Supporting Information

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SI Materials and Methods

Formation of Alginate Gels for Mechanical Testing and Release Studies. Medical grade, high guluronic acid-content, high M_r alginate (MVG) was purchased from FMC Biopolymers. Alginates were dialyzed against water, filtered (0.22-μm mesh), freeze dried, and resuspended into Dulbecco's PBS (dPBS; Invitrogen). For drug, protein, and plasmid DNA (pDNA) release studies, the alginate polymers first were combined with the molecule of interest to facilitate noncovalent interactions. Next, alginate was cross-linked with calcium sulfate ($CaSO_4$; Sigma) to a final concentration of 50 mM Ca^{2+} and 20 mg/mL MVG within gels, and a biopsy punch was used to obtain gels of a defined size and shape (12.2-mm diameter and 2-mm thickness). Before release and mechanical property studies, hydrogels were equilibrated overnight in serum free DMEM without phenol red (Invitrogen).

For in vitro ultrasound pulsing studies, gels were transferred to 15-mL tubes filled with dPBS with an additional 0–13.3 mM Ca^{2+}. Gels were subjected to ultrasound treatment (9.6 mW/cm^2, 5 min-pulses, once per hour). The power and time of ultrasound application were chosen based upon measurements of the temperature of media subjected to ultrasound, and within the range used here, temperature change did not exceed controls (controls were kept at 37 °C; ultrasound was done at room temperature, and the media temperature remained <32 °C).

In Vitro Hydrogel Mitoxantrone Release Studies. For release studies, mitoxantrone first was combined with high M_r alginate and allowed to mix for 20 min to facilitate noncovalent interactions. Next, the gel/drug mixture was cross-linked with $CaSO_4$ to a final concentration of 50 mM Ca^{2+} and 20 mg/mL alginate within the hydrogels. The final concentration of mitoxantrone was 850 μg/mL of gel. Ultrasound was applied at 9.6 mW/cm^2 for 5 min at intervals of 1 h. Release of mitoxantrone was measured colorimetrically with a BioTek plate reader (650 nm).

Shear-Thinning Hydrogel Formulation for in Vivo Studies. For in vivo studies, an injectable formulation of alginate was used. This injectable formulation is a binary combination of low M_r MVG and was prepared by irradiating medical grade MVG at 5 Mrad with a cobalt source (1). A binary formulation of 20 mg/mL low M_r MVG with 0.5 mg/mL unmodified MVG was mixed with mitoxantrone (850 μg/mL of gel), then cross-linked with 4% wt/vol $CaSO_4$ (1.22 M). During in vitro release studies with this gel formulation, we found that a 2.5-min ultrasound exposure time facilitated digitally controlled release without altering the baseline release levels (Fig. S1).

A total mass of 85 μg drug was delivered per injection, as this yields a systemic dose of ~3.1 mg/kg, within the dosage range previously shown to reduce xenograft tumor burden in nude mice treated i.v. with mitoxantrone (2, 3). Because free mitoxantrone is cleared rapidly from plasma, <5% is found at a xenograft tumor site (2, 3), and exhibits cytotoxic effects in the heart and other organs (4), locally deployed mitoxantrone gels were chosen for this study; by placing an equivalent amount of drug at the tumor site, therapeutic levels of drug should be achieved. Based on in vitro kinetics, diffusion-based release should be completed over the time frame of these studies.

To estimate the amount of mitoxantrone released in vivo, we used the release rates from the in vitro testing (Fig. S1). The amount released equals the ultrasound accelerated release rate (0.63 μg/min) by time (2.5 min), which gives 1.575 μg. To estimate the volume over which this is spread, we take the area measurements for the extent of in vivo mitoxantrone release (from Fig. 2 *D* and *E*) and the diameter for the average tumor size measured in mice treated with mitoxantrone-laden hydrogels and daily ultrasound (from Fig. 4*D*), and use these to calculate the volume of an outer ellipsoid; the inner ellipsoid is based on injected gel volume (100 μL). Subtraction of these gives the volume over which the drug is spread: 415 μL. Thus, the concentration is given as 3.8 μg/mL. This value is the same order of magnitude as condition II in the table in Fig. 3*E*, and as this is an averaged value, localized concentrations will be higher, possibly reaching the higher values tested in Fig. 2 *D* and *E*. From the non–ultrasound-stimulated gels, the rate at which drug travels through the s.c. tissue (average distance to outer mitoxantrone border/time) is estimated at 120 μm/h; thus, the duration these values remain elevated may reasonably be expected to last for timescales at least as long as those in table 2e.

In Vitro Hydrogel Cytokine and DNA Release Studies. For release studies of cytokines and pDNA, these biomacromolecules first were combined with high M_r alginate and allowed to mix for 20 min to facilitate noncovalent interactions. Next, the gel/molecule mixture was cross-linked with $CaSO_4$ to a final concentration of 50 mM Ca^{2+} and 20 mg/mL alginate within the hydrogels. The final concentration of each bioactive agent within gels was as follows: SDF-1α (Peprotech), 20 μg/mL of gel; ^{125}I-VEGF-165 (VEGF-165 was obtained from the Biological Resources Branch of the National Cancer Institute), 60 μg/mL of gel; and pDNA (gWiz GFP plasmid DNA, Aldevron), 100 μg/mL of gel. Ultrasound was applied at 9.6 mW/cm^2 for 5 min at intervals of 2 h (SDF-1α) or 24 h (pDNA).

^{125}I-VEGF-165 release was detected by using standard radiometric methods (Fig. S2). Release of SDF-1α was measured by ELISA (R&D Systems) and that of pDNA by PicoGreen assay (Invitrogen).

Analysis of Power Delivered by Ultrasound Transducer. Because the power delivered by the sonicator—as displayed on its screen—assumes 100% efficiency, this value cannot be used to predict the ultrasound intensity accurately (2). To improve the approximation of the ultrasound intensity, we measured the pressure profiles of the ultrasound tips (3-mm and 12-mm diameter operating at 20 kHz using a Sonics Vibra-Cell VCX120 sonicator) used in the study in a free field (water bath) using a Dapco needle hydrophone (Dapco Industries Inc.) positioned at the same location relative to the ultrasound tip as the hydrogel center. The chosen ultrasound intensities were based on previous work applying ultrasound for drug release (5, 6). The pressure profiles were converted to the frequency domain by using fast Fourier transforms, and for 20-kHz frequency and its harmonics (up to 200 kHz), the pressure values were used to approximate the ultrasound intensity, I, according to the equation

$$I = \sum \frac{p^2}{2\rho c},$$

where p is the peak pressure at each frequency, ρ is the density of water (1,000 kg/m^3), and c is the speed of sound in water (1,480 m/s). The precise ultrasound intensity for the in vitro and in vivo experiments depends on the experimental setup and location within the field and thus may differ from these measurements.

In Vitro Naproxen Release Studies. High molecular weight chitosan (Sigma) was dissolved in 0.3 M NaCl containing 1% acetic acid (pH 2.5) to 40 mg/mL. Naproxen (Sigma) was dissolved into 100% EtOH to 50 mg/mL, and this stock solution was diluted further in PBS and mixed with the chitosan to a final concentration of 20 mg/mL chitosan and 5 mg/mL naproxen. This solution was poured into a 2-mm–high mold, and the chamber containing the molded gel was filled with a 50-mg/mL solution of tripolyphosphate (TPP) dissolved in water. Care was taken to avoid deforming the molded polymer when pouring TPP.

After allowing the gels to cure overnight, 12.2-mm–diameter disks were formed with a biopsy punch and allowed to equilibrate into the TPP buffer described above for 10 h. The disks then were washed in phenol red-free αMEM (MediaTech) and blotted gently to remove excess TPP/naproxen. Ultrasound-triggered release studies were performed while the gels were incubated in serum and phenol red-free αMEM containing 0.1% sodium azide (Sigma).

Following the release studies, the solutions were transferred to a quartz cuvette and analyzed for fluorescence spectra with a Jovin Horiba SpectraMax fluorescence spectroscope. Based on the fluorescence spectrum of naproxen in αMEM, the emission wavelength of 390 nm was used to detect concentration based on a standard curve prepared with serial dilutions of naproxen in αMEM.

Studies on the Mechanism of Ultrasound-Induced Drug Release. Having established the utility of ultrasound treatment of drug-laden, ionically cross-linked alginate hydrogels toward on-demand delivery (main text), we next investigated possible mechanisms of ultrasound-triggered release. The extent of mitoxantrone release depended strongly on the geometry of hydrogels, suggesting that surface-mediated delivery was the primary means of release (7) (Fig. S3), and increased concurrently with applied ultrasound power (Fig. S4). However, measurements of the temperature of media surrounding gels immediately before and after ultrasound treatments revealed no significant temperature change. Even if subtle temperature changes were to occur during ultrasound, the increase in release rate during applied ultrasound—on the order of 10-fold for the lowest ultrasound power level applied—is inconsistent with temperature changes as a predominant means of enhancing release, as this would have required a much more dramatic, observable temperature change. Thus, although locally cavitation may increase temperature (8), a simple change in diffusion coefficient due to temperature change alone cannot explain the dramatic increase in release rate during ultrasound.

In vivo, shear-thinning gels stayed grossly intact even after daily ultrasound for 1 wk, suggesting that these materials, although biodegradable in vivo (9), stay intact long enough to act as a stable depot of drug (Fig. S4).

As ultrasound-mediated release did not appear to be related to temperature changes, we hypothesized that ultrasound-triggered drug release might result from two alternate kinetic processes: disruption of the gel structure (increasing the effective surface area for drug efflux from the gel) and accelerated disassociation of polymer-bound drug (increasing the soluble pool of drug available for release; Fig. S5). The relationship between the strength of drug–polymer interaction and release via ultrasound was investigated by using pDNA condensed with varying amounts of poly(ethylene imine) (PEI). Increasing the overall positive charge on PEI–pDNA condensates, by increasing the stoichiometric ratio of PEI to pDNA in condensates, strengthened their ionic interactions with the negatively charged polymer network, and markedly diminished plasmid release during ultrasound (Fig. S5). Interestingly, the ratio of PEI–pDNA released via ultrasound to PEI–pDNA released via diffusion alone at a fixed PEI/pDNA charge ratio diminished as the charge ratio increased (Fig. S5). Although these observations do not rule out the possibility that ultrasound can accelerate drug/polymer dissociation, the data are more consistent with the hypothesis that ultrasound initiates release via transient gel disruption. To directly confirm a role for gel disruption in drug release, release studies were performed with trypan blue, a small molecule that does not interact with alginate. Although the baseline release rate for trypan blue from alginate was high, owing to the absence of molecular interactions with the polymers and trypan blue's small size relative to the gel pores, the rate of trypan blue release markedly increased during ultrasound treatment (Fig. S5). This finding also is consistent with transient gel disruption as a predominant mechanism for drug release.

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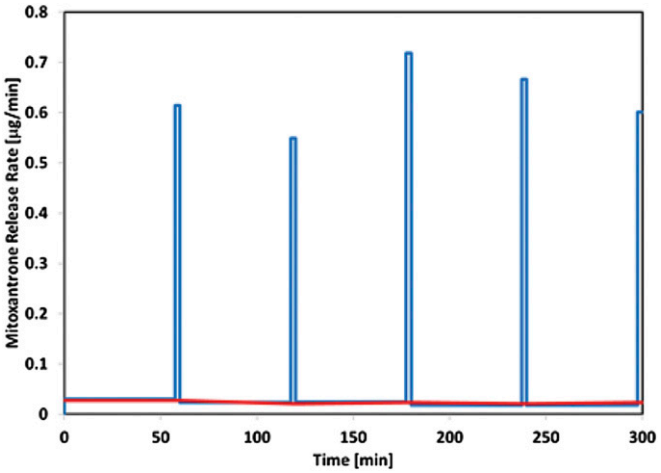


Fig. S1. Reversible, ultrasound-triggered release from shear-thinning, injectable alginate hydrogels used for in vivo studies. Analysis of the mitoxantrone release rate at baseline (without ultrasound) and during ultrasound pulsation for 2.5 min, once every hour for 5 h (five ultrasound cycles), for either ultrasound-treated gels (blue) or control gels that were not exposed to ultrasound (red). Note that the release rate during ultrasound and at baseline does not increase with repeated ultrasound treatment, consistent with the assumption that these hydrogels stay intact during ultrasound treatment. The graph represents the mean of $n = 3$ hydrogels.

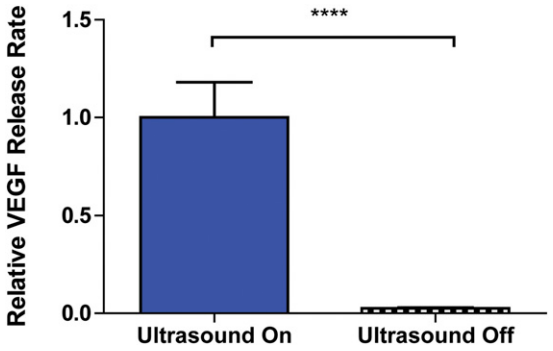
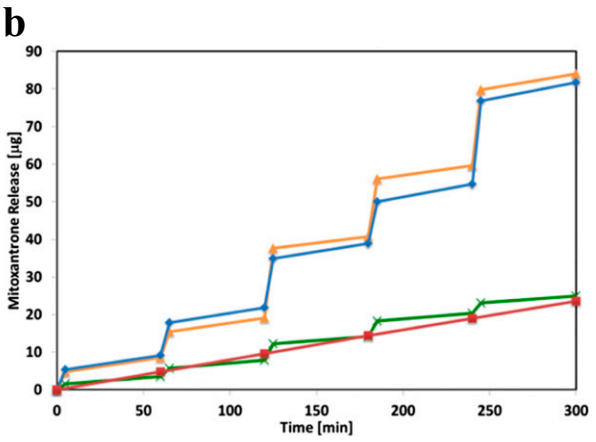


Fig. S2. Ultrasound-mediated VEGF-165 release from alginate hydrogels. Relative release rates of ^{125}I-labeled VEGF-165 from alginate gels, either with or without ultrasound treatment. Error bars are SD, $n = 5$. ****$P < 0.001$.

a

Curve Color	Ultra-sound?	Cylinder Diameter (mm)	Cylinder Height (mm)	Surface Area (mm^2)
Red	No	12.7	2	333
Blue	Yes	12.7	2	333
Orange	Yes	12.7	3	373
Green	Yes	8	5	226



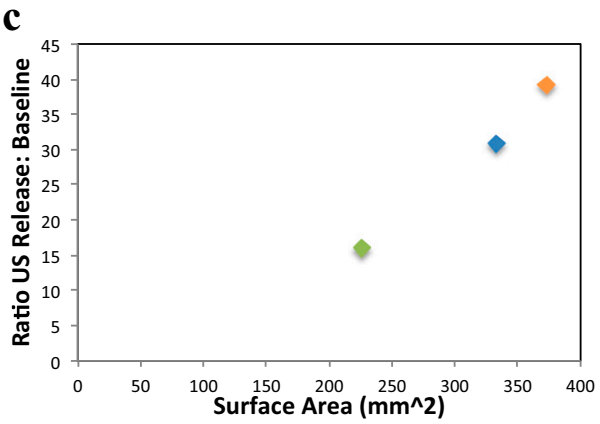
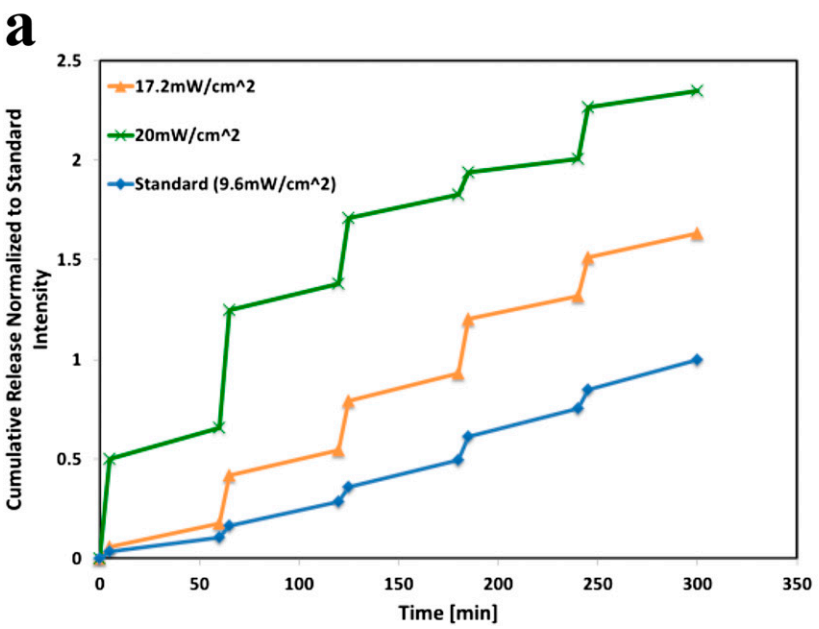


Fig. S3. Ultrasound triggers surface release from alginate hydrogels. (*A*) Table describing the geometry of alginate gels used in the release study. (*B*) Cumulative mitoxantrone release from alginates with different geometry, treated with 9.6 mW/cm^2 ultrasound. (*C*) Plot of the fold-increase rate during ultrasound of the different gels in *A* and *B* as a function of the gel surface area, indicating strong correlation. For all studies in this figure, ultrasound was applied for 5 min each hour for 5 h.



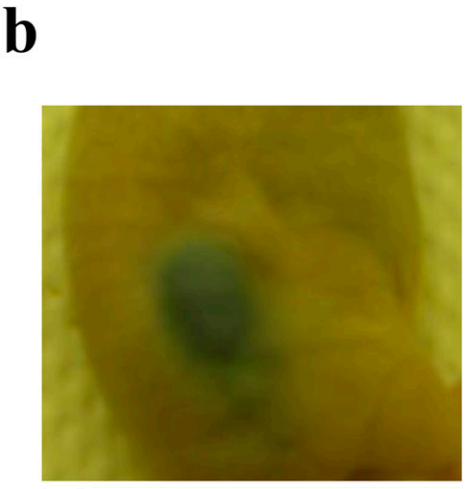


Fig. S4. Limitations on power applied to alginate hydrogels to elicit digitally controlled release. (*A*) Cumulative release—normalized to "standard treatment"—over time of mitoxantrone from hydrogels treated with 17.2 mW/cm^2 (orange curve), 20 mW/cm^2 (green curve), or standard ultrasound treatment (9.6 mW/cm^2, blue curve). Ultrasound was applied for 5 min each hour. Note that the slope of the curve for baseline release changes, and the dose delivered by ultrasound pulsing becomes more variable, when ultrasound power is increased to 20 mW/cm^2. (*B*) Representative image of a mitoxantrone-laden, shear-thinning, injectable hydrogel that was injected s.c., adjacent to a tumor and then subjected to daily ultrasound treatments of 2.5 min.

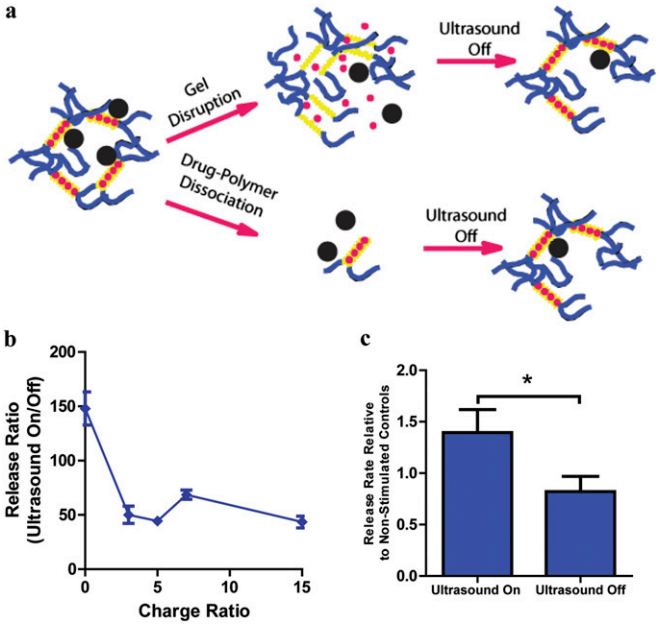


Fig. S5. Data supporting transient gel disruption as a mechanism for ultrasound-mediated drug delivery. (*A*) Proposed mechanisms for ultrasound-mediated release from reversibly cross-linked hydrogels. Drug molecules (pink pentagons) may be released by a transient loss of calcium (black ●) cross-links and gel disruption (*Upper*) or by release of polymer-bound drug (*Lower*). (*B*) Ratio of the release rate of PEI pDNA condensates of varying PEI/pDNA charge ratio from calcium alginate hydrogels during ultrasound treatment vs. when the ultrasound stimulus was removed. Increasing the PEI/pDNA charge ratio increases the strength of ionic association with alginate polymers. (*C*) Release rates for trypan blue from alginate gels during and after ultrasound treatment. Ratios were calculated by normalizing the rate of release for samples treated with ultrasound to the rate of release for matched alginate gels that were never subjected to ultrasound. Error bars are SD, $n = 3–5$. $^*P < 0.05$.

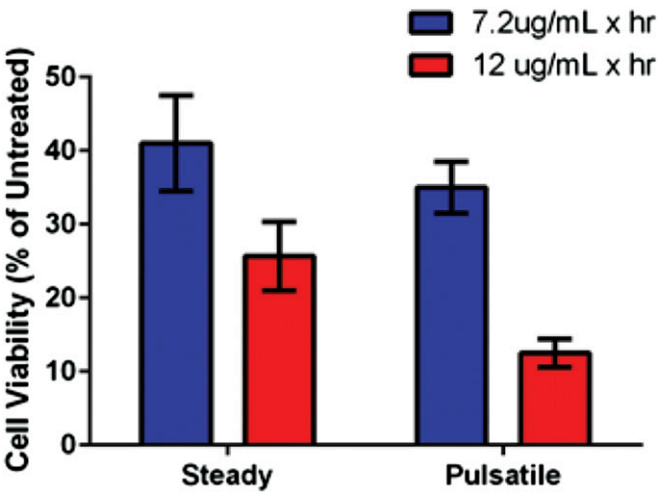


Fig. S6. Breast cancer cells are sensitive to cumulative drug dose. MDA-MB-231 cells were exposed to mitoxantrone in either steady or pulsatile fashion by media exchange. Cells were treated for either 36 h or 72 h with drug so that the accumulated product of drug concentration × time was either 7.2 μg/mL × h (blue) or 12 μg/mL × h (red). As shown, pulsatile delivery alone was not more effective at diminishing cancer cell viability, as both the delivery profile and the total drug exposure had effects on cell viability. Error bars: SD, $n = 4$.

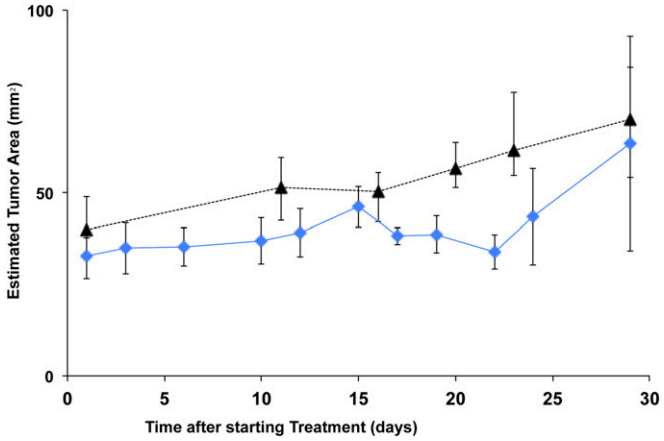


Fig. S7. Drug-laden drug gels treated with ultrasound become ineffective when the drug payload has been depleted. Growth curves for human MDA-MB-231 breast cancer xenografts in Nu/J mice. Tumors were left untreated (black curve) or treated with a combination of mitoxantrone-laden hydrogels and daily ultrasound (blue curve). Without the addition of a second mitoxantrone-laden hydrogel on day 14, the tumors in the treatment group rebounded in growth, which illustrates the timescale of drug delivery. The gels that were not stimulated to undergo burst release with ultrasound in the long-term study are expected to release drug for a longer period.

Table S1. Detailed information on the treatment regimens for short-term xenograft tumor growth studies

Treatment received	Mitoxantrone dose, μg	Administration site and days	Ultrasound*	Alginate†
Tail vein injection	82.5	i.v., day 0	N	N
Ultrasound + single local injection	82.5	Peritumoral, day 0	Y	N
Mitoxantrone-laden gel	82.5	Peritumoral, day 0	N	Y
Ultrasound with alginate gel	—	Peritumoral, day 0	Y	Y
Mitoxantrone-laden gel + ultrasound	82.5	Peritumoral, day 0	Y	Y
Daily local injection	5.8 (per day)‡	Peritumoral, days 0–7	N	N

*Y = 2.5 min at 120 mW/cm^2 on days 1–7.
†Y = 100 μL of alginate gel [20 mg/mL low M_r MVG with 0.5 mg/mL unmodified MVG cross-linked with 4% wt/vol $CaSO_4$ (1.22M)].
‡Equivalent to 2-wk dosing totaling 82.5 μg.