used to obtain z_3 (sample thickness). Lastly, to obtain z_3, “z_1 + z_2 + z_3” was subtracted by z_1 and z_2.

\[
\frac{z_1 + z_3}{c_{\text{sample}}} - \frac{z_1 + z_2 + z_3}{c_{\text{water}}} = \frac{z_1 + z_2 + z_3}{c_{\text{measured}}}
\]

(1)

Ultrasound attenuation of the sample (αsamp(f)) as a function of frequency in units of dB/cm was obtained using Fourier analysis of the signals collected from the speed of sound measurements [6]. Following Eq. (2), \(\langle V_{\text{ns}}(f, z) \rangle\) is the average spectral voltage obtained exclusively from the aluminum plate, \(\langle V_{\text{ns}}(f, z) \rangle\) is the average spectral voltage obtained from the aluminum plate with the sample, \(z_2\) is the sample thickness in units of cm, and \(\alpha_{\text{ns}}(f)\) is the ultrasound attenuation of water as a function of frequency in units of dB/cm.

\[
\alpha_{\text{samp}}(f) = \frac{10}{z_2} \log_{10} \left( \frac{\langle V_{\text{ns}}(f, z) \rangle}{\langle V_{\text{ns}}(f, z) \rangle} \right)^{10^{-\alpha_{\text{ns}}(f)}}
\]

(2)
2.3. Measurement of optical absorption and scattering properties of silk phantoms

Optical absorption and reduced scattering coefficients of silk were obtained using a single integrating sphere spectrophotometry system. Total transmittance and diffuse reflectance measurements were performed by placing samples at the entrance and exit ports of the integrating sphere (4 P-GPS-033-SL, LabSphere). Two spectrometers (HR2000, Ocean Optics and DS-ImGaAs-S12, StellarNet) were attached to the integrating sphere using optical fibers (M59L01, Thorlabs) to detect light in the 400–1100 nm and 900–1700 nm spectral ranges. Light from a halogen lamp (HL-2000, Ocean Optics) was focused onto the samples with a spot size of 3.9 mm in diameter. Transmittance through air and reflectance from a > 99% Spectralon sample were used as references. To obtain the optical properties from spectrophotometer measurements, an inverse Monte Carlo algorithm was utilized as previously described elsewhere [32]. This technique allowed for measurements of the optical absorption and reduced scattering coefficient under the assumption of the Henyey-Greenstein scattering phase function. To ensure complete coverage of the integrating sphere ports during measurements, cylindrical scaffolds were made by pouring silk solution into 35 mm diameter petri dishes to exceed the 12.7 mm diameter of the sample port and 25.4 mm diameter of the exit port. After soaking in PBS for 48 h, the scaffolds were cut into 500 µm and 1 mm thick slices using a vibratome (VT1200S, Leica). Measurements of dry specimens were not performed on phantoms. At each wavelength, 25 A-lines at three different positions were collected. The collected photoacoustic signals were processed on a custom written MATLAB code that involved using a Hilbert transform to obtain the signal envelope. For further visualization, ICG placed beneath the aforementioned scaffold was imaged at the peak of absorption of 800 nm to demonstrate the comparably low photoacoustic response from the silk phantom.

2.4. Ultrasound and photoacoustic imaging of silk phantoms

To confirm the ultrasound attenuation coefficient of silk phantoms, both transducers were scanned across the phantom at a step size of 100 µm while collecting 50 A-lines per position. The length of the scan was set to 10 mm to exceed the boundaries of the scaffold which was later removed while post-processing the data for display. Three separate slices (separated by 500 µm) were collected from each phantom. To support the measurement of optical absorption and reduced scattering coefficient, the PA signal of strong optical absorbers were compared when measured with and without the silk scaffold. The first absorber was 0.3 mm pencil lead which ideally displays a flat photoacoustic spectral response in the NIR while the second absorber was indocyanine green (ICG) dye, a common photoacoustic contrast agent, at a concentration of 10 µM filled into a polyethylene tube with an inner and outer diameter of 1.40 mm and 1.90 mm, respectively. Both were placed in direct contact underneath a 4% silk scaffold situated into the previously described 3D-printed mount. The silk scaffold was cut down in size to dimensions of 15 mm x 3.5 mm x 3.5 mm to reduce the amount of scattered light allowing for imaging with and without the scaffold to be performed at the same receiver gain. Photoacoustic imaging was performed using a custom built acoustic-resolution photoacoustic microscope using an OPO (Phocus HE Benchtop, OPOTEK) as its tunable source as previously reported [1]. The transducer and receiver electronics were similar to those mentioned in Section 2.3, but used the OPO Q-switch output as the main trigger for data acquisition. Wavelengths in the 690–950 nm range at 10 nm increments, chosen based on the peak absorption range of ICG, were used for photoacoustic imaging. The light was delivered to the sample via seven multi-mode fibers concentrically situated around the transducer. The fibers were oriented to aim light at the transducer focus (1 in.). The maximum fluence at 690 nm was approximately 30 mJ/cm² which is above the ANSI standard of 20 mJ/cm² [33], but was determined to be acceptable as imaging was performed on phantoms. At each wavelength, 25 A-lines at three different positions were collected. The collected photoacoustic signals were processed on a custom written MATLAB code that involved using a Hilbert transform to obtain the signal envelope. For further measurement of mechanical properties of gelatin, agar, and silk phantoms

Freshly made rectangular gelatin, agar, and silk scaffolds were submerged in DI water and 1x PBS, respectively, for 48 h in a 4 °C refrigerator. Afterwards, the phantoms were removed from the refrigerator and left to rest at room temperature (21 °C) for 1 h prior to testing. The volume of phantoms was then measured with calipers prior to loading onto a compression test machine (Instron 3366, Instron). Max compression was set to approximately 15% (~1 mm) of their respective heights without preloading. Chart speed was set to 1 mm/min. The time-dependent extension and force signals were recorded digitally through Instron’s Bluehill software. All measurements were performed at room temperature.

Young’s modulus (Eq. 3) was calculated using the slope of the stress-strain curves obtained by processing the raw data from the compression machine. The system provides force versus extension curves which were converted to stress versus strain curves by dividing the force by the contact area on the phantoms and dividing the extensions by the original phantom height. To calculate the slope, a linear fit using the MATLAB function polyfit was applied to the first 10% strain of the stress-strain curve. The average of the slopes obtained from the fit was reported as the Young’s modulus of the material. Statistical significance amongst different groups was calculated using GraphPad Prism.

E = \frac{\sigma}{\varepsilon} \frac{F/A}{\Delta H/L} \quad \text{(3)}

3. Results

3.1. Speed of sound and ultrasound attenuation of silk phantoms

The speed of sound as a function of time for all silk concentrations (4%, 6%, 8%) is shown in Fig. 3. During the first week, the lowest concentration of 4% displays an average speed of sound of 1580 m/s which is close to heart muscle (1561 m/s) [34], kidney medulla (1564 m/s) [34], and spleen (1568 m/s) [34]. When using higher concentrations between 6% and 8%, the 1610 m/s to 1664 m/s speed of sound, respectively, starts to resemble cartilage (1640 m/s) [35], cervix tissue (1629 m/s) [34], and certain layers of the skin (1595 - 1645 m/s) [36]. Overall, a general trend is seen in which higher silk concentrations display a faster speed of sound. This is to be expected as higher
concentrations of silk have an increased matrix density, and correspondingly, a decreased PBS or water volume. Over the one-month period, the speed of sound for all silk concentrations showed no significant change upon performing a two-way ANOVA with a Tukey multiple comparison test.

Ultrasound attenuation of different silk concentration blocks (15 mm x 5 mm x 5 mm) measured immediately after preparation using the aforementioned transducers with clinical and preclinical frequencies are shown in Fig. 4a. Displayed are only the frequencies within the individual – 6 dB bandwidth. Overlaid are the ultrasonic attenuation of water and different tissue types including heart muscle, skin, breast, and liver [37]. Silk phantoms exhibit a frequency-dependent relationship following:

\[ \alpha(f) = Af^n \]  

(4)

In Eq. (4), \( \alpha(f) \) represents the frequency-dependence of ultrasound attenuation in units of dB/cm, \( A \) is the scaling constant and acoustic attenuation coefficient with units dB/(cm·MHz), and \( n \) is the exponent with no units. The least-squares regression fits of the data set displayed in Fig. 4a have their coefficients listed in Fig. 4b. Overall, a general trend was seen in which higher concentrations of silk correlated with higher ultrasound attenuation coefficients. This is to be expected because as previously stated, higher silk concentrations correspond to a higher matrix density and therefore, lower water or PBS volume. As silk displays a higher attenuation coefficient than water for both clinical and preclinical frequency bandwidths, an increase in its density will increase its attenuation coefficient. Upon measuring the attenuation coefficient for a one-month period, all silk concentrations (4%, 6%, 8%) showed no significant change after performing a two-way ANOVA with a Tukey multiple comparison test as shown in Fig. 4c.

3.2. Optical absorption and scattering properties of silk phantoms

Based off the tested acoustic properties, 4% silk displays characteristics that closely match common tissue types. While this is likewise observed in 6% and 8% silk, they correspond to harder tissues with higher speed of sound and attenuation. This may be desirable in some cases, such as using 8% silk to mimic the attenuation of heart muscle, but as they represent a smaller percentage of tissue types, their use is limited. Therefore, further analysis of silk was performed exclusively with 4% silk as it will presumably be the most used concentration for future experiments as it displays a more usable attenuation for ultrasound and photoacoustic imaging.

The optical properties of 4% silk hydrated with PBS are summarized in Fig. 5. The absorption coefficient displays a relatively flat curve as a function of wavelength in the visible spectrum at approximately 0.6 cm\(^{-1}\). In this visible range, PBS has low absorption, approximately 100x less than the measured silk plus PBS values near the UV, and 2x less near the NIR. This is expected as PBS has a continuously increasing absorption coefficient in the visible spectrum. In the NIR, the absorption coefficient of silk remains relatively constant until 1100 nm at which it increases to a peak of 1.4 cm\(^{-1}\) at 1200 nm. Past this range, PBS displays significant absorption due to water and therefore dominates the trends. The reduced scattering coefficient continuously decreased in the visible spectrum which follows many tissue trends due to less scattering at longer wavelengths. However, the trend to decrease was moderate, starting at 18 cm\(^{-1}\) and ending at 15 cm\(^{-1}\) in the range 400–700 nm. Towards the NIR, the reduced scattering coefficient continually...
decreased to approximately 13 cm\(^{-1}\), but at a slower rate than in the visible spectrum. From these measurements, both optical absorption and reduced scattering fall within the range of many tissue types including the brain, heart, lung, prostate, and skin [38].

### 3.3. Ultrasound and photoacoustic imaging of silk phantoms

B-mode ultrasound images of mouse kidney (Fig. 6a) and 4% silk phantom (Fig. 6b) are shown for both transducers with clinical and preclinical center frequencies. The echoes displayed by silk qualitatively exhibit a similar speckle pattern as those observed in the mouse kidney. Additionally, as expected, the clinical frequency displayed large speckle patterns while preclinical frequencies displayed smaller speckle patterns. These signals correspond to the porous features of silk phantoms that facilitate cell proliferation and invasion and hence is an optimal tissue engineering scaffold. A recent study by Milazzo et al. demonstrated with micro-CT and SEM images that silk scaffolds have an irregular sponge shape characterized by alveolar structures and pores of various diameters while also possessing a relatively smooth surface [39].

**Fig. 5.** Absorption coefficient and reduced scattering coefficient of 4% silk phantom. Error bars (SEM) were calculated from 6 samples (3 samples each of 0.5 mm and 1 mm thickness).

**Fig. 6.** a) B-mode images of mouse kidney and b) 4% silk phantom for clinical (5 MHz) and preclinical (25 MHz) frequencies. Scale bar = 1 mm. c) Signal amplitude of 4% silk as a function of depth for the 5 MHz (red line) and 25 MHz (green line) frequencies respectively.

**Fig. 7.** a-b) Photoacoustic signal measured with and without 4% silk phantom for a) pencil lead and b) ICG (10 \(\mu\)M) dye in a polyethylene tube. Error bars (SEM) were calculated from three separate readings. c) Ultrasound and d) photoacoustic (\(\lambda = 800\) nm) image of 10 \(\mu\)M ICG in a tube under a 4% silk phantom. Scale bar = 1 mm.
3.4. Mechanical properties of silk phantoms

Comparison of volume and Young’s modulus in the various concentrations of silk phantoms over a two-week period is provided in Fig. 8. Change in phantom volume measured over a one-month period. Error bars (SEM) were calculated from 3 samples. ***p = 0.0005 and ****p < 0.0001.

Fig. 8. Change in phantom volume measured over a one-month period. Error bars (SEM) were calculated from 3 samples. ***p = 0.0005 and ****p < 0.0001.

Fig. 9. a) Young’s modulus of various biological tissues and the tested phantom materials. b) Change in Young’s modulus of various phantom materials over a one-month period demonstrating the stability of Young’s modulus in silk phantoms. Error bars (SEM) were calculated from 3 samples. *p < 0.05 and **p < 0.01.

Silk was an optimal tissue mimicking phantom material for US and PA imaging. The advantages and disadvantages of silk-based phantom materials compared to water-based and oil-based phantom materials are summarized in Table 1. Silk can be molded into various shapes similar to other phantom materials while maintaining a longer shelf life similar to oil-based materials. The required preparation time was 5 days to go from cocoons to end stage phantoms, but can be reduced to 1 day by using solution extracted in advance which can be stored in 4°C for up to one month. The primary advantages of silk as a phantom material resides in its intrinsic acoustic and optical properties being similar to human tissue. Scattering materials like titanium dioxide that are generally added to water-based or oil-based phantom materials do not need to be added to silk phantoms. Addition of these optical and acoustic scatters in water-based materials can make them incompatible for cell seeding,

supplementary data Fig. S1. Given that 4% silk has similar optical and acoustic properties to human tissue, this silk concentration was compared to other standard phantom materials (gelatin and agar) over a one-month period. The change in volume of the gelatin, agar, and silk phantoms over a one-month period is shown in Fig. 8 with data represented as mean +/- standard error (SEM). The gelatin and agar concentrations were selected based on literature in which these concentrations demonstrated similar properties to human tissue. The volume of gelatin increased over the measurement duration which is believed to have occurred due to swelling as phantoms were stored in water. Upon performing a two-way ANOVA with a Dunnet’s multiple comparison test, the 16% gelatin concentration showed a significant change in volume over each week compared to the original value. A change in volume was not observed in agar and silk phantoms with any significance.

Young’s modulus measured on day 0 (48 h after submersion in PBS or water) is shown in Fig. 9a. Gelatin, agar, and silk expectedly displayed a higher Young’s modulus at higher material concentrations. When plotted against various biological tissues, the materials span the mid-range of tissue stiffness, extending from 10’s to 100’s of kPa [41]. Over the one-month period, all phantom materials experienced no significant change in Young’s modulus (Fig. 9b). However, between the weeks, upon performing a two-way ANOVA with a Dunnet’s multiple comparison test, the 16% gelatin showed a significant change in Young’s modulus between week 0 and week 3, and the 2% agar showed an increase in Young’s modulus between week 0 and week 1. All silk measurements show no significant change in Young’s modulus. In general, this was expected as gelatin and agar phantoms swelled during storage (gelatin more than agar) resulting in an increased volume and consequently, a change in mechanical properties. This effect was not observed for the silk phantoms.

4. Discussion and conclusions

Silk was an optimal tissue mimicking phantom material for US and PA imaging. The advantages and disadvantages of silk-based phantom materials compared to water-based and oil-based phantom materials are summarized in Table 1. Silk can be molded into various shapes similar to other phantom materials while maintaining a longer shelf life similar to oil-based materials. The required preparation time was 5 days to go from cocoons to end stage phantoms, but can be reduced to 1 day by using solution extracted in advance which can be stored in 4°C for up to one month. The primary advantages of silk as a phantom material resides in its intrinsic acoustic and optical properties being similar to human tissue. Scattering materials like titanium dioxide that are generally added to water-based or oil-based phantom materials do not need to be added to silk phantoms. Addition of these optical and acoustic scatters in water-based materials can make them incompatible for cell seeding,
unlike silk phantoms that can be used for long-term cell culture [42–44]. By changing silk concentration and other fabrication methods, the acoustic and optical properties of the phantoms can also be modulated. It should be noted that in the study above, a freeze-drying approach was utilized to fabricate the silk phantoms, however, by utilizing different methods (e.g., such as water annealing), the final silk product can also be made transparent [45–47]. This allows for a wider range of tissue mimicking such as the cornea which is optically clear, as well as the fabrication of new biocompatible photonic devices [48–51]. Furthermore, silk can be functionalized with various fluorescence and optical dyes [52]. The use of dyes to manipulate optical absorption of silk phantoms and their effect on cell adhesion and growth will be investigated in future studies.

Table 1 summarizes the advantages and disadvantages of different base materials and silk. Adapted from Fonseca et al. [5].

| Matrix [References] | Advantages | Disadvantages |
|---------------------|------------|---------------|
| **Water-based materials** (gelatin, agarose, and polyvinyl alcohol cryogel) [5, 9] | • Largely optically transparent  
• Relative ease of preparation  
• Relatively similar speed of sound as tissue  
• Dyes and scatterers can be easily incorporated  
• Supports cell growth | • Acoustically transparent, need to add acoustic or optical scatterers for contrast  
• Some materials require a long time for cross-linking (e.g., polyvinyl alcohol) while others have minimal flexibility to form thin structures (e.g., gelatin)  
• High susceptibility to physical damage  
• Low temperature stability at physiological temperatures, causing loss of structural integrity |
| **Oil-based materials** (paraffin gel wax, polyvinyl chloride-plastisol (PVCP), and styrene-based copolymers (SEBS)) [5,24,53] | • Long shelf-life and good structural rigidity  
• Moldable into various shapes and sizes | • Non-trivial fabrication procedures  
• Additional acoustic and optical scatterers need to be added for contrast  
• High temperatures used in fabrication can damage additives used for optical absorption  
• Higher acoustic attenuation and Young’s modulus than human tissues  
• Cannot incorporate cells or engineered tissues |
| **Silk** | • Resistant to swelling and damage and structurally more rigid than water soluble phantoms  
• Intrinsic acoustic and optical scattering properties similar to tissue  
• Biocompatible for hosting cells and long-term cell culture  
• Ability to mold into different shapes and sizes and also 3D print into complex tissue structures  
• Ability to functionalize silk proteins with dyes | • Non-trivial preparation involving several steps that can lead to end-product variations |

Table 2 summarizes the speed of sound, acoustic attenuation coefficient, and Young’s modulus at listed concentrations. Values are dependent on: * 6% to 12% (w/w) gelatin, ** temperature 10-30 °C, *** number of freeze cycles, **** 60% concentration of cellulose to polyvinyl chloride-plastisol (PVPC), + Ratio Resin/Plasticizers, ++ Ratio Softener/PVC Resin.

| Phantom | Concentration (%) | Speed of sound (m/s) | Acoustic attenuation coefficient (dB/(cm•MHz)) | Young’s modulus (kPa) | References |
|---------|------------------|----------------------|-----------------------------------------------|----------------------|------------|
| Gelatin | 4-12 (w/w)       | 1500-1525            | 0.015-0.053                                   | 9.77-35.79*          | [6,11]     |
|         | 1520-1650        |                      | 0.12-1.5                                      |                      | [9]        |
| Agarose* | 3 (w/w)         | 1531-1553            | 0.047-0.253                                   | -                    | [13,14]    |
|         | 1498-1600        |                      | 0.04-1.40                                     |                      | [9]        |
| Polyvinyl alcohol (PVAc)** | 10 (w/w)  | 1520-1540            | 0.075-0.22                                    | -                    | [16]       |
|         | 1520-1610        |                      | 0.07-0.35                                     |                      | [9]        |
| Paraffin Gel Wax | 0-8 (w/w) | 1449-1443            | 0.073-2.663                                   | 17.4±1.4             | [21]       |
| Polyvinyl chloride-plastisol (PVCP)**** | 50-70 (v/v)* | 1400±20              | 0.146-0.381                                   | 772±155-2913±624 (GPa) | [22-24]    |
|         | 5-20 (v/v)**     | 1408-1402            | 0.67-0.55                                     | -                    | [5]        |
|         | 0.50 (v/v)**     |                      | 45.6                                           |                      | [10]       |
| Styrene-ethylene/butylene-styrene copolymer (SEBS) | 10-15 (w/w) | 1450-1460            | 0.18-0.224                                    | 5.2-7.6              | [19]       |
|         | 2-6 (w/w)        | 1420-1450            | 0.5-2                                          |                      | [25]       |
| Silk    | 4 (w/v)          | 1580                 | 1.153                                          | 29.2                 |            |
|         | 6 (w/v)          | 1610                 | 1.446                                          | 65.6                 |            |
|         | 8 (w/v)          | 1664                 | 2.131                                          | 121.6                |            |
those of human tissue such as brain, heart, lung, prostate, and skin while possessing low absorption. Silk phantoms overall showed greater stability in both volume and Young’s modulus when compared to gelatin and agar phantoms over a one-month period. Furthermore, the silk was resistant to swelling and damage when compared to other materials. However, despite these advantages against the other materials presented in Table 1, silk is challenging to work with due to the non-trivial fabrication process. One such challenge is ensuring that all silk co-contras are sourced from the same domesticated silkworm species to assure consistent preparation and processing [54]. Additionally, the porous structure of the silk material can lead to trapped air bubbles that can show up as artifacts in US and PA images. To remove the trapped air bubbles, the silk phantom needs to be submerged for 48 h, however, a vacuum chamber may be used to bypass this step. Despite these minor drawbacks, silk-based phantoms provide biocompatibility and can be tuned with simple surface modifications to provide a wide range of tissue mimicking phantoms and scaffolds that are conducive for cell adhesion, proliferation, migration, and differentiation. Our future studies will explore the biophysical influence of incorporating cells in varying concentrations of silk protein. The acoustic, optical, and mechanical properties of silk were tested in addition to its ultrasound and photoacoustic imaging response which were confirmed to be similar to human tissues. Moreover, silk-based materials can be stored long-term without change in its biophysical properties. Our future work will involve testing the proposed material with the inclusion of dyes and additional scatterers to determine the limits of fine-tuning the material’s characteristics and additionally, its stability and impact on cell seeding and long-term cell growth. In summary, silk-based materials offer many advantages such as long-term storage and biocompatibility, and has the potential to be an important part of the tissue mimicking phantom material list utilized to assess the performance of ultrasound and photoacoustic systems.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.pacs.2022.100416.

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