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Pipe Phantoms with Applications in Molecular Imaging and System Characterization

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Abstract

Pipe (vessel) phantoms mimicking human tissue and blood flow are widely used for cardiovascular related research in medical ultrasound. Pipe phantom studies require the development of materials and liquids that match the acoustic properties of soft tissue, blood vessel wall, and blood. Over recent years, pipe phantoms have been developed to mimic the molecular properties of the simulated blood vessels. In this paper, the design, construction, and functionalization of pipe phantoms are introduced and validated for applications in molecular imaging and ultrasound imaging system characterization. There are three major types of pipe phantoms introduced: a gelatin-based pipe phantom, a polydimethylsiloxane (PDMS)-based pipe phantom, and the “Edinburgh Pipe Phantom” (EPP). These phantoms may be used in the validation and assessment of the dynamics of microbubble-based contrast agents and, in the case of a small diameter tube phantom (EPP), for assessing imaging system spatial resolution/contrast performance. The materials and procedures required to address each of the phantoms are described.

Index Terms

Ultrasound; pipe (vessel) phantom; gelatin; polydimethylsiloxane (PDMS); ultrasound molecular imaging

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I. Introduction

IN medical ultrasound, pipe (vessel) phantoms are designed to mimic the major features of a blood vessel wall and blood flow, allowing precisely-controlled and well-characterized realistic data to be collected by an ultrasound system under examination. Pipe phantoms can be used quantitatively and qualitatively for cardiovascular related research. For example, the vessel geometry can be tailored to a range of morphologies to examine, in a parametric sense, the impact of varying levels of occlusion and tortuosity. More rigorously, they can be used quantitatively, for example, in the evaluation of the error in flow estimation by making available a reliable “gold standard” for flow conditions that cannot be practically achieved in an *in vivo* examination. Pipe phantoms for quantitative use should be constructed with materials that match the acoustic properties of tissues. In addition, in studies involving blood flow, the liquid flowing through the pipe phantoms should match the acoustic properties and flow properties (e.g. viscosity) of blood. Early pipe phantom developments focused on providing realistic spectral Doppler waveforms [1]. However, they could not be used quantitatively since the phantom materials were not acoustically tissue-matched [2]–[4]. Later efforts of pipe phantom studies focused on the development of phantom materials which match the acoustic properties of soft tissue [2]–[4], fluids which match the acoustic and viscous properties of blood [5]–[7], and materials which match the acoustic properties of blood vessel wall [8]–[10]. Recently, due to the development of ultrasound molecular imaging with microbubble contrast agents, pipe phantoms have been required to mimic the molecular properties of the blood vessels [11]–[14]. A molecular targeted (i.e. receptor mimic) coating may be applied to a phantom wall to model the simulated blood vessel wall [11], [13], [15]–[18].

In this paper, we describe designs and construction protocols for three different classes of phantoms: gelatin-based pipe phantom, polydimethylsiloxane (PDMS)-based pipe phantom, and Edinburgh Pipe Phantom (EPP). The materials and corresponding processes required to address each of the phantoms are described. The pipe phantoms described here have applications in the acoustical and optical assessment of the dynamics of microbubble contrast agents (with and without molecular targeting) and, in the case of a small diameter tube phantom (EPP), for assessing imaging system spatial resolution performance. We use the term “pipe” in place of “vessel” because the geometries presented are invariably smooth, circular cross-section voids with uniform diameter – i.e. pipe-like. In addition, the key intersection between the two categories of phantoms, which are flow phantoms for molecular imaging and EPP for system characterization, is their similar physical appearance as straight pipe.

Tissue-mimicking gelatin/agar materials have been used to construct phantoms for studies of medical ultrasound [19], [20] for several decades. Both the acoustic and mechanical properties of the gelatin-based phantoms have been investigated to construct phantoms possessing acoustical properties similar to those of human tissue [21]. For ultrasound molecular imaging in large blood vessels, wall-less gelatin-based pipe phantoms have been fabricated to mimic blood vessels [12], [14], [18], [22]–[24]. Functionalized pipe phantoms are required to mimic a stimulated blood vessel with molecular markers associated with specific disease (e.g. vascular cell adhesion molecule 1 (VCAM-1) and P-selectin for

cardiovascular diseases) expressed on the surface of the inner blood vessel wall [17], [25]. Additionally, measurements of the density of surface molecules coated on the flow channel wall are essential in the validation of quantitative measurements derived using ultrasound molecular imaging [26]. Consequently, accurate and reliable coating methods and associated quantification methods in pipe phantoms are of vital importance.

Graphite or aluminum powder is introduced in gelatin- or agar-based phantoms to act as scatterers in order to introduce attenuation similar to that obtained from soft tissue and a speckle pattern in the ultrasound image [21], [27]. Phantoms containing graphite or aluminum powders are generally opaque. Even without graphite or aluminum, the optical imaging distance is very short in gelatin/agar-based phantoms. In some studies, such as those involving measuring microbubble response to acoustic radiation forces [28], [29], the objects need to be observed optically via microscopy. Typically, small diameter (200 μm) transparent tubing is used for optical imaging with application of acoustic radiation forces [28]–[30]. However, there are no adequate transparent wall-less pipe phantoms for use in ultrasound that mimic large blood vessels. On the other hand, PDMS is widely used in microfluidics due to its good optical transparency and straightforward, robust fabrication process [31]–[33]. Consequently, the use of PDMS as a material for pipe phantom construction is particularly advantageous for applications where optical observation is necessary and modest deficiencies in terms of acoustic match to human tissue can be tolerated [34].

In this paper, the design, construction, and functionalization (where necessary) of three different types of pipe phantoms are introduced and validated for medical ultrasound research. The importance of this paper involves addressing the gap between the simplified experimental description found in standard articles and the exact experimental technique, such as fine detail of experimentation. Consequently, using the descriptive methods included in this article, researchers may obtain high quality results with minimal expenditure on materials and experimentation. By including a methodical description of materials and methods, the risk of performance variability and/or poor performance in use is minimized.

IMPORTANT NOTICE: Readers are reminded that in the methods presented, toxic materials are sometimes employed that require a functional fume hood. It is also necessary that readers check for compliance with all local, provincial, national and trans-national regulations relating to safety and environmental protection. In addition, sensible precautions are necessary when handling materials that are any combination of: hot, bulky, heavy, or hazardous.

It should be noted that some suppliers identified in this paper operate internationally and some do not. In many cases, local alternative suppliers can be found for those not operating internationally. Many of the larger international suppliers to the scientific community sell the same products using identical, or similar, part numbers from nationally-based distribution systems.

II. Wall-less Gelatin-based Pipe phantom

A. Section Overview

In this section, we introduce a custom designed phantom holder and associated procedures to reliably construct wallless gelatin-based pipe phantom containing a number of unique shape flow channels.

B. Materials

C. Equipment

D. Construction Procedure

1. Prepare the degassed deionized (DI) water (Table I) by letting the DI water sit for 24 h. Other methods can be used to accelerate this process, including using a heater or in-line degasser.
2. Clean the phantom holder (Table II) and glass rods (Table II) with 70% ethanol (Cat. No. BP2818-4, Fisher Scientific, Fair Lawn, NJ, USA) followed by sufficient DI water to remove surface dirt and grease. Wipe with lint free wipers (Kimtech Science, Kimberly-Clark, Roswell, GA, USA). Place them in oven (Table II) and let them dry completely at 60 °C for 30 min. **CRITICAL:** Failure to rigorously clean the glass rods may result in a rough channel surface. The phantom holder is custom designed to have a width of 9.0 cm, a height of 5.5 cm, a length of 27.5 cm, and a distance of 2.5 cm between adjacent flow channel holes (Fig. 1a). Standard female luer-lock connectors are integrated into the one-piece design for each flow channel hole (Fig. 1b). The phantom holder is made of high-strength heat-resistant Somos NanoTool material. In this design, 10 flow channels could be formed using this phantom holder.
3. Place the glass rods horizontally in the phantom holder to mold the flow channels (Fig. 1c). Place the phantom holder with glass rods in a plastic bucket, then fill the gap between the phantom holder and bucket with ice (Fig. 1d).
4. Heat degassed DI water in a beaker using the stirring hotplate (Table II). Cover the beaker with aluminum foil to reduce evaporation. Keep water stirring using a magnetic stir bar to ensure uniform heat distribution. The volume of water is 1.2-fold of the phantom volume. Monitor the temperature of water using a digital thermometer (Table II). When the temperature reaches 80 °C, turn the hotplate off, remove the aluminum foil cap, but keep water stirring using magnetic stirrer.
5. Mix gelatin (5.55%, w/w), agar (2.02%, w/w), and graphite (1.61%, w/w) in a clean plastic weigh dish and pour them very slowly into the beaker (Fig. 1e) (Table I). Agitate the water with the spoon as necessary, otherwise the mixed powder can be sucked into the vortex and become attached to the stirrer bar. Keep the liquid mass stirring at a high speed to ensure uniform distribution.

Monitor the temperature of the liquid mass using a digital thermometer.

CRITICAL: Pouring the powder too quickly or stirring the liquid mass too slowly may lead to agglomeration of the powder.

6. To improve the long-term durability of the gelatin-based phantom, antibacterial agents (e.g. sodium azide at a concentration of 0.1%) can be added to the mixture to act as a preservative [35].
7. When its temperature decreases to 50 – 60 °C, slowly pour the liquid mass into the phantom holder to the designated level. CRITICAL: If the temperature is too high (e.g. 70 °C) when pouring, an acoustically transparent layer without any graphite (typically 1 – 3 mm thick) will be formed underneath the top surface of the phantom and flow channels due to graphite sedimentation effects during the long cooling process. If the temperature is too low (e.g. 40 °C) when pouring, there is high risk to damage the inner surface of flow channels when pulling out the glass rods later. In addition, more air bubbles may be trapped in the phantom due to high viscosity of the liquid mass.
8. Immediately after the pouring step, the air bubble layer on the surface of the phantom is carefully wiped off using the lint free wipers (Fig. 1f). This step should be completed within 5 min after pouring while the viscosity of the liquid mass is still relatively low. CRITICAL: Failure to completely wipe off the air bubbles on top surface of the phantom may lead to the generation of a high attenuation air bubble layer with bright reflections.
9. Let the phantom cool down in the ice bucket for 1.5 h until the phantom surface is solidified. Cover the phantom surface with DI water to prevent drying. Let the phantom cool down for another 2 h until it is completely solidified.
10. Very slowly manually extract the glass rods by pulling in one direction. Do not rotate the glass rods. Flush the flow channels with degassed DI water for 3 min. Then examine the flow channels optically and under ultrasound to verify the presence of a smooth, specular reflecting, inner surface. CRITICAL: Pulling out the glass rods too fast, or rotating the glass rods may damage the inner surface – resulting in a rough surface.
11. Fill the flow channels with degassed DI water, and use plug caps to seal the channels. Cover the phantom surface with DI water, and then transfer it to the refrigerator for storage at 4 °C.

After completion of all the construction steps, the phantom is ready for experiments. Saline or blood mimic fluid can be used in the flow system. The entire flow systems, including the open-loop constant flow system and the close-loop pulsatile flow system are shown in Fig. 2. For the constant flow system (Fig. 2a), a syringe pump is used to provide constant flow rate. However, the running time is limited by the maximum volume of the syringe. For the pulsatile flow system (Fig. 2b), a pulsatile pump is used to provide pulsatile flow. In addition, B-mode image of the gelatin-based pipe phantom filled with DI water is shown in Fig. 3(a). Fig. 3(b) depicts the gelatin-based pipe phantom filled with biotinylated microbubbles. The vessel wall was coated with streptavidin (described in IV). Adherent

microbubbles were attached to the bottom vessel wall due to molecular binding. Fig. 3(c) shows the cross-sectional view of the gelatin-based pipe phantom.

III. Wall-less Polydimethylsiloxane (PDMS)-based Pipe phantom

A. Section Overview

In this section, polydimethylsiloxane (PDMS) is introduced to construct wall-less pipe phantoms in larger diameters (few millimeters) due to its good optical transparency and simple, robust fabrication process [31]–[33]. However, when using an unfilled PDMS, it is necessary to be aware of the tradeoff being made in terms of acoustic properties. The density, attenuation and velocity parameters of unfilled PDMS are poorly matched to human tissue [36]. However, for cases with a short path length and where optical imaging compatibility is essential, the tradeoff is warranted. Polyacrylamide (PAA) hydrogel also has good optical transparency and suitable acoustic properties. However, the fabrication process is more complicated and involves legitimate health/safety concerns [37]. The introduced PDMS-based pipe phantoms may be used for studying microbubble binding dynamics using the co-axial acoustic and optical imaging system [34].

B. Materials

C. Equipment

D. Construction Procedure

1. Methodically clean the glass rods (similar to steps in II.D.2). Place the clean glass rods (Table II) horizontally in a clean Petri dish (Cat. No. 09-720-500, Fisher Scientific, Fair Lawn, NJ, USA) to mold the bottom part of the flow channels (Fig. 4a). The distance between two consecutive glass rods is 1 cm. **CRITICAL:** Fail to rigorously clean the glass rods may lead to rough channel surface of the phantom.
2. Mix Sylgard 184 base and curing agent (Table III) by weight at a ratio of 10:1.1 in a clean plastic weigh dish at room temperature. Stir the base and curing agent mixture thoroughly using a plastic rod for at least 3 min. Air bubbles are generated during the mixing process.
3. Pour the PDMS mixture into the Petri dish with glass rods (Fig. 4b). For the top cover of the flow channels, 1 mm-thick PDMS layer is poured into another Petri dish.
4. Place the Petri dishes with PDMS mixture in a vacuum chamber connected to a vacuum line (approximately 6.7 kPa) of the fume hood to degas for 30 min, or until most of the air bubbles disappear. Slowly vent the vacuum chamber.
5. Place the Petri dishes with PDMS mixture in a vacuum oven (Table IV) with temperature set to 70 °C and cure them for 30 min.

6. In a dust-free environment, break the sides of the Petri dishes and carefully peel the PDMS disk off the bottom of Petri dishes (Fig. 4c). For the Petri dish with glass rods, place the PDMS disk with glass rods side (binding side) facing up, and use a razor blade to cut the PDMS disk into blocks with desired dimensions. Each PDMS block contains one glass rod. Then cut open the PDMS along the glass rod and carefully remove the glass rod from the PDMS block (Fig. 4d). The flow channels are formed in these PDMS blocks used as bottom layer. For the Petri dish with top cover PDMS layer, place the PDMS disk with contact side (binding side) facing up, and cut the top cover PDMS layer into rectangular shape with same dimensions of bottom PDMS blocks. Keep both the bottom and top cover PDMS blocks in separate clean and covered Petri dishes to avoid contamination of its surface by dust. **CRITICAL:** Avoid touching the binding sides of both bottom and top cover PDMS blocks. Contamination of the binding sides may lead to failure at the plasma binding step.
7. Apply plasma to both the top cover and bottom PDMS blocks, with binding sides facing up, simultaneously using a laboratory corona treater (Table IV) for 8 min. Sweep the head of the corona treater to cover all surfaces of PDMS blocks to ensure uniform distribution of plasma. Thereafter, bind together the bottom and top cover PDMS blocks with binding sides facing each other and with finger pressure for 10 s to form the phantom (Fig. 4e). Leave the phantom in room temperature for 30 min to ensure the firm binding. **CRITICAL:** The plasma treatment time of the treater are critical parameters and optimized based on our setup. Under treatment or over treatment of plasma may lead to unsuccessful binding of PDMS. Instrumentation-based optimization of plasma treatment parameters may be required. The bonding step can also be completed faster using an oxygen plasma oven/etcher. For example, place the PDMS blocks in an oxygen plasma oven (March II, Nordson March, Concord, CA, USA) for oxidation at 800 mTorr of oxygen gas and 30 W of power for 30 s [32], [33].
8. Insert connection tubing (Table IV) into the two ends of the phantom. Apply hot-melt glue on the connections using a glue gun (Table IV). Leakage is examined by injecting DI water into the phantom using a syringe pump at a flow rate of 75.4 mL/min (e.g. 10 cm/s with 4 mm-diameter in our settings). This testing flow rate should be the highest flow rate in the future experiments.

After completion of all the construction procedures, the PDMS-based pipe phantom is ready for experiments. It can be used in both the constant and pulsatile flow systems shown in Fig. 2. In addition, B-mode image of the PDMS-based pipe phantom filled with DI water is shown in Fig. 5(a). Fig. 5(b) shows the optical transparent PDMS-based pipe phantom with 4-mm flow channel diameter. In Fig. 5(c), a microscopy image depicts the adherent microbubbles at the bottom vessel wall of the PDMS-based pipe phantom. Details of how to take the photo can be found in previous studies [34].

IV. Molecular coating and quantification

A. Section Overview

In ultrasound molecular imaging, functionalized pipe phantoms are frequently constructed to study the binding dynamics between targeted microbubbles and stimulated blood vessel wall [11], [13], [14], [17], [26]. Streptavidin coated on the flow channel wall is used to mimic the molecular targets expressed by endothelial cells (e.g. VCAM-1 in cardiovascular diseases). Biotin molecules on the microbubble shell are used to mimic the antibodies (e.g. anti-VCAM-1 in cardiovascular diseases) targeted to the molecular targets of interest (e.g. VCAM-1). Consequently, molecular coating and quantification are introduced on pipe phantoms. It is recognized that the straight pipe phantoms presented in this study are less than perfect analogs of human diseased vessels. However, animal disease model surrogates also present immense challenges due to immense variability in molecular marker concentration, difficulties in quantification of molecular marker concentration, and presence of confounding challenges (e.g. vessel geometry, presence of other molecular markers, and possible less than perfect binding specificity). Therefore, it is beneficial to study the binding dynamics of microbubbles firstly in simple straight vessels for better controlled experiments.

B. Materials

C. Equipment

D. Molecular Coating – Gelatin-based Pipe Phantom

1. Repeat step II.D.1) to prepare degassed DI water.
2. Prepare the 5% (w/w) bovine serum albumin (BSA) (Table V) solution in DI water. Let the solution sit for at least 60 min at a temperature of 4 °C to eliminate most of the air bubbles.
3. For targeted flow channels coating with streptavidin, prepare the streptavidin (Table V) solution in DI water at an incubation concentration of 50 µg/mL (improved binding efficacy may be achieved by using a higher incubation concentration). Let the solution sit for 60 min at a temperature of 4 °C.
4. Flush the flow channels of gelatin-based pipe phantom with degassed DI water for 1 min. Use compressed air to blow out the residual water inside the flow channels. Seal one end of the flow channels using the plug caps (Table VI).
5. Slowly inject the streptavidin solution into the flow channels using a syringe (10 mL) with needle (20 gauge) from the open end. Avoid the generation of an air plug in the flow channels. When the flow channels are full, seal the open end with plug caps. **CRITICAL:** The injection volume should match the volume of the flow channel. If the former is less than the latter, an air plug is formed. Use compressed air to blow out the residual solution inside the flow channel. Repeat

this step to refill the flow channel. If the former is greater than the latter, leakage may occur due to a broken flow channel or loose plug cap.

6. Cover the gelatin-based pipe phantom surface with DI water, and then store it in the refrigerator for incubation at a temperature of 4 °C for 12 h.
7. Before the experiments, remove the plug caps and use compressed air to blow out the residual streptavidin solution in the flow channels. Seal one end of the flow channels using the plug caps. Use the same methods in step IV.D.5) to fill the flow channels with 5% (w/w) BSA solution, and incubate the flow channels for 10 min to prevent non-specific adhesion. **CRITICAL:** BSA is used to block the non-specific binding sites on the inner surface of flow channels. Fail to complete this step may lead to significant level of non-specific adhesion of microbubbles.

For control flow channels coated with BSA, follow steps IV.D.4) and IV.D.5) to fill the flow channels with 5% (w/w) BSA solution. Repeat step IV.D.6) to incubate the flow channels.

E. Molecular Coating – PDMS-based Pipe Phantom

1. Repeat step II.D.1) to prepare degassed DI water.
2. Repeat steps IV.D.1) to IV.D.3) to prepare BSA and streptavidin solutions.
3. Flush the flow channels of PDMS-based pipe phantom with degassed DI water for 1 min. Use compressed air to blow out the residual water inside the flow channels.
4. For targeted flow channels coating with streptavidin, slowly inject the streptavidin solution into the flow channels using a syringe (10 mL) with needle (20 gauge) from the connection tubing. When the flow channels are full, seal the connection tubing using binder clips. Avoid generation of an air plug in the flow channels (steps in IV.D.5).
5. Store the PDMS-based pipe phantom in the refrigerator for incubation at a temperature of 4 °C for 12 h.
6. Before the experiments, remove the binder clips and use compressed air to blow out the residual streptavidin solution in the flow channels. Use the same methods in step IV.D.5) to fill the flow channels with 5% (w/w) BSA solution, and incubate the flow channels for 10 min to prevent non-specific adhesion. **CRITICAL:** BSA is used to block the non-specific binding sites on the inner surface of flow channels. Fail to complete this step may lead to significant level of non-specific adhesion of microbubbles.
7. For control flow channels coating with BSA, follow steps IV.D.4) and IV.D.5) to fill the flow channels with 5% (w/w) BSA solution. Repeat step IV.D.6) to incubate the flow channels.

F. Quantification of Molecular Coating – Gelatin-based Pipe Phantom

1. Prepare the solution of Eu^{3+} -labelled streptavidin in DI water at the desired incubation concentration (typically 1–50 $\mu\text{g/mL}$, represented by C_1). Let the solution sit for 60 min at a temperature of 4 $^{\circ}\text{C}$.
2. Repeat steps IV.D.1) to IV.D.6) to incubate the flow channel for 12 h at a temperature of 4 $^{\circ}\text{C}$ using Eu^{3+} -labelled streptavidin solution.
3. Remove the plug caps and pour out the Eu^{3+} -labelled streptavidin solution. Use DI water to rinse the flow channel to remove excess free streptavidin. Use compressed air to blow out the residual solution inside the flow channel. Seal one end of the flow channel using a plug cap.
4. Slowly inject 1 mL enhancement solution (Table V) into the flow channel using a syringe (10 mL) with needle (20 gauge) from the open end. Then, immediately seal the open end with plug cap.
5. Invert the flow channels back and forth every 5 s for 1 min to let the enhancement solution absorb all Eu^{3+} on the inner surface of the flow channel. Thereafter, remove the plug caps and collect the enhancement solution from the flow channel. **CRITICAL:** The time for the enhancement solution to absorb the Eu^{3+} is critical in quantification of Eu^{3+} on inner surface of the flow channel. It is anticipated that the measured Eu^{3+} (obtained using spectrofluorometry) is overestimated. The average pore size in agarose gelatin-based pipe phantom was approximately 200 nm [38], [39]. Presumably, Eu^{3+} -labelled streptavidin molecules could permeate the phantom through the holes on surface during incubation. As a result, when washing the phantom channels, enhancement solution would take away Eu^{3+} from not only the streptavidin molecules attached onto the channel surface, but also a portion of streptavidin molecules embedded inside the phantom within a certain depth. However, the streptavidin molecules embedded inside the phantom cannot participate in the binding process with targeted microbubbles.
6. Transfer 200 μL of the collected enhancement solution to the microplate and use the dissociation-enhanced time-resolved spectrofluorometer (Table VI) to measure the fluorescence level (represented by I_1 on the spectrofluorometer unit).
7. Dilute the Eu^{3+} -labelled streptavidin in the enhancement solution at a concentration of 50 $\mu\text{g/mL}$ as the reference solution. Transfer 200 μL of the reference solution to the microplate and use the spectrofluorometer to measure the fluorescence level (represented by I_2 on the same unit as used for I_1 above).
8. The surface concentration (represented by C_S in the unit of molecule/ μm^2) of Eu^{3+} -labelled streptavidin in the flow channel is calculated by the following equation:

$$C_S = 1.6 \times 10^8 \left(\frac{I_1}{DLI_2} \right) \quad (1)$$

where L and D represent length and diameter of the flow channels in the unit of mm, respectively. Note that the molecular weight of streptavidin is assumed to be approximately 6×10^4 g/mol. A detailed derivation of Equation (1) is presented in Appendix.

9. Repeat steps IV.F.1) to IV.F.8) in 5 different phantom flow channels to decrease measurement error.

Repeat steps IV.F.1) to IV.F.9) at different incubation concentrations (e.g. 1 – 50 $\mu\text{g/mL}$) to achieve the calibration curve between surface concentration (C_S) and incubation concentration (C_I) of Eu^{3+} -labelled streptavidin.

V. Edinburgh Pipe Phantom (EPP)

A. Section Overview

The Edinburgh Pipe Phantom (EPP) is designed for the performance assessment of ultrasound grey-scale images. Measurement of the imaged lengths of a series of anechoic pipes enables the calculation of the resolution integral which is a measure of the ratio of penetration to resolution for the imaging system. Two other imaging parameters are also defined: depth of field (L_r) and characteristic resolution (D_r) such that $R = L_r/D_r$ [40]. The EPP consists of a block of tissue mimicking material (TMM) containing a series of fluid-filled pipes which are molded into the TMM during manufacture. The TMM recipe is described by [41] and has acoustical properties similar to those of soft tissue with speed of sound of 1540 m/s and attenuation coefficient of 0.5 dB/cm/MHz. Pipe sizes are chosen to provide equally spaced data points on the resolution integral graph from which L_r and D_r can be calculated [42]. The location of each pipe is shown in the schematic diagram in Fig. 6. This figure also illustrates the dimensions of the box required for manufacture of the EPP. An isometric view and B-mode image of the EPP are shown in Fig. 7. The pipes are inclined at an angle of 40 degrees to the vertical and have diameters ranging from 0.42 mm to 7.9 mm. The majority of pipes are molded on stainless steel rods of outer diameter corresponding to standard needle gauges. During manufacture the rods are held in place by a rigid former (not shown in Fig. 6). Once the TMM has set, the phantom is immersed in a TMM maintenance fluid, the rods are withdrawn and the TMM maintenance fluid fills the pipes and additionally provides coupling between the phantom and transducer under test. The fluid is a water/glycerol and antibacterial solution, the addition of the latter fluid minimizing the likelihood of mold growth on the phantom.

Additionally, a smaller version of this phantom with pipes as small as 64 μm has been manufactured to assess high frequency clinical and preclinical scanners up to 55 MHz [42].

B. Manufacture of the Edinburgh Pipe Phantom

Table VII details the ingredients, weight concentration and distributor of each of the ingredients required in the manufacture of the agar-based TMM. Table VIII describes the equipment required to make the EPP and the personal protective equipment (PPE) required. The following are the procedures to follow to manufacture the phantom assuming that the container is of the same dimensions as that illustrated in Fig. 6.

Preparation of TMM:

1. Place the rods into the former (Table VIII), and place the former into the pipe phantom container (Table VIII). Insert spacer between end of former and container. Place pipe phantom container with rods, former and spacer into a refrigerator at 4 °C for 12 h.
2. The following morning fill the water bath with hot water, using a kettle as required. Set the heater temperature to 96 °C.
3. The quantity of TMM needed to fill the EPP container and a small sample jar can be found in Table IX. This table also shows the breakdown of ingredients.
4. Set up the precision balance (Sartorius 1202 MP, Table VIII) in the fume cupboard, use calibrated weights to ensure it is working correctly. Place the ceramic pot on the balance and tare. Also place the mixing beaker (Table VIII) in the fume cupboard.
5. Set up the heavy weight balance (Ohaus Scout Pro 6 kg, Table VIII) near a sink. Place the cooking beaker (Table VIII) and the rinsing beaker (Table VIII) nearby.
6. Weigh the correct quantity of water into the cooking beaker (Table VIII). From the cooking beaker, pour 1000 mL and 250 mL of water into the rinsing and mixing beakers, respectively.
7. Return the cooking beaker to the scales and tare.
8. Using eye protection, weigh the 99% glycerol (Table VII) into the cooking beaker and tare. Repeat for the 10% Benzalkonium chloride solution (Table VII).
9. *Using PPE gloves, transport the 0.3 μm aluminum oxide to the fume cupboard; using a plastic spoon measure the correct weight into the ceramic pot. Note that with the fume cupboard on the balance will become less accurate due to air movement. Temporarily stop transferring and turn off the fume cupboard if needed, to get a stable reading.
10. *Empty the ceramic pot into mixing beaker (Table VIII), cleaning as much of the particles as possible into the beaker with a dry paper towel.
11. *Ensure ceramic pot and spoon are clean. Repeat steps V.B.9) and V.B.10) for the 3 μm aluminum oxide (Table VII) and silicon carbide.
12. The fume cupboard can be turned off while the agar is measured. Once measured, keep the agar separate for now.
13. Place the cooking beaker in the water bath. Have a colleague hold the beaker down or weigh it down with a metal bar as it will float until the remaining fluid is poured into it.
14. Thread the stirrer (curved-paddle) through the central hole in the water bath lid and secure it in the overhead motor. Place the lid in the raised position.

Turn on the fume cupboard during the steps identified with an “”.

15. Adjust the stirrer and motor so that the paddle of the stirrer is close to the bottom of the cooking beaker but can rotate freely without touching the beaker. Set the stirrer to speed A – 120 rpm. (Table X).
16. *Mix the aluminum and silicon carbide particles (Table VII) in the mixing beaker thoroughly to a smooth paste with the plastic spoon.
17. *Add more water from the rinsing beaker until the mixing beaker has 500 mL. Then using the hand-held blender mix the solution for 1 to 2 min to break up clumps into a fine solution.
18. Turn the fume cupboard off, transfer and pour the contents of the mixing beaker into the cooking beaker. Use the remaining water in the rinsing beaker to wash all the particles from the blender, the spoon and the mixing beaker into the cooking beaker.
19. Top up the level of the water bath so that it matches that of the TMM within the cooking beaker.
20. Slowly sprinkle the agar particles into the cooking beaker. Agitate the water with the plastic spoon as necessary, otherwise agar can be sucked into the vortex and become attached to the stirrer shaft.
21. Lower the water bath lid, using the guide pins to aid alignment of the cooking beaker. Ensure these fit well.
22. When the temperature of the TMM reaches 95 °C start the timer and set stirrer speed as per row B in Table X.
23. After 70 min set the heater to 45 °C and the stirrer speed as per row C in Table X. The following procedure is followed for cooling and pouring the TMM.
24. Connect the cold water tap to the inlet of the heat exchanger and place the outlet pipe in the sink.
25. Turn on the tap so there is a steady but low flow through the system.
26. Allow the water bath and TMM to cool. They should both level off at 45 °C after around 30 min assuming a temperature of 20 °C.
27. When TMM reaches 45 °C, set the temperature control to 40 °C and monitor TMM temperature continuously.
28. Condensation will have formed on the underside of the Perspex lid, carefully lift and dry with paper towels to prevent excess water dripping into the TMM.
29. Two staff members are strongly advised for the remaining steps. Set up the phantom container on a trolley adjacent to the water bath and have paper toweling ready.
30. When the temperature of the TMM reaches 41 °C switch off the stirrer and with the aid of a colleague separate the stirrer from the motor unit and remove the water bath lid.

31. Begin agitating the TMM with the stirrer manually as the cooking beaker is removed from the water bath and place onto the paper towels. The agar solidifies to gel at 38 – 39 °C, depending on whether it is stationary or moving.
32. Remove any froth from the top of the TMM with the plastic spoon.
33. Remove phantom container with rods inserted from fridge. Spray each pipe with aerosol freezer.
34. Pour the TMM steadily into the desired phantom container and accompanying sample pot. Avoid creating air bubbles where possible by avoiding direct pouring onto the rods in the container. While pouring, the container should be tilted initially so that it can be filled by pouring onto the inner side surface. As pouring continues reduce the tilt until the container is vertical. Fill the container to 30 mm from the top.
35. Allow the TMM to solidify in a place protected from disturbance.
36. After 1 h, add TMM maintenance fluid to the container so that top of the TMM is covered, and leave 12 h to fully solidify.

C. Preparing Phantom for Use

1. Remove spacer from end of tank.
2. Add more TMM maintenance fluid to a level above the scanning surface.
3. Clear excess TMM debris and residue from area vacated by the spacer.
4. Carefully extract the rods one at a time, topping up with TMM maintenance fluid as required.
5. Remove the former, again keep TMM maintenance fluid level above scanning surface.
6. Clear excess TMM debris from the end of the phantom and, using a razor blade, trim off excess TMM from top of pipes to ensure level scanning surface.
7. Fit a 50 micron Polymethylpentene (TPX) tray into top of phantom to protect the scanning surface.
8. Scan to check integrity of phantom with high and low frequency probes (typically 3 MHz and 10 MHz center frequencies).

D. Care and Maintenance of Edinburgh Pipe Phantom (This procedure should be followed every 2 to 3 months)

1. Remove lid and wipe off residue
2. Remove the TPX layer and wash under the tap.
3. Replace if worn, damaged or very dirty.
4. Using a syringe, suck up all the floating debris. Attaching a tube to the end of the syringe allows access to the bottom of the tank.

5. Refill phantom with TMM maintenance fluid.

E. Instructions on How to Manufacture the TMM Maintenance Fluid

1. Weigh 1000 g of water into the cooking beaker (Table VIII) and tare.
2. Using eye protection, weigh 143.9 g of 99% glycerol (Table VII) into the cooking beaker and tare.
3. Weigh 58.71g of 10% Benzalkonium chloride solution (Table VII).
4. Stir well.

VI. Discussion

A. Purpose and Application of Each Type of Phantom

Three types of phantoms were introduced in this study, including the gelatin-based pipe phantom, PDMS-based pipe phantom, and Edinburgh Pipe Phantom. The gelatin-based pipe phantom with molecular targeting coating is designed and constructed to mimic large blood vessels in ultrasound molecular imaging. It can be used for regular Doppler imaging, but the main purpose is to study the microbubble dynamics under the influences of molecular binding forces, flow shear forces, and acoustic radiation forces. It has many applications in ultrasound molecular imaging including prototyping of new pulse sequence and testing of new designs of targeted microbubbles. The PDMS-based pipe phantom is also designed for mimicking large blood vessels in ultrasound molecular imaging, but with high transparency to perform optical observation. It can be used for simultaneous acoustic and optical assessment of microbubble dynamics inside the flow channel. The Edinburgh Pipe Phantom with small diameter tubes is designed to assess the spatial resolution and contrast quality of imaging system. It can be used for performance evaluation and validation of ultrasound scanners. In summary, the characteristics and suitable applications of each phantom is summarized in Table XI. The characteristics listed in this table need to be considered when choosing or designing a phantom to fit the specific purposes.

B. Acoustic Properties of the Phantoms

Based on Table XII, TMM of the EPP has the best tissue-matching acoustic properties [41]. The measured acoustic properties of the gelatin-based phantom are similar to those of tissue. The speed of sound and attenuation can be adjusted by changing the weight ratio of gelatin, 1-propanol, and graphite, respectively [43]. The PDMS-based phantom has the poor tissue-matching acoustic properties. The measured acoustic properties of the PDMS-based phantom are similar to those of silicone [37].

C. Limitations

This paper does not address anatomic phantoms, typically including wire targets and “lesions” comprising regions possessing calibrated differences in backscatter level. These phantoms are important in the fields of design and validation of anatomic imaging systems and are addressed in other literature [44]. Furthermore, these more common phantom

designs are readily available commercially so there is relatively little need to develop a laboratory “in-house” approach to phantom fabrication.

Both the gelatin-based and PDMS-based phantoms designed for ultrasound molecular imaging are straight-tube wall-less phantoms. They can be used under either constant or pulsatile flow conditions. We prefer this design for our studies for the following two reasons: (1) The rod withdrawal approach to fabrication of smooth and straight flow channels is simple and effective. Tortuous and branching vessels are more difficult to make. (2) Using a simple geometry allows the wall shear conditions to be accurately estimated based on diameter and peak or mean flow velocity. Therefore, better controlled experiments can be achieved. It is a worthwhile future goal to extend the method to more complex geometries.

To properly estimate the binding dynamics of microbubbles with targeted vessel wall in realistic physiological conditions, the realistic anatomy/geometry of blood vessels, viscoelasticity of vessel wall, rheology of blood flow, and flow conditions need to be taken into consideration. The phantoms listed in this study are based on simplified models of blood vessels and flow properties. More advanced pipe phantoms can be developed to provide more realistic estimation of binding dynamics of microbubbles. In addition, the pipe phantoms listed in the flow phantoms are mainly targeted at large arteries or veins with diameter around 4 mm. Diameter of the pipes can be changed to fit different applications, for example, microvasculature.

The feasibility and stability of the gelatin-based and PDMS-based pipe phantoms are demonstrated in previous studies [25], [26], [34]. The variabilities of results in gelatin-based pipe phantoms are greater than those in PDMS-based pipe phantoms. In order to achieve statistically significant results, we recommend the experiments to be repeated in 10 different flow channels. For the coating quantification methods introduced in this paper, relative surface concentration of molecules can be measured accurately [26]. However, due to the reasons mentioned in IV.F.5), the absolute surface concentration of molecules can hardly be measured accurately.

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APPENDIX

A detailed derivation of Equation (1): The inner surface area of the flow channel is: 3.14DL (mm^2). The total number of Eu^{3+} (equals to the total number of Eu^{3+} -labelled streptavidin on the inner surface of flow channel) in the 1 mL collected enhancement solution after wash is: $C_s \times 3.14\text{DL} \times 10^6$. Since only 200 μL of the collected enhancement solution (total volume is 1 mL) was used for fluorescence measurement, measurement I_1 is proportional to the total number of Eu^{3+} in the 200 μL collected enhancement solution: $I_1 \sim C_s \times 3.14\text{DL} \times 10^6 \times 0.2$.

$$I_1 \sim 6.28 \times 10^5 C_s DL \quad (2)$$

For the reference measurement, the mass of Eu^{3+} -labelled streptavidin in the 200 μL enhancement solution is: $200 \mu\text{L} \times 50 \mu\text{g/mL} = 1^{-5} \text{ g}$. Consequently, the total number of Eu^{3+} (equals to the total number of Eu^{3+} -labelled streptavidin) in the 200 μL enhancement solution is: $1^{-5} / (6 \times 10^4) \times N_A$. The molecular weight of streptavidin is approximately $6 \times 10^4 \text{ g/mol}$, and N_A is the Avogadro's number ($6.02 \times 10^{23} \text{ mol}^{-1}$). The reference measurement I_2 is proportional to the total number of Eu^{3+} : $I_2 \sim 1^{-5} / (6 \times 10^4) \times N_A$.

$$I_2 \sim 1.00 \times 10^{14} \quad (3)$$

Based on Equation (2) and (3), we have:

$$\frac{I_1}{I_2} = \frac{6.28 \times 10^5 C_s DL}{1.00 \times 10^{14}} \quad (4)$$

Equation (1) can be derived directly from Equation (4).

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Figure 1.

Photographs of the major steps involved in the fabrication of wallless gelatin-based pipe phantom. (a) Phantom holder. (b) Zoom-in of the female luer-lock fittings on the phantom holder incorporated into the holder directly via the 3D printing process. (c) Glass rods were placed in the phantom holder with plug caps. (d) Phantom holder was placed in the ice bucket. (e) Mixture of gelatin, agar, and graphite. (f) Surface air bubbles were wiped clean.

Figure 2. Schematic illustrations of the flow system for gelatin-based and PDMS-based pipe phantoms. (a) Open-loop constant flow system with syringe pump. (b) Close-loop pulsatile flow system with pulsatile flow pump. The gelatin-based and PDMS-based pipe phantoms can be used in both constant flow and pulsatile flow systems.

Figure 3.

Images of the gelatin-based pipe phantom. (a) B-mode image showing the flow channel filled with DI water. (b) B-mode image showing the flow channel filled with biotinylated microbubbles. The vessel wall was coated with streptavidin. After acoustic radiation forces applied, adherent microbubbles were attached to the bottom vessel wall. (c) Photo showing the cross-sectional view of the gelatin-based pipe phantom.

Figure 4.
Schematic illustration of the procedures for fabricating PDMS-based pipe phantom.

Figure 5.

Images of the PDMS-based pipe phantom. (a) B-mode image showing the flow channel filled with DI water. (b) Photo showing the transparent PDMS-based pipe phantom. Three flow channels were built in the phantom. (c) Microscopy image showing the adherent microbubbles on the bottom vessel wall.

Figure 6.
Schematic of the EPP with position and size of pipes also shown.

Figure 7.
(a) Isometric view, and (b) B-mode image of the EPP.

Table I

Materials for the gelatin-based pipe phantom

Material	Weight (%)	Manufacturer and Details
DI water	90.82	
Gelatin	5.55	Type B, Cat. No. G7-3, Fisher Scientific, Fair Lawn, NJ, USA
Agar	2.02	Cat. No. AC400400010, Acros Organics, Geel, Belgium
Graphite	1.61	< 20 μm , Cat. No. 282863-1KG, Sigma-Aldrich, St. Louis, MO, USA

Table II

Equipment for the gelatin-based pipe phantom

Equipment	Manufacturer and Details
Phantom holder	Custom-designed. Applied Rapid Technologies, Fredericksburg, VA, USA.
Glass rod	Heat-resistant borosilicate, Cat. No. 8496K12, diameter = 4 mm, McMaster-Carr, Robbinsville, NJ, USA
Oven	VWR-1400E, VWR, Radnor, PA, USA
Stirring hotplate	Cat. No. 11-520-49SH, Fisher Scientific, Fair Lawn, NJ, USA
Digital thermometer	Cat. No. 14-648-46, Fisher Scientific, Fair Lawn, NJ, USA

Table III

Materials for the PDMS-based pipe phantom

Material	Weight (%)	Manufacturer and Details
Polydimethylsiloxane (PDMS) base	90.1	Sylgard 184 Silicone Elastomer Kit, Dow Coming, Midland, MI, USA
PDMS curing agent	9.9	Sylgard 184 Silicone Elastomer Kit, Dow Coming, Midland, MI, USA

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Table IV

Equipment for the PDMS-based pipe phantom

Equipment	Manufacturer and Details
Vacuum oven	VWR-1400E, VWR, Radnor, PA, USA
Corona treater	BD-20, Electro-Technic Products, Chicago, IL, USA
Connection tubing	4 mm inner diameter, Cat. No. 5186T13, McMaster-Carr, Robbinsville, NJ, USA
Glue gun	Cat. No. 75215A65, McMaster-Carr, Robbinsville, NJ, USA

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Table V

Materials for the molecular coating and quantification

Material	Manufacturer and Details
Bovine serum albumin (BSA)	Cat. No. A7906-100G, Sigma-Aldrich, St. Louis, MO, USA
Streptavidin	Cat. No. AS-60659, AnaSpec, Fremont, CA, USA
Eu ³⁺ -labelled streptavidin	DELFA Eu-N1, Prod. No. 1244-360, PerkinElmer, Waltham, MA, USA
Enhancement solution	DELFA, Prod. No. 1244-105, PerkinElmer, Waltham, MA, USA

Table VI

Equipment for molecular coating and quantification

Equipment	Manufacturer and Details
Plug cap	Male luer-lock fitting, Cat. No. 51525K38, McMaster-Carr, Robbinsville, NJ, USA
Microplate spectrofluorometer	SPECTRAmax GEMINI XS, Molecular Devices, Sunnyvale, CA, USA

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Table VII

Weight concentration of ingredients used to manufacture the tmm

Ingredients	Weight (%)	Manufacturer and Details
DI water	78.83%	
Glycerol	11.21%	99% pure, (G7757) Sigma-Aldrich Company Ltd. The Old Brickyard, New Road, Gillingham, Dorset, UK Merck Agar-Agar technical (111925) (53648 5K) VWR International Ltd. Hunter Boulevard, Magna Park, Lutterworth, Leics, UK
Agar	3%	
Al ₂ O ₃ powder	0.95%	3 µm, Logitech Ltd. Erskine Road, Old Kilpatrick, Glasgow, Scotland
Al ₂ O ₃ powder	0.88%	0.3 µm, Logitech Ltd. Erskine Road, Old Kilpatrick, Glasgow, Scotland
SiC powder	0.53%	400 grain, Logitech Ltd. Erskine Road, Old Kilpatrick, Glasgow, Scotland
Benzalkonium chloride (C ₆ H ₅ CH ₂ N(CH ₃) ₂ Cl)	4.6%	10% solution, (09621) (50% solution, diluted in-house to 10%) Sigma-Aldrich Company Ltd. The Old Brickyard, New Road, Gillingham, Dorset, UK

Table VIII

Equipment required to manufacture epp

Equipment	Manufacturer and Details
Overhead stirrer	IKA EUROSTAR power control-visc IKA Werke GmbH & Co. KG, Staufen, Germany
Thermoregulator	Techne TE-10D Techne Ltd. Duxford, Cambridge, UK
6 kg weighing balance	Ohaus Scout Pro Ohaus Corp. Pine Brook NJ. USA
300 g weighing balance	Sartorius 1202 MP Sartorius AG Goettingen, Germany
Mixing beaker, 2000 mL	Standard lab ware Pyrex glass, VWR INTERNATIONAL
Cooking beaker, 4000 mL	Stainless steel beaker with handle and spout, VWR INTERNATIONAL
Rinsing beaker, 200 mL	VWR INTERNATIONAL
Plastic spoon	VWR INTERNATIONAL
Ceramic pot	VWR INTERNATIONAL
Digital thermometer	TENMA 72-2060 RS Components Ltd. Birchington Road, Corby, Northants, NN17 9RS, UK
Hand-held blender	e.g. Moulinex
Rubber hose/siphon	Custom design to meet user's need
Phantom container / TMM mold	Custom design to meet user's need
Former	Custom design to fit phantom container
Water bath	Custom design to meet user's need
Fume cupboard/Safety goggles / Disposable gloves	Personal Protective Equipment (PPE)

Table IX

Required quantities of tmm and constituent ingredients for Edinburgh Pipe Phantom

Phantom Type	Edinburgh Pipe Phantom
Volume (g)	3150
Water (g)	2623.76
99% Glycerol (g)	377.56
10% Benzalkonium Chloride (g)	154.04
SIC-powder, 400 grain (g)	17.64
Al ₂ O ₃ – powder, 3 micron (g)	31.50
Al ₂ O ₃ – powder, 0.3 micron (g)	29.30
Agar (g)	100.17

Table X

Guide to RPM Settings during TMM making

Speed setting	When used	RPM stirrer
A	Mixing of ingredients “Pre-cooking phase”	120
B	TMM temperature 96 °C “Cooking phase”	110
C	When cooling “Post Cooking phase”	100

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Table XI

Summary of the gelatin-based phantom, PDMS-based phantom, and Edinburgh Pipe Phantom

	Gelatin-based pipe phantom	PDMS-based pipe phantom	Edinburgh Pipe Phantom
Tissue mimicking material	Gelatin	PDMS	TMM
Optical transparency	Opaque	Transparent	Opaque
Tissue matching performance (Table XII)	Good	Poor	Good
Circulation flow type	Constant and pulsatile	Constant and pulsatile	NA (static)
Molecular coating feasibility	Yes	Yes	NA
Purpose	Mimic blood vessel	Mimic blood vessel	Assess system resolution
Application	Doppler / molecular imaging	Doppler / molecular imaging	Spatial/contrast imaging performance evaluation / validation

Table XII

Properties of gelatin-based phantom, pdms-based phantom, and Edinburgh Pipe Phantom

	Gelatin-based pipe phantom	PDMS-based pipe phantom	Edinburgh Pipe Phantom [41]
Speed of sound (m/s)	1554 ± 82 (4 – 12 MHz)	981 ± 72 (5 – 13 MHz)	1541 ± 3 (3 – 10 MHz)
Attenuation (dB/cm/MHz)	0.54 ± 0.04 (4 – 8 MHz)	4.0 ± 0.5 (5 – 13 MHz)	0.5 ± 0.03 (3 – 10 MHz)