A simple method for determining the coagulation threshold temperature of transparent tissue-mimicking thermal therapy gel phantoms: Validated by magnetic resonance imaging thermometry

N. Patrik Brodin

*Institute for Onco-Physics, Albert Einstein College of Medicine, Bronx, New York 10461
and Department of Radiation Oncology, Montefiore Medical Center, Bronx, New York 10461*

Ari Partanen

Clinical Science MR Therapy, Philips, Andover, Massachusetts 01810

Patrik Asp

Liver Research Center and Radiation Oncology, Albert Einstein College of Medicine, Bronx, New York 10461

Craig A. Branch

Department of Radiology, Albert Einstein College of Medicine, Bronx, New York 10461

Chandan Guha and Wolfgang A. Tomé^{a)}

*Institute for Onco-Physics, Albert Einstein College of Medicine, Bronx, New York 10461
and Department of Radiation Oncology, Montefiore Medical Center, Bronx, New York 10461*

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Purpose: Tissue-mimicking thermal therapy phantoms that coagulate at specific temperatures are valuable tools for developing and evaluating treatment strategies related to thermal therapy. Here, the authors propose a simple and efficient method for determining the coagulation threshold temperature of transparent thermal therapy gel phantoms.

Methods: The authors used a previously published gel phantom recipe with 2% (w/v) of bovine serum albumin as the temperature-sensitive protein. Using the programmable heating settings of a polymerase chain reaction (PCR) machine, the authors heated 50 μ l gel samples to various temperatures for 3 min and then imaged them using the BioRad Gel Doc system to determine the coagulation temperature using an opacity quantification method. The estimated coagulation temperatures were then validated for gel phantoms prepared with different pH levels using high-intensity focused ultrasound (HIFU) heating and magnetic resonance imaging (MRI) thermometry methods on a clinical MR-HIFU system.

Results: The PCR heating method produced consistent and reproducible coagulation of gel samples in precise correlation with the set incubation temperatures. The resulting coagulation threshold temperatures for gel phantoms of varying pH levels were found to be 44.1 ± 0.1 , 53.4 ± 0.9 , and 60.3 ± 0.9 °C for pH levels of 4.25, 4.50, and 4.75, respectively. This corresponded well with the coagulation threshold temperatures determined by MR-thermometry, with coagulation defined as a 95% decrease in T2 relaxation time, which were estimated at 53.6 ± 1.9 and 62.9 ± 2.4 °C for a pH of 4.50 and 4.75, respectively.

Conclusions: The opacity quantification method provides a fast and reproducible estimate of the coagulation threshold temperature of transparent temperature-sensitive gel phantoms. The temperatures determined using this method were well within the range of temperatures estimated using MR-thermometry. Due to the specific heating capabilities of the PCR machine, and the robust determination of coagulation threshold temperatures based on the statistically significant increase in the opacity of gel samples, coagulation temperatures can be determined more precisely and with less variability compared to MRI-based methods. © 2016 American Association of Physicists in Medicine. [<http://dx.doi.org/10.1118/1.4941361>]

Key words: thermal therapy, focused ultrasound, MRI, tissue-mimicking phantoms

1. INTRODUCTION

Thermal therapy delivered via focused ultrasound, oscillating magnetic particles, microwaves, lasers, or radiofrequency ablation has emerged as an alternative or complement to invasive surgery or ionizing radiation and chemotherapy,

especially in the last few decades.¹ The increased interest in high-intensity focused ultrasound (HIFU) and other thermal therapy modalities has led to the development of many different alternatives for tissue-mimicking phantoms (TMPs) to be used for quality assurance, research, training, and treatment optimization purposes.^{2–8} For HIFU applications,

such phantoms provide the visualization or measurement of the heated area or volume within a setting that resembles the acoustic and thermal properties of, e.g., muscle, internal organs, or skin. Two key properties to be determined using TMPs are the geometrical size and location of the heating and the temperatures obtained within the heated region.

The development of magnetic resonance imaging (MRI) techniques has allowed for precise delivery and temperature monitoring of modern thermal therapy. As a result, MRI-guided HIFU is currently being investigated for a number of indications in cancer treatment as this can be delivered as a highly focused, noninvasive therapy that is not dose limiting.⁹ Some TMPs provide visual feedback of the heated region within the phantom whereas others require external measurements using, e.g., MRI.⁴ A common method for assessing the temperature achieved within the TMP is observing the coagulation of temperature-sensitive proteins within the phantom. For this type of TMP, it is important to determine the coagulation threshold temperature, which can be done using MR-thermometry and T2 mapping, as previously published for polyacrylamide gel phantoms.⁷ Other methods for determining coagulation threshold temperatures include spectrophotometry, which has been shown to be feasible for phantoms containing thermochromic dye that transiently changes color at a certain threshold temperature.¹⁰

Depending on the application, the desired coagulation threshold temperatures of the TMPs may vary, something that can usually be achieved by altering the biochemical properties of the gel phantom. Although MRI-based methods have been shown to be robust and reproducible for determining coagulation threshold temperatures, it can be time consuming, expensive, or not readily available and as such might not be optimal for continuous quality assurance or for the assessment of new gel recipes, or thermal therapy regimens.

Here, we present a simple method for determining the coagulation threshold temperature of transparent tissue-mimicking thermal therapy gel phantoms using controlled heating of gel samples and quantifying the resulting increase in opacity of the heated samples using image processing tools. In addition, we validate the results of this method using the focused heating and MRI-thermometry capabilities of the Philips Sonalleve clinical MR-HIFU system. We also provide an example of the application of this coagulation threshold temperature determination method and its usefulness for evaluating and comparing various focused ultrasound treatment parameters.

2. METHODS AND MATERIALS

2.A. Temperature sensitive gel phantom

We used a recipe for a tissue-mimicking gel phantom containing 2% (w/v) of bovine serum albumin (BSA) as the temperature-sensitive protein, which was previously published by McDonald *et al.*⁷ For the purpose of this study, we required a phantom with adjustable coagulation temperature, which for the chosen phantom can be accomplished by altering pH levels. McDonald *et al.* explored several different acoustic attenuation modifiers, and they recommend using

Intralipid® (30% v/v)⁷ due to maximum MRI T2 contrast between coagulated and uncoagulated samples. Here, we chose to use the recipe with corn syrup (60% w/v), approximating the acoustic attenuation properties of muscle, as our studies required the phantom to be transparent. McDonald *et al.* presented the coagulation temperature evaluation of the Intralipid-containing phantom as a function of pH level, although for our purposes we needed to determine the coagulation threshold temperature for the corn syrup based gels used in our ultrasound studies.

2.B. Determining the coagulation threshold temperature by opacity quantification following sequential polymerase chain reaction (PCR) heating

MRI systems may not be readily available for most research labs and may thus be inconvenient to determine the coagulation threshold temperature of the gel phantoms. Therefore, we propose to use the sequential heating capabilities of a PCR machine, a device that is available in most research labs, to heat small samples of gel to various temperatures and determine when coagulation occurs. Right before the larger gel phantoms were cast and placed at 4°C to harden, we transferred aliquots of gel to PCR tubes (eight tubes connected in one strip) pipetting 50 μ l of gel into each tube (cf., Fig. 1). We generated gel batches of pH levels 3.96, 4.10, 4.25, 4.50, and 4.75, respectively, in order to determine how the resulting coagulation threshold temperatures depend on pH, when estimated using our proposed method.

The PCR machine was then programmed to heat the tubes for 3 min to increasing temperatures from left to right, and once the coagulation threshold temperature was reached the gel became cloudy and opaque. Determining the coagulation threshold temperature purely by visual examination is subjective and as such we employed a quantitative

FIG. 1. Images of heated gel samples demonstrating the strict temperature dependent coagulation. For clarity, the images are shown with inverted pixel intensities (white = highest values, black = lowest values) and as such increasing coagulation results in higher pixel values. The pH of these gel samples was 4.50 and the corresponding heating temperatures are given in °C with an example of the drawn ROI shown as a white rectangle. Scale bar = 5 mm.

statistical method to determine what would be defined as the point where coagulation starts to occur. The heated PCR tubes were imaged using a Bio-Rad Gel Doc system (Bio-Rad, Hercules, CA) and the accompanying IMAGE LAB software, ensuring that no pixels were saturated, providing quantifiable images of the gel opacity at different heating temperatures. To ensure reproducibility, the heating and imaging procedure was repeated for at least two separate gel batches for each studied *pH* level. Figure 1 shows two examples of these images with corresponding heating temperatures. A dedicated analysis program was developed using MATLAB (Mathworks Inc., Natick, MA) to quantitate the opacity of the gel samples, in which rectangular regions of interest (ROIs) were drawn across all samples in a series (cf., Fig. 1). Pixel values of the centermost 15×15 pixel elements for each tube were automatically recorded when drawing the ROIs. The repeatability of the pixel measurements was determined by measuring the same set of heated samples four consecutive times, which showed a standard deviation of less than 1% in the resulting average pixel values for each sample.

The mean and standard deviation of the pixel values were then computed for the image samples relating to the corresponding heating temperatures, using the MATLAB program. As the absolute pixel intensity depends on the exposure time of the images, we derived the coagulation threshold temperatures based on the statistically significant increase in opacity compared to the samples where there was clearly no coagulation of the BSA within the gel. The threshold pixel intensity value was determined by a statistically significant increase in opacity, which was set as the high end of the 95% confidence interval of the pixel values in the uncoagulated samples, i.e., the statistical significance level was set to 5%. To evaluate the effect of the chosen significance level on the resulting coagulation threshold temperature, we performed a sensitivity analysis setting the corresponding significance level to either 10% (lower pixel intensity threshold) or 1% (higher pixel intensity threshold), respectively.

2.C. Validating coagulation temperature measurements using a MR-HIFU system

To validate the coagulation threshold temperatures estimated using our PCR heating method, we employed the Sonalleve MR-HIFU system (Philips Healthcare, Vantaa, Finland). HIFU exposures (sonications) were delivered to the gel phantoms while simultaneously acquiring temperature maps calculated from MR images using the proton resonance frequency shift (PRFS) method.¹¹ The resulting change in T2 relaxation time in the heated areas of the phantom was then quantified, similar to the method used by McDonald *et al.*⁷ As PRFS-based MR-thermometry only measures relative temperature change, the temperature of the gel phantom prior to the first sonication was measured using a thermocouple thermometer. To allow for good acoustic coupling during the sonications, we assembled a phantom setup consisting of commercial ultrasound gel pads (Aquaflex gel pad, Parker Labs, Fairfield, NJ) sandwiching the temperature-sensitive gel (approximately 7.5 mm thick) between layers of ultrasound gel pads, as shown in Fig. 2. Absorber gels were placed following the last ultrasound gel layer (based on the HIFU being delivered from beneath the phantom) to reduce the ultrasound energy reflected back to the temperature-sensitive gel. The plastic holder was then filled with degassed water, as to remove infiltrating air pockets that would compromise the acoustic coupling.

An Achieva 3.0 T MRI system (Philips Healthcare, Best, Netherlands) with a dedicated HIFU small animal coil¹² was used to acquire images for planning, as well as monitoring temperature elevations during the ultrasound exposures (setup illustrated in Fig. S1 of the supplementary material).²⁰ Prior to ultrasound exposures, T1-weighted planning images were acquired as a 3D sagittal stack, as shown in Fig. 2. MR-thermometry was performed during the HIFU sonications using dynamic fast field echo-echo planar imaging (FFE-EPI). One coronal and one sagittal image were acquired per dynamic scan repetition and automatically positioned

FIG. 2. Assembly of ultrasound gel phantom for MR-HIFU experiments, showing the phantom components on the left hand panel and a corresponding T1-weighted MR image on the right. Scale bar = 7.5 mm.

perpendicularly and parallel to the HIFU beam axis. The same imaging parameters were used for both slices, with TR = 36 ms, TE = 16 ms, spatial acquisition resolution $1.4 \times 1.4 \text{ mm}^2$, slice thickness = 5 mm, and 19.5° flip angle, resulting in an acquisition time of 3 s per dynamic scan. For HIFU sonications, the applied parameters were frequency = 1.2 MHz, acoustic power of 40–80 W, and sonication cells (circular sonication targets) of 4 or 8 mm diameter. We completed this validation experiment for two separate batches of gel phantom, one with pH 4.50 and one with pH 4.75, performing eight separate sonications on each gel to test the reproducibility of the measured coagulation threshold temperatures. Each sonication was performed to a specified cutoff temperature ranging from 52.5 to 62 °C for the pH 4.50 gel and from 55 to 65 °C for the pH 4.75 gel.

After the ultrasound exposures, T2 maps for quantifying the change in T2 relaxation times were acquired using an eight-channel clinical wrist coil (Philips Healthcare, Best, Netherlands), since the use of this coil allowed for an improved signal-to-noise ratio. T2 maps of coronal stacks consisting of 3 slices per gel were acquired using a multiecho sequence with 16 echo times ranging from 9 to 144 ms. T2 maps were acquired using 2D multislice turbo spin echo sequences with in-plane acquisition resolution of $1.0 \times 1.0 \text{ mm}^2$, 1 mm slice thickness with a slice gap of 0.6 mm, TR = 4206 ms, two signal averages, and a flip angle of 90° .

Using our analysis program, we then compared the maximum temperature maps from the sonications with the corresponding T2 relaxation times of the heated regions. This was done by manually selecting the heating centroid on the T2 maps as well as in the temperature maps for each sonication cell. The change in T2 relaxation time or temperature, respectively, was then automatically measured from these images along radial line profiles starting at the center point of the target cell. A total of 90 line profiles spanning 360° was automatically drawn and the average value of these resulted in, respectively, either the T2 relaxation time, or the temperature, as a function of distance from the heating centroid in each cell. The coagulation threshold temperatures of the two different gels were then determined based on the T2 changes and compared to the coagulation threshold temperatures estimated from the opacity quantification imaging. Here, the coagulation threshold temperature depends on the applied T2 threshold value, and as such we compared several methods for defining this threshold value, including the method used by McDonald *et al.*⁷

2.D. Example application of the proposed method to evaluate HIFU treatment protocols using a small animal therapeutic ultrasound system

Having determined the coagulation threshold temperature of the gel phantoms, we then used them to estimate the temperature increase resulting from focused ultrasound treatment protocols with varying sonication parameters. Here we used the therapy imaging probe system (TIPS, Philips Research North America, Briarcliff Manor, NY)¹³ that is used for

small animal ultrasound therapy studies in our lab. Ultrasound protocols of continuously decreasing intensity were applied to the tissue-mimicking gel phantom to determine at which parameter settings the achieved temperature was below the previously determined coagulation threshold temperature. The starting temperature of the gel was approximately 35 °C as it was kept in direct contact with a water bath monitored to be at consistently this temperature using a thermocouple. All sonications were delivered as a 5×5 square of sonication points with 1.2 mm spacing in between, 75% duty cycle, ultrasound frequency 1 MHz, and pulse repetition frequency of 100.

3. RESULTS

3.A. Coagulation threshold temperature from the opacity quantification method

Using the opacity quantification method and the 5% statistical significance level, we determined the coagulation threshold temperature for gel batches of pH 3.96, 4.10, 4.25, 4.50, and 4.75. Which samples were uncoagulated was determined based on visual examination of the pixel intensity data and typically the two or three lowest temperature samples were uncoagulated. This resulted in coagulation threshold temperatures in agreement with what could roughly be estimated from visual inspection, as for the example of pH 4.75 shown in Fig. 3. The general behavior of pixel intensity change

Fig. 3. Coagulation threshold temperature determined by a statistically significant increase at the 5% level compared to the average pixel intensity of all uncoagulated samples for a gel batch with pH 4.75. The dashed lines mark the intersection on the curve for the pixel intensity threshold defining the coagulation threshold temperature. Visually this corresponds to coagulation manifesting between samples 4 and 5 from the left, where the temperature is given below in °C. The uncertainty bars represent ± 1 standard deviation in pixel intensity value for each sample. Scale bar = 5 mm.

TABLE I. Coagulation threshold temperatures estimated for various gel batches using the opacity quantification method based on statistically significant increase in pixel intensity. Values are presented as average and standard deviation for at least two separate gel batches at each pH level.

Gel phantoms	Coagulation threshold temperature (°C): 5% significance level	Coagulation threshold temperature (°C): 10% significance level	Coagulation threshold temperature (°C): 1% significance level
pH 3.96	43.3 ± 0.8	42.9 ± 0.7	43.9 ± 1.0
pH 4.10	41.4 ± 1.0	41.2 ± 1.1	41.9 ± 0.9
pH 4.25	44.1 ± 0.1	43.8 ± 0.2	44.6 ± 0.2
pH 4.50	53.4 ± 0.9	53.1 ± 0.9	53.8 ± 0.8
pH 4.75	60.3 ± 0.9	60.0 ± 0.9	60.8 ± 1.0

as a function of temperature was consistent across the various pH levels tested (cf., Fig. S2 of the supplementary material).²⁰

The resulting coagulation threshold temperatures, including those estimated in the sensitivity analysis, are summarized in Table I for gel phantom batches with the various pH levels.

The effect on coagulation threshold temperature from changing the pH level that we found in this study is presented in Fig. 4. There is a clear linear trend of increasing coagulation threshold temperature with increasing pH, at pH levels above 4.25, something that was also seen for the phantom recipe used by McDonald *et al.*⁷ For lower pH levels there was no clear trend for coagulation threshold temperature as a function of pH level. The lower pH levels of 3.96 and 4.10 tested in this study resulted in slightly opaque gels, with some coagulation already at gel preparation. As such, while the opacity quantification method appears to work well for determining the coagulation threshold temperature for these lower pH values, these may not be usable in thermal therapy studies reliant on visual determination of coagulation within the gel phantoms.

FIG. 4. The determined coagulation threshold temperature as a function of pH level. The uncertainty bars represent ± 1 standard deviation of gel batches at the various pH levels, including the variation from the threshold value sensitivity analysis. As the data seem to present two distinctly different behaviors, separate linear regression models were fitted for the lower and higher pH values, respectively, weighted by the inverse standard deviation of each point. The correlation between pH and coagulation threshold temperature in these linear models is shown as R -squared correlation coefficients.

3.B. MR-HIFU validation of coagulation threshold temperatures

Based on the phantom setup described in Sec. 2, we performed HIFU sonications during continuous PRFS-based MR-thermometry for one gel phantom with pH 4.50 and one with pH 4.75. There were some issues sonicating certain target cells caused by, e.g., small air bubbles resulting in inadequate acoustic coupling, and as such the heating for these cells was inconsistent and hence they were excluded from further analysis. The average sonication time for all analyzable target cells was 56 ± 12 s, and the corresponding average heating rate (measured for the central 5×5 pixels of a target cell) was 0.8 ± 0.2 °C/s. The visual coagulation results and corresponding change in a T2-weighted MR image are shown in Fig. 5, where there is clear agreement between the location, shape, and size of the coagulated region and the reduction in T2 signal intensity.

By coregistering the data from the maximum temperature maps and the T2 maps and rescaling them to the same spatial resolution, we plotted the change in T2 relaxation time against the increase in temperature for each of the sonication cells. To determine the T2 relaxation time threshold value for coagulation, we used the method presented by McDonald *et al.*,⁷ defining it as 95% of the drop in T2 relaxation time between the highest and lowest T2 relaxation time for each cell. We also determined what we consider to be a likely underestimation as well as overestimation of the coagulation threshold temperature, in order to get a reasonable range for the coagulation threshold temperature estimate. The underestimated value was obtained by using the largest T2 relaxation time that matched the contour outline of the coagulated areas in the T2 maps when used as a threshold (cf., Fig. S3 of the supplementary material).²⁰ The overestimated value was obtained by defining the threshold as the T2 relaxation time that corresponded to the region where the T2-curves began to flatten out (defined as when the change in T2 relaxation time per 1 °C change in temperature was less than 0.25 ms). The results for these different methods of defining the coagulation threshold temperatures are shown in Fig. 6.

The data for the last sonication cells had to be excluded for each gel as the phantoms had heated up during the experiments and as such the relative change in temperature gave a false indication as the starting temperature, determined

FIG. 5. Results of MR-HIFU sonications on a *pH* 4.50 gel phantom. Sonications were performed sequentially in the clockwise direction starting with cell 1, with total acoustic power ranging from 40 to 80 W at a frequency of 1.2 MHz. The left hand panel shows a photo of the temperature-sensitive gel post sonication and the right hand panel shows the corresponding T2-weighted image acquired with TE = 144 ms and TR = 4206 ms. The cells excluded from further analyses due to suboptimal heating were cell 1, cell 2, and cell 6. Scale bars = 5 mm.

at the beginning of the experiment, was no longer correct. The data for all cells, including the cells with bad heating and incorrect starting temperature, are presented as Fig. S4 of the supplementary material,²⁰ where this effect can be seen. This problem was seen for the cells sonicated about 45 min after the first sonication.

The results comparing the coagulation threshold temperatures determined by the opacity quantification method and the independent validation based on the MR-thermometry are presented in Table II.

Since the 67% confidence intervals (± 1 standard deviation) clearly overlap, the coagulation threshold temperatures estimated by the opacity quantification method are well within the range of those estimated using the MR methods, with especially good agreement for the *pH* 4.50 gel phantom. In both cases the coagulation threshold temperatures from the opacity quantification method are well within the under-

and overestimation limits estimated using the MR methods, which also holds true for the temperatures from the sensitivity analysis in the opacity quantification method.

3.C. Direct validation of PCR heated gel samples through MR imaging

As an attempt at a more direct comparison of the coagulation threshold temperatures obtained using the opacity quantification technique and the corresponding temperatures estimated based on the change in T2 relaxation times, we performed MR imaging on a set of PCR heated gel samples to obtain their T2 maps. The aim of this experiment was to analyze the estimated coagulation threshold temperature from the T2 maps and then directly compare it to the temperature found using the opacity quantification method. This was done by filling the PCR tubes completely with gel aliquots and

FIG. 6. The change in T2 relaxation time in the T2 maps shown as a function of temperature for each of the accurately heated cells for the *pH* 4.50 gel phantom (a) and the *pH* 4.75 gel phantom (b). The coagulation threshold temperatures estimated using the three different methods are shown including the T2 relaxation time threshold value for the contour outline method. Cells 6, 7, and 8 for the *pH* 4.50 gel and cells 6 and 7 for the *pH* 4.75 gel were excluded as the gel phantoms had heated up during the course of the experiment and as such the relative temperature increase measured by MR-thermometry was no longer accurate. Please note that for cell 1 and 4 in (b) the maximum temperature is reached before the curves flatten out so the flat curve method essentially estimates the coagulation threshold temperature as the maximum heating temperature.

TABLE II. Estimates of mean coagulation threshold temperatures using the opacity quantification method compared to the MR-thermometry methods given as average value ± 1 standard deviation.

Method for determining coagulation threshold temperature	Coagulation threshold temperature ($^{\circ}\text{C}$) pH 4.50	Coagulation threshold temperature ($^{\circ}\text{C}$) pH 4.75
Opacity quantification (5% significance level)	53.4 ± 0.9	60.3 ± 0.9
MR: 95% T2 drop method	53.6 ± 1.9	62.9 ± 2.4
MR: contour outline method (underestimation)	49.5 ± 1.0	56.6 ± 2.6
MR: flat curve method (overestimation)	56.2 ± 2.3	63.9 ± 2.0

then placing them in a water-filled phantom holder that was assembled for this purpose, to minimize the image artifacts related to air surrounding the tubes. T2 maps were acquired using the same wrist coil as in the previous experiments due to the high signal-to-noise ratio achievable with this coil. However, due to the small size of the sample tubes and the image artifacts created by the plastic material of the tubes surrounding the gel samples, we were unable to acquire reliable T2 maps, with image noise overshadowing any discernable change in T2 relaxation time related to the different heating temperatures.

3.D. Evaluation of HIFU treatment protocols using the gel phantoms with a small animal HIFU system

Using the gel phantoms investigated here, we evaluated various ultrasound treatment protocols with respect to the induced temperature increase. The results are presented in Fig. 7 for protocols delivering continuously less acoustic energy with each step, until the gel was no longer coagulated. We could clearly see that both the coagulated region and the intensity of the coagulation were diminished as the total

FIG. 7. Ultrasound protocols investigated using a gel phantom with pH 4.50, which should have a coagulation threshold temperature of 53°C according to our results. Coagulation can be seen as white cloudy areas, where the total acoustic power (in W) and exposure time (in ms) settings varied according to the indications in the figure.

acoustic power and/or delivery times were decreased, until coagulation was no longer achieved. As such, the presented method can be used to optimize thermal therapy treatment protocols with respect to the expected temperature increase, as illustrated in Fig. 7 for the case of HIFU sonication.

4. DISCUSSION

A simple and time efficient opacity quantification imaging method has been presented for measuring the coagulation threshold temperature of transparent temperature-sensitive tissue-mimicking gel phantoms for thermal therapy studies. For pH levels of 4.25 and higher we found a linear relationship of increasing coagulation threshold temperature with increasing pH, consistent with the results found by McDonald *et al.*⁷ Here, we employed corn syrup as the acoustic attenuation modifier and found a coagulation threshold temperature of $53.4 \pm 0.9^{\circ}\text{C}$ for pH 4.5, which can be compared to the coagulation temperature of 55°C found by McDonald *et al.* for the same pH, although for phantoms containing Intralipid instead of corn syrup.⁷

The threshold value for coagulation in the PCR heated gel samples was determined based on a statistical cutoff. This appears to be a robust method as the sensitivity analysis showed that varying the statistical threshold value affects the resulting coagulation temperature by less than 1.0°C . When validating the coagulation temperatures found by the opacity quantification method using MR-guided HIFU sonication and the resulting change in T2 relaxation times, the opacity quantification based coagulation threshold temperatures were well within the estimates provided by the MR-thermometry. Compared to the HIFU heating method, there was little variation in the heating of the gel samples using the opacity quantification method, which can be attributed to the precise and reproducible heating achieved with a PCR machine (as stated by the manufacturer). Potentially, more accurate MR-thermometry measurements could be achieved by reference-less or selfreferencing PRFS MR-thermometry methods, to further limit the uncertainty related to the gradually increasing temperature of the gel phantom during the experiment.¹⁴ It should be noted that the temporal resolution of the MR-thermometry method used is $1/3$ Hz, which can be compared to the rate of heating which was on average 0.8°C/s , leading to an effective resolution of about 2.4°C for measuring the temperature increase. This resolution should, however, have little effect on the presented results as the maximum intensity projection temperature maps were used for the analysis (i.e., the highest achieved temperature for each pixel was used).

The method of heating between the PCR machine and the subsequent ultrasound studies is quite different, with the focused ultrasound providing rapid heating of a small volume for a short duration (typically within seconds). In this study we programmed the PCR machine to use a 3 min heating cycle, so heating during a considerably longer time compared to a HIFU sonication. However, once the BSA in the gel phantom has reached a certain temperature and then coagulated or not coagulated based on the temperature, we expected there to be

little effect of continued exposure to the same temperature. We confirmed this by varying the PCR heating time to 5 min instead of 3 min, and saw no difference in the coagulation of samples exposed to the different times (data not shown). There was, however, some visible difference in coagulation between a 1 min heating and a 3 min heating, suggesting that a long enough time has to be used to allow the PCR heating to achieve the intended temperature uniformly in the respective gel samples. This was expected since protein coagulation depends not only on the maximum temperature achieved but also on the time the protein is exposed to a certain temperature.^{15–17} It should be mentioned that using a PCR machine to sequentially heat the gel samples is just one application of the proposed method, and any controlled procedure for heating the samples should be compatible with the opacity quantification technique.

The central part of a heated region will likely have a higher level of coagulation compared to the outer area. As such, a method for estimating the coagulation threshold temperature that is based solely on evaluating the coagulated area (such as the contour outline method) will underestimate the temperature needed to induce coagulation compared to quantitative analysis methods such as the 95% T2 drop method, or the opacity quantification imaging method.

One limitation of the proposed method for estimating coagulation threshold temperature is of course that it can only be used for transparent gel phantoms. If using an opaque gel phantom, one possibility for getting around this limitation could be to use a temperature-sensitive protein that produces a clear change in color of the coagulated area. It seems, however, that the opacity quantification method may be useful even for slightly opaque gel phantoms, such as the phantoms with pH 3.96 and pH 4.10 used in this study, where coagulation can no longer be determined visually. Although we focused on TMPs containing proteins as the temperature-sensitive agent in this study, it is reasonable to assume that our method should in principle also be applicable to reusable phantoms such as those containing nonionic surface-active agents (NISAAs)^{18,19} or thermosensitive dyes.¹⁰ Investigation into whether our proposed method could also be applicable for these types of TMPs would provide an interesting expansion of the current study.

Characterizing thermal therapy protocols prior to preclinical or clinical investigation is essential in order to ensure accurate thermal energy delivery. As such, the appropriateness of any given tissue-mimicking gel phantom used for simulating the effects of thermal therapy *in vivo* will have to be assessed depending on the study in question. However, the proposed opacity quantification method for estimating the coagulation threshold temperature should be applicable to any transparent temperature-sensitive gel phantom.

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^{a)} Author to whom correspondence should be addressed. Electronic mail: wolfgang.tome@einstein.yu.edu; Telephone: +1-718-405-8560.

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