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Supporting Information

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Non-Invasive and In Situ Characterization of the Degradation of Biomaterial Scaffolds by Volumetric Photoacoustic Microscopy**

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Experimental Section

Materials: Gelatin (type A, from porcine skin), sorbitan monooleate (Span® 80), and toluene (99.8%) were all obtained from Sigma-Aldrich and used as received for fabricating the gelatin microspheres with uniform sizes. PLGA (lactide:glycolide = 50:50 and 75:25), and 1,4-dioxane (99.8%) were also obtained from Sigma-Aldrich and used as received for fabricating the scaffolds. The water used in all fabrications was obtained by filtering through a set of cartridges (Millipore).

Preparation of inverse opal scaffolds: PLGA inverse opal scaffolds were fabricated according to our previously reported protocols.[1b, 6c, 6d, 9] Briefly, uniform gelatin microspheres (about 1.5 wt.%) were collected as a suspension in methanol in a 50-mL centrifuge tube. The tube was gently tapped to pack the microspheres into a cubic close-packed lattice. The lattice was placed in an oven heated at 70 ^{o}C for 1 h to induce necking between the adjacent microspheres. After cooling to room temperature, the lattice was carefully harvested using a spatula, placed on a filter paper to remove methanol, and then infiltrated with a PLGA solution in 1,4-dioxane (30 wt.%, containing a mass ratio of 1:1 of the two types of PLGAs, 50:50 and 75:25). After removing the excess PLGA solution with filter paper, the pellet containing PLGA solution was frozen in a refrigerator (-20 ^{o}C) for 5 h, and then lyophilized in a freeze-dryer (Labconco) overnight. The sample was immersed in ethanol, vacuumed briefly to eliminate air bubbles trapped in the sample, and placed in 900 mL of water heated at 45 ^{o}C under magnetic stirring for 3–4 h to dissolve the gelatin microspheres. To fabricate formazan-doped scaffolds, 0.5 wt.% (*i.e.*, 1.67 wt.% to PLGA) MTT formazan (Sigma-Aldrich) was added into the PLGA solution in 1,4-dioxane and this solution was used to infiltrate the gelatin lattice. All the other procedures were kept the same as for making plain scaffolds. The scaffolds were then stored in PBS (Invitrogen) prior to use.

Animals and implantation of scaffolds: All animal experiments were performed in accordance with protocols approved by the Washington University Department of Comparative Medicine and the Animal Studies Committee. Athymic nude mice 4–5 weeks old were obtained from Harlan and housed under specific pathogen-free conditions in the animal facility at Washington University. All the scaffolds were sterilized in 70% ethanol for at least 2 h prior to implantation. The scaffolds were then implanted subcutaneously in the ears of the mice.[6c] Briefly, animals in all groups were anesthetized by administration of gaseous isoflurane (2%,

Butler Inc., Dublin) and aseptically prepared. One parasagittal incision was made on the top side of the ear of each animal approximately 0.5 cm to the head. Subcutaneous pockets were created lateral to the incision by using blunt dissection. One scaffold was inserted into the subcutaneous pocket using a pair of tweezers (one scaffold per mouse). The skin incision was then closed with 9-0 Ethilon suture (Ethicon) and secured using GLUture topical tissue adhesive (Abott Laboratories).

Degradation of inverse opal scaffolds: For *in vitro* experiments, inverse opal scaffolds immersed in 5 mL of either PBS or PBS containing 0.025 wt.% lipase (Sigma-Aldrich) at 37 ^{o}C. The medium was changed every day. One group of scaffolds (N = 4) was fixed onto glass slides using a small amount of epoxy (one scaffold per slide), and was subjected to non-invasive PAM imaging. The scaffolds in the other group (N = 3 for each time point) were used for mass loss measurement. Briefly, three scaffolds were removed from the medium each week, washed thoroughly with water, and allowed to air-dry. The mass of each dry scaffold was measured using a microbalance (Cahn C-30), then, each scaffold was dissolved in 0.2 mL 1,4-dioxane, and absorbance at 562 nm was obtained by a spectrophotometer (Tecan Infinite 200). The amount of MTT formazan was calculated according to a calibration curve. For *in vivo* experiments, one group of implanted scaffolds (N = 3) was monitored non-invasively using PAM, and the scaffolds in the other group (N = 3 for each time point) were used for mass loss measurement. Three scaffolds were explanted each week and freeze-dried. Each scaffold/tissue construct was dissolved in dichloromethane (DCM), and the insoluble parts were removed by centrifugation. The DCM solution containing dissolved PLGA was freeze-dried and the mass was measured using a microbalance. To determine the background mass caused by soluble tissue components, ear tissue samples with approximately the same volume were subjected to the same analysis.

Photoacoustic microscopy (PAM) and signal processing: Two PAM systems were used for this study: optical-resolution PAM (OR-PAM) and acoustic-resolution PAM (AR-PAM). For PAM, the optical and ultrasonic foci are configured coaxially and confocally. For the OR-PAM, the optical focal spot size was approximately 5 μm, much smaller in diameter than the acoustic focus, so the lateral resolution was predominantly determined by the optical focal spot size, the so-called optical resolution. For the AR-PAM, the optical focal spot size was 2 mm in diameter in a clear medium, much larger than the ultrasonic focus, so the lateral resolution was predominantly determined by the ultrasonic focus, the so-called acoustic resolution.

OR-PAM was used to monitor the degradation of scaffolds *in vitro*.[13] Briefly, The nanosecond pulsed laser beam from a tunable dye laser (CBR-D, Sirah) pumped by a Nd:YLF laser (INNOSLAB, Edgewave) was tightly focused into the sample by an optical objective (NA = 0.1 in air). An optical-acoustic beam combiner composed of a layer of silicone oil sandwiched by two prisms was used for the coaxial and confocal alignment of the optical illumination and acoustic detection. The resultant ultrasound waves were first focused by a concave acoustic lens (NA = 0.5 in water) ground into the bottom of the combiner, and then detected by a wideband ultrasonic transducer (V214-BB-RM, Olympus-NDT), which had a central frequency of 50 MHz and a 6-dB nominal bandwidth of 70%. A correction lens offset the optical aberration of the prism. The OR-PAM could achieve a lateral resolution of approximately 5 μm, an axial resolution of approximately15 μm, and a penetration depth of about 1 mm.

AR-PAM was used to monitor the degradation of scaffolds *in vivo*.[14] Briefly, optical excitation was provided by the same laser system as for OR-PAM, and the photoacoustic signals were detected using an ultrasonic transducer. The light was coupled into a multimode optical fiber (M30L02, Thorlabs) and reshaped by a conical lens to form a ring pattern on the tissue surface. The ring-shaped light pattern was then weakly focused into the sample by an optical condenser, the light focus overlapped with the tight detection ultrasonic focus. The AR-PAM could achieve a lateral resolution of approximately 45 μm, an axial resolution of approximately 15 μm, and a penetration depth of about 3 mm, when an ultrasonic transducer with a central frequency of 50 MHz was used. Alternatively, when using an ultrasonic transducer with a central frequency of 20 MHz, the AR-PAM had a lateral resolution of approximately 80 μm, an axial resolution of approximately 30 μm, and a penetration depth of up to 5–10 mm.

All volumetric data were processed using custom MATLAB (Mathworks) programs.

Scanning electron microscopy (SEM): SEM (Nova NanoSEM 2300, FEI) was used to characterize the gelatin lattices and the PLGA inverse opal scaffolds. Prior to imaging, the samples were sputtered with gold for 60 s. Images were taken at an accelerating voltage of 5 kV.

Statistics: All the results were presented as mean ± standard error for each experimental group. Four scaffolds were monitored for *in vitro* PAM imaging, and the other three scaffolds were used for mass measurement at each time point. Three mice were monitored for *in vivo* PAM imaging, and the other three mice were sacrificed for histology analyses at each time point.

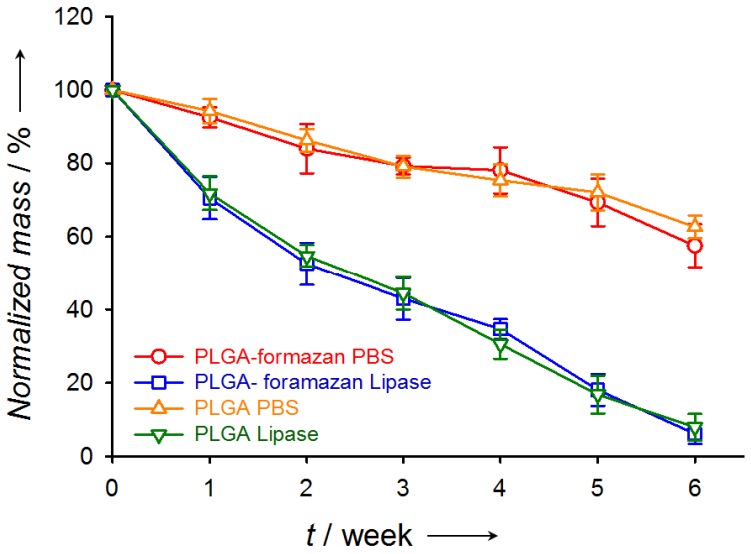

Figure S1. Degradation of pristine and MTT formazan-doped PLGA inverse opal scaffolds in PBS and lipase solution, as measured by the mass loss assay. The addition of MTT formazan did not alter the degradation profiles of the scaffolds.

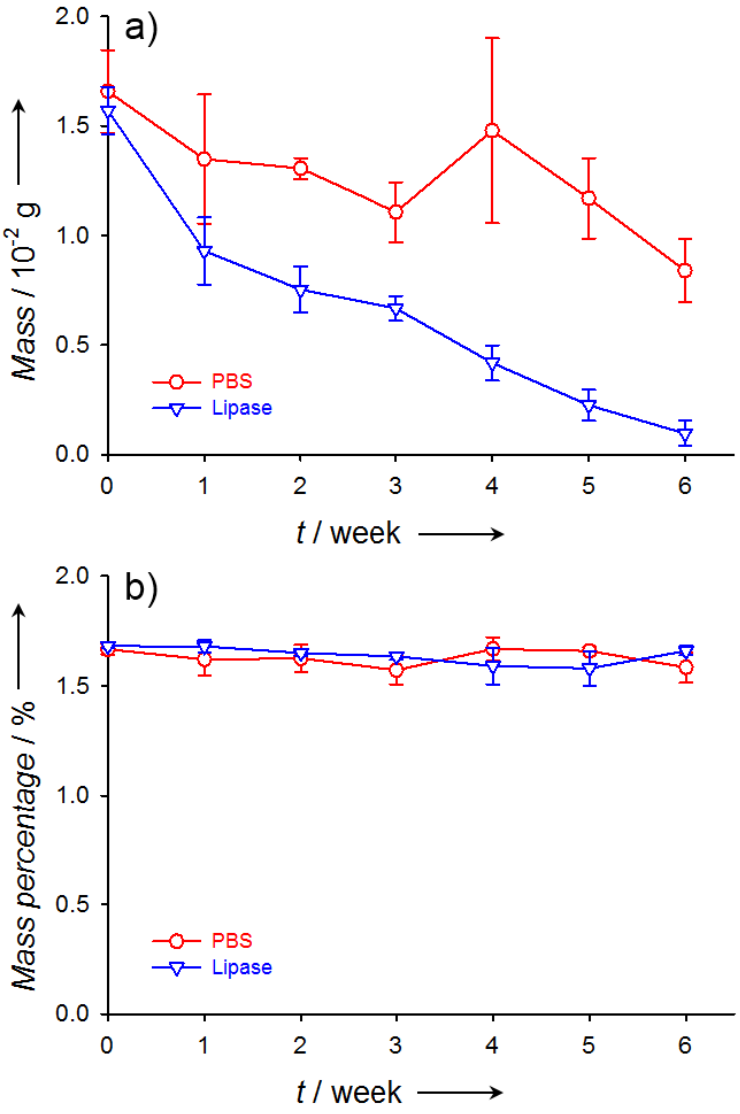

Figure S2. a) The change in the mass of MTT formazan as a function of degradation time for the scaffolds in PBS, in the absence and presence of lipase, respectively. b) The change in the mass percentage of MTT formazan relative to the scaffolds as a function of degradation time of the scaffolds in PBS, in the absence and presence of lipase, respectively. The amount of MTT formazan remained almost unchanged during the period of 6 weeks, indicating that essentially no dye leaked out from the scaffold during incubation.

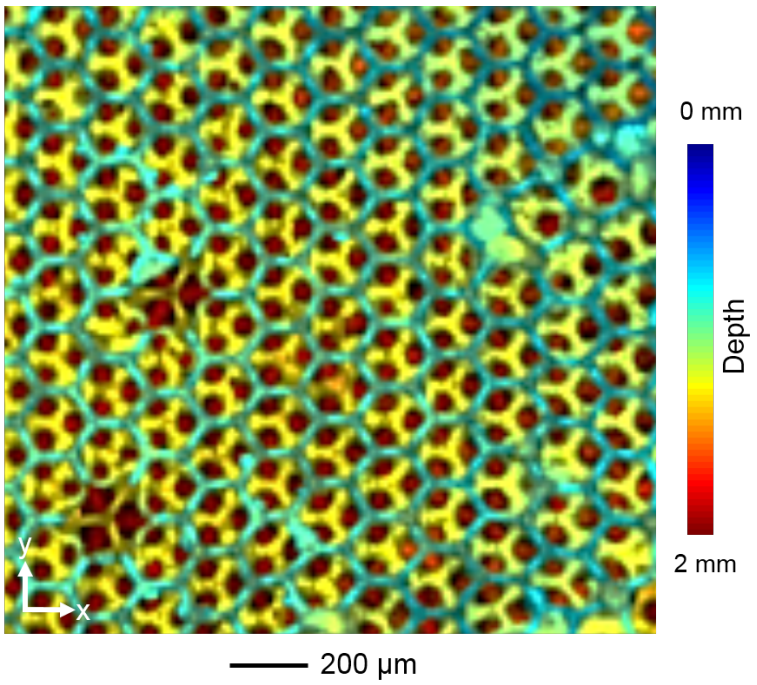

Figure S3. An OR-PAM MAP image of a PLGA inverse opal scaffold doped with MTT formazan, showing the uniform pores and interconnecting windows. The image was color-coded by depth of maximum. MAP stands for "maximum amplitude projection".

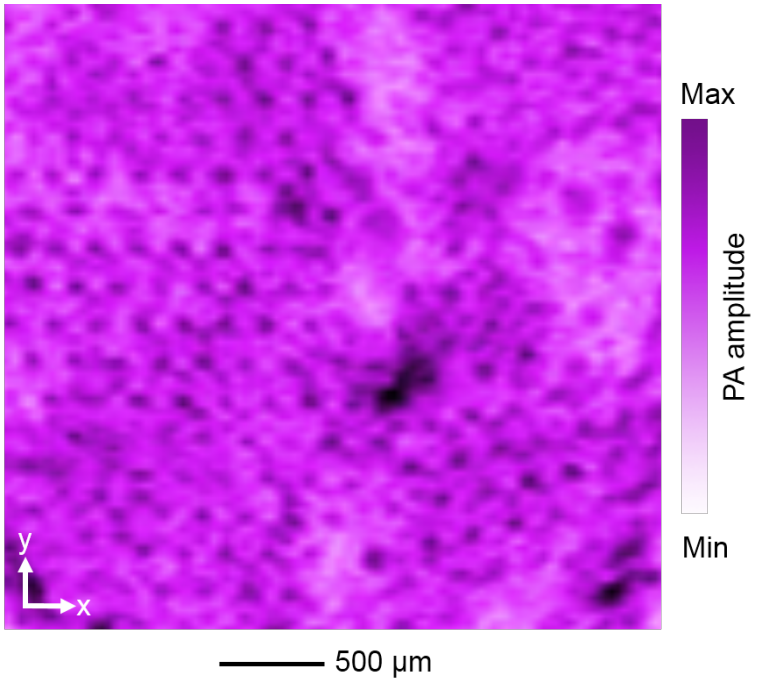

Figure S4. A coronal AR-PAM MAP image of a PLGA inverse opal scaffold doped with MTT formazan, showing the porous structure. MAP stands for “maximum amplitude projection”.

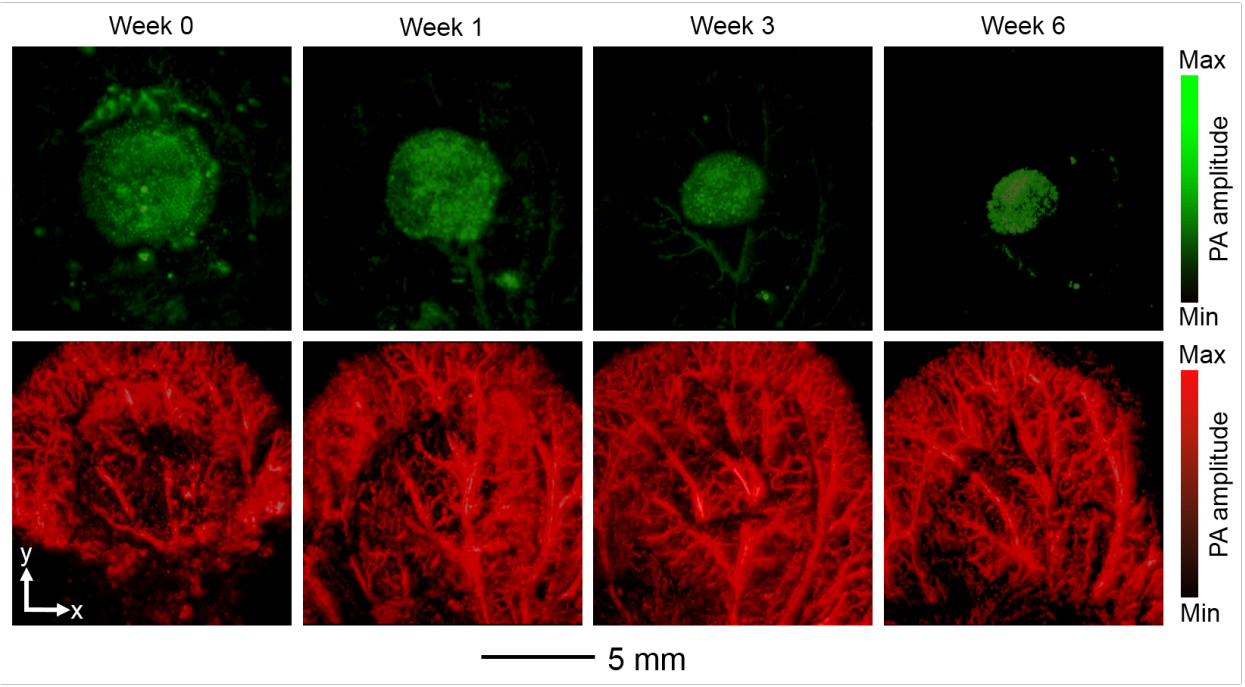

Figure S5. Coronal AR-PAM MAP images separately showing the implanted PLGA inverse opal scaffold and the blood vessels in the ear of a nude mouse at weeks 0, 1, 3, and 6 post implantation. MAP stands for "maximum amplitude projection".

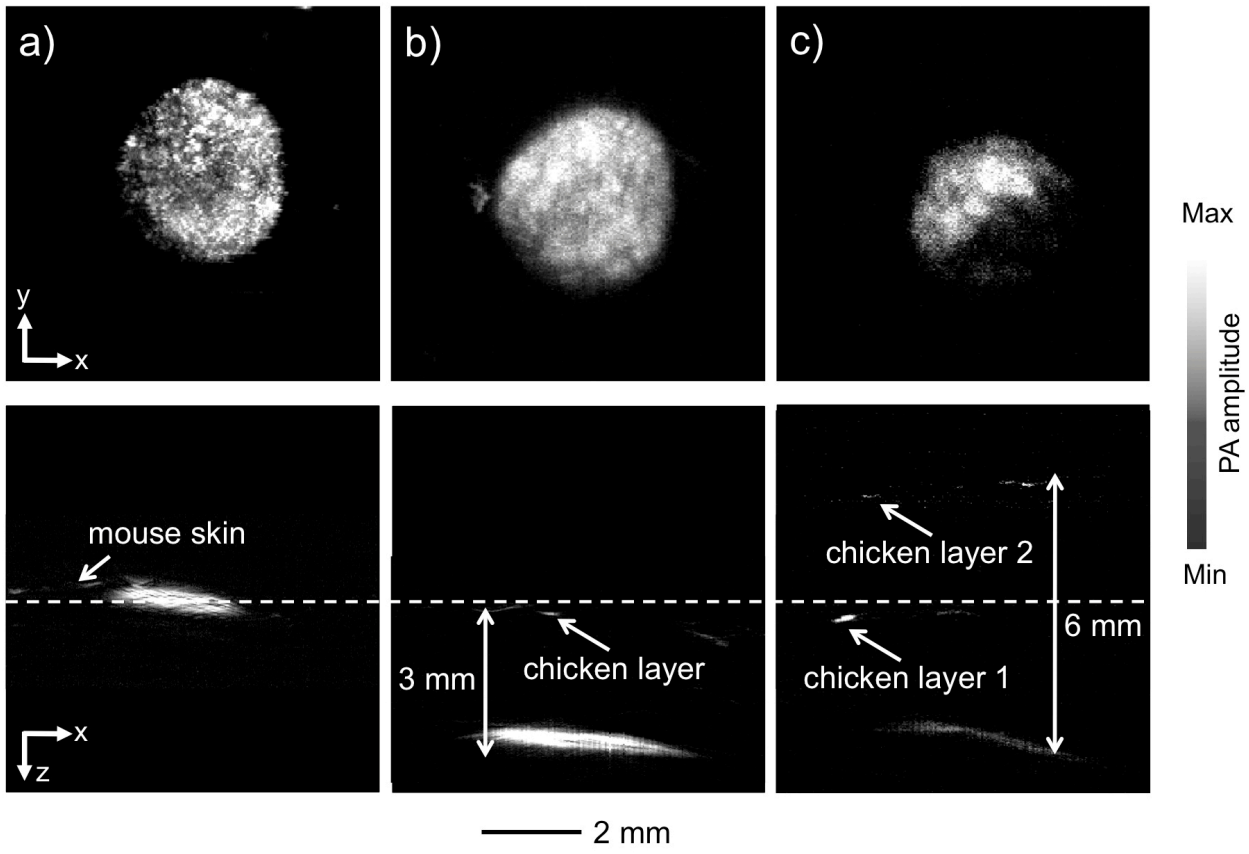

Figure S6. a–c) Coronal (top row) and sagittal (bottom row) PAM MAP images showing the PLGA inverse opal scaffold subcutaneously implanted at the dorsal site of a nude mouse, at week 2 post implantation, with (a) 0, (b) 1, and (c) 2 layers of chicken breast overlaid on top of the skin. MAP stands for "maximum amplitude projection".

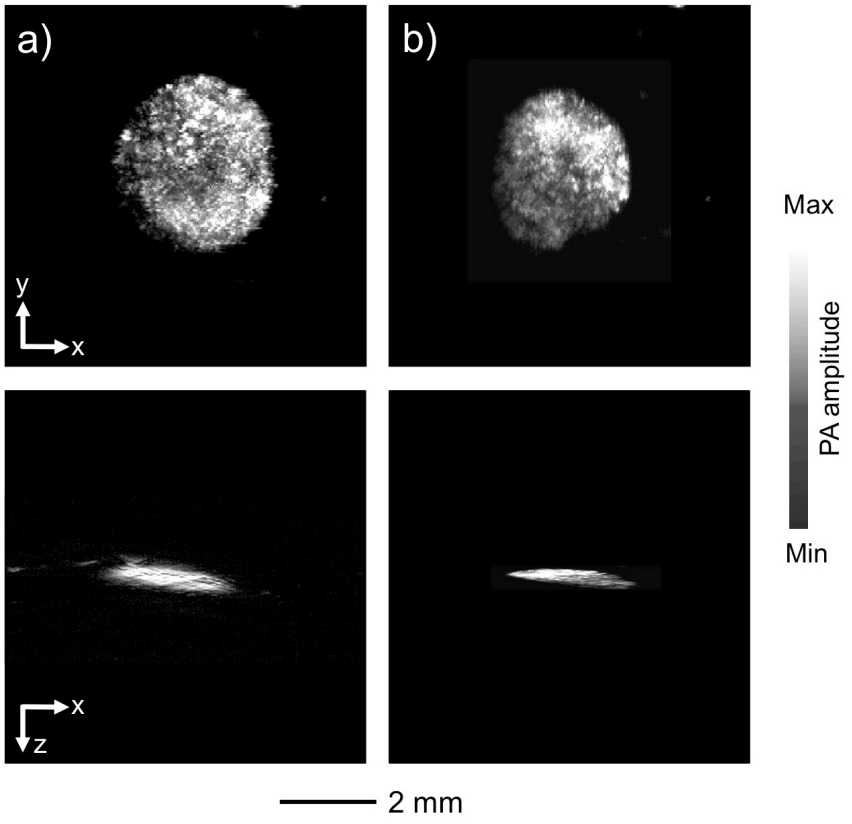

Figure S7. Coronal (top row) and sagittal (bottom row) PAM MAP images showing the PLGA inverse opal scaffold subcutaneously implanted at the dorsal site of a nude mouse, at (a) week 2 and (b) week 4 post implantation. MAP stands for "maximum amplitude projection".

Movie S1. A 3D co-registered dual-wavelength AR-PAM movie showing the scaffold and the vasculature simultaneously at week 0 post implantation, in the ear of a nude mouse.

Movie S2. A 3D co-registered dual-wavelength AR-PAM movie showing the scaffold and the vasculature simultaneously at week 1 post implantation, in the ear of a nude mouse.

Movie S3. A 3D co-registered dual-wavelength AR-PAM movie showing the scaffold and the vasculature simultaneously at week 3 post implantation, in the ear of a nude mouse.

Movie S4. A 3D co-registered dual-wavelength AR-PAM movie showing the scaffold and the vasculature simultaneously at week 6 post implantation, in the ear of a nude mouse.