Ultrasound-triggered local anaesthesia

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On-demand relief of local pain would allow patients to control the timing, intensity and duration of nerve blocks in a safe and non-invasive manner. Ultrasound would be a suitable trigger for such a system, as it is in common clinical use and can penetrate deeply into the body. Here, we demonstrate that ultrasound-triggered delivery of an anaesthetic from liposomes allows the timing, intensity and duration of nerve blocks to be controlled by ultrasound parameters. On insonation, the encapsulated sonosensitizer protoporphyrin IX produced reactive oxygen species that reacted with the liposomal membrane, leading to the release of the potent local anaesthetic tetrodotoxin. Repeatable ultrasound-triggered nerve blocks were achieved in vivo, with the nerve-block duration depending on the extent and intensity of insonation. There was no detectable systemic toxicity and tissue reaction was benign in all groups. On-demand, personalized local anaesthesia could be beneficial for the management of relatively localized pain states and could potentially minimize opioid use.

Current treatments of perioperative and other forms of relative acute pain rely heavily on opioids and local anaesthetics. Opioids are systemic therapies associated with clouding of the sensorium and the potential for tolerance, addiction, diversion and overdose. Local anaesthetics are effective in perioperative and other forms of acute pain and can be applied throughout a tissue (infiltration local anaesthesia) or around a specific nerve or group of nerves (regional anaesthesia). In the case of regional anaesthesia, local anaesthetics can be effective even when applied some distance from the injured area or surgical site. However, free local anaesthetic solutions tend to be relatively brief in duration of effect. Formulations have been developed that provide approximately one week of nerve block following a single administration1,2. However, drug release from such systems cannot be adjusted in response to changes in the patient’s needs. Safe and on-demand local pain relief that allows patients to control the timing, intensity and duration of analgesia according to their changing needs and conditions would have a marked impact on the management of acute pain and could reduce or obviate the use of opioids.

Remotely triggered drug-delivery systems have been developed that address the need to modulate pharmacological effects in real time. We have developed injectable on-demand local anaesthetic systems3,4 that can be triggered by near-infrared light. The difficulty with light as a trigger is that its penetration of tissue may be limited5. Although this limitation may be overcome by increasing the irradiance, there is a risk of causing burns6,7. Ultrasound is non-invasive, can penetrate deeply into tissues and can be applied in a focused manner so that the energy applied in the surrounding non-targeted tissues is minimized8,9. Ultrasound is already common in clinical practice for both diagnostic and therapeutic purposes. Nerve-block injections are widely guided by ultrasound in the clinical setting and are associated with a reduction in injection-related complications10 and an increase in the success rate compared with injections without ultrasound guidance11. An on-demand nerve-block system that could use readily available ultrasound clinical devices might ease clinical translation of on-demand drug-delivery systems in pain management.

Many of the current ultrasound-triggerable drug-delivery systems, such as micelles12, liposomes13, composites14 and hybrid15 materials, are responsive to the thermal and mechanical effects of ultrasound waves. Ultrasound may also be used to carry out chemical reactions—a process known as sonochemistry16. Sonodynamic therapy has been proposed as a strategy analogous to photodynamic therapy, in which a sonosensitizer is activated by acoustic energy to generate reactive oxygen species (ROS). However, the generation of ROS by this effect for triggering the release of drugs remains largely unexplored17.

In this study, we report a liposome-based system that provides ultrasound-triggered, repeatable, on-demand local anaesthesia (Supplementary Scheme 1). Ultrasound energy encountering the liposomes caused a sonosensitizer to release ROS, peroxidating unsaturated lipids in the bilayers and leading to the release of local anaesthetics, which induced nerve block. We selected protoporphyrin IX (PPIX) as the sonosensitizer18. PPIX is a naturally occurring intermediate of heme biosynthesis19 and the active component of the prodrug 5-aminolevulinic acid, which is approved by the US Food and Drug Administration20. The hydrophilic site 1 sodium channel blocker tetrodotoxin (TTX) was chosen as the encapsulated local anaesthetic due to its ultrahigh potency and minimal myotoxicity21 and neurotoxicity22. Clinical trials of systemic TTX for cancer pain have previously demonstrated the potential safety of this drug23,24.

Results

Liposome preparation and in vitro evaluation. PPIX was encapsulated in liposomes consisting of the unsaturated lipid

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1,2-dilinoleoyl-sn-glycero-3-phosphocholine (DLPC), the saturated lipid 1,2-distearyl-sn-glycero-3-phosphocholine (DSPC), 1,2-distearyl-sn-glycero-3-phosphatidyglycerol (DSPG) and cholesterol. Their mean size was 3.1 ± 0.9 µm (Supplementary Fig. 1). Ultrasound application did not induce statistically significant changes in lipidosome size ($P = 0.95$, $n = 4$; Supplementary Fig. 1).

ROS production under insonation was measured by the fluorescent indicator 5-(and-6)-carboxy-2',7'-dichlorodihydro fluorescein diacetate (carboxy-H2DCFDA). Carboxy-H2DCFDA was added in the liposome solution and the production of ROS on insonation was measured (Fig. 1a). Maximal ROS production occurred at a PPIX loading of 0.3% (mg PPIX/mg (PPIX + lipid) × 100%) and was associated with lipid peroxidation. The extent of peroxidation (see Methods) increased as the duration of insonation increased (Fig. 1b). The effect of PPIX loading on the ability of ultrasound to trigger drug release was evaluated in liposomes encapsulating the fluorescent dye sulforhodamine B (SRho) (Lipo–PPIX–SRho; Fig. 1), as reported previously. SRho was loaded into the liposomes by hydrating the liposomal lipid cake with a highly concentrated SRho aqueous solution. The dye was encapsulated at a concentration at which self-quenching resulted in low fluorescence. Once SRho was released, its fluorescence increased, and this was measured to determine SRho release. The greatest amount of dye release under ultrasound application (3 W cm−2, continuous application, 1 MHz, 10 min) occurred at 0.3% loading (Fig. 1c). In liposomes made with the saturated lipid DSPC instead of the unsaturated and peroxidizable DLPC, the dye release from insonation was greatly reduced (DPLC negative group in Fig. 1c). Consequently, 0.3% PPIX was used in all subsequent experiments.

The release of dye from Lipo–PPIX–SRho was dependent on the frequency, duration, intensity and duty cycle of insonation (Fig. 1d–f). Based on these results, an ultrasound frequency of 1 MHz, an intensity of 3 W cm−2 and a 100% duty cycle were used for subsequent experiments unless otherwise stated. Two durations of insonation were used: 10 min, to examine the effect of a single pulse on cargo release, and 5 min to assess the effect of repeated pulses (the shorter duration resulted in less release and therefore allowed more triggered events). Dye release could be repeatedly triggered from liposomes by repeated insonation (3 W cm−2, 1 MHz, 5 min; Fig. 2a), with up to four triggerable events releasing 4.3 ± 0.8%, 4.5 ± 0.9%, 8.2 ± 2.1% and 6.0 ± 0.9%, respectively.

To demonstrate that the generation of ROS was the likely mechanism of triggered release, we irradiated Lipo–PPIX–SRho (PPIX 0.9 µg ml−1; see Methods for details) with 400 nm light, which can cause PPIX to generate ROS26. Lipo–PPIX–SRho irradiated at 5 W cm−2 for 10 min released 9.3% ± 1.8% of the dye payload (Supplementary Fig. 2). Lipo–SRho irradiated under the same conditions released very little dye (Supplementary Fig. 2).

The mechanical properties of blank liposomes (Lipo) and liposomes loaded with PPIX (Lipo–PPIX) were assessed by atomic force microscopy (AFM). Liposomes were immobilized on a 3-triethoxysilylpropylamine-functionalized silicon wafer and their elastic moduli were measured (see Methods for details). The mean elastic modulus of Lipo was 20.3 ± 6.2 kPa, and that of Lipo–PPIX was 217.3 ± 71.1 kPa ($P$ value comparing the two = 0.00079). These results demonstrated that the addition of PPIX to the liposomal formulation increased the elastic modulus of the liposomal bilayers; that is, it made them less susceptible to the mechanical effects of insonation. These results suggest that the release of TTX from Lipo–PPIX was more likely to have been attributable to ultrasound-induced sonochemistry than to the mechanical effects of ultrasound.

The dye-filled liposomes were injected subcutaneously to screen whether the ultrasound-triggered response would function in vivo.
Ultrasound-triggered TTX release in vitro. TTX was encapsulated in PPIX-loaded liposomes (Lipo–PPIX–TTX; Fig. 3a) with a mean size of 2.8 ± 0.5 μm. The loading efficiencies of TTX and PPIX were 22.2 ± 0.2% and 79.1 ± 8.5%, respectively (Supplementary Table 1), which were comparable to those reported in previous studies of TTX-loaded liposomes.

To confirm that TTX release was also triggerable from PPIX-loaded liposomes, the release of TTX in vitro was assessed by dialysing 150 μl of Lipo–PPIX–TTX against 14 ml of phosphate-buffered saline (PBS; Fig. 3b,c). Within the first 4 h, 7.1 ± 1.0% of TTX was released, followed by a slower release of approximately 0.4% per hour (Fig. 3b). Ultrasound (3 W cm⁻², 1 MHz, 10 min) applied at the 5 h time point induced the release of 5.4 ± 2.6% of TTX over the next 4 h (Fig. 3b). Without ultrasound, 1.4 ± 1.0% was released over the same time period. Liposomes loaded with TTX but without PPIX (Lipo–TTX) showed comparable TTX loading to Lipo–PPIX–TTX (Supplementary Table 1), but TTX release was faster in the absence of PPIX (19.6 ± 5.7% in the first 4 h, after which it levelled off; Fig. 3c). Ultrasound applied to Lipo–TTX at the 5 h time point did not trigger TTX release. Comparing the release profiles of Lipo–PPIX–TTX and Lipo–TTX, we found that in the first hour, the release from Lipo–TTX (Fig. 3c) was more than double that from Lipo–PPIX–TTX (P = 0.02; Fig. 3b), and by 2 h the difference was almost three-fold (P < 0.001). These results show that PPIX was necessary for ultrasound-triggered TTX release.

Lipo–PPIX–TTX and Lipo–TTX (TTX liposomes without PPIX) were insonated, and TTX was extracted as described and quantified by enzyme-linked immunosorbent assay. Insonation, and the resulting production of ROS, did not affect the concentration of TTX in Lipo–PPIX–TTX (Supplementary Fig. 3).

Ultrasound-triggered sciatic nerve blockade in vivo. Liposomes were injected at the rat sciatic nerve and nerve blockade was assessed by neurobehavioural testing using a modified hot-plate test (see Methods). In these experiments, the principal metric of nerve block was the latency (that is, the length of time for which a rat would leave its hindpad on a hotplate; 2 s was baseline and 12 s was maximal). In general, a block was considered successful if the latency was greater than 7 s. The duration of the nerve block was defined as the time for thermal latency to return to 7 s—the midpoint between baseline and maximal block.

Lipo–PPIX–TTX induced an initial nerve block lasting 8.3 ± 4.7 h (n = 4; Supplementary Table 2 and Fig. 4a). Insonation (3 W cm⁻², 1 MHz, 10 min) after the thermal latency returned below 4 s resulted in a return of the nerve block for 0.7 ± 0.2 h. A second application of ultrasound, which was applied after the thermal latency again returned to ≤ 4 s, caused a return of nerve block for 0.2 ± 0.2 h (Fig. 4a). No nerve block was observed after a third ultrasound application. There was no animal mortality or increase in latency in the non-injected extremity (an established metric of systemic toxicity). However, 50% of animals injected with Lipo–TTX (three out of six) died 3–10 h after injection, and four out of six rats showed an increase in latency in the non-injected extremity, which was possibly a reflection of the more rapid TTX release in this group (Fig. 3c). In the three animals that survived (Fig. 4b), Lipo–TTX induced an initial nerve block of 17.2 ± 11.3 h, but insonation after the thermal latency returned below 4 s did not induce a renewed nerve block (Fig. 4b; P = 0.001 between the nerve-block durations of Lipo–PPIX–TTX and Lipo–TTX after insonation) or dye release (Supplementary Fig. 4), demonstrating that PPIX was necessary for insonation-induced nerve blocks and cargo release.

To assess the possibility that a formulation component other than TTX was causing the nerve blocks, rats were injected at the sciatic nerve with PPIX-loaded liposomes without TTX (Lipo–PPIX; Supplementary Fig. 5). The initial injection did not induce a nerve block, nor did insonation 8 h after the injection, demonstrating that TTX was necessary for nerve block to occur. Animals directly treated with ultrasound in the absence of formulation administration did not develop a nerve block upon insonation (Supplementary Fig. 6).

To further demonstrate that the ability of Lipo–PPIX–TTX to trigger a nerve block was due to ultrasound causing liposome lipid peroxidation, and no other effects of ultrasound, we injected rats at the sciatic nerve with TTX-containing liposomes (Lipo–DSPC–TTX) that did not contain PPIX or DLPC (DLPC was replaced by DSPC, which cannot be peroxidated) and therefore could not be triggered in vitro (Supplementary Fig. 7a). Lipo–DSPC–TTX caused an initial nerve block of 6.1 ± 3.6 h, which was comparable to the duration of initial block caused by Lipo–PPIX–TTX, but insonation did not induce a nerve block (Supplementary Fig. 7).

To further enhance the number and duration of ultrasound-triggerable nerve blocks, we co-administered dexmedetomidine (DMED)-loaded liposomes (Lipo–DMED; see Supplementary Fig. 8 for a cryo-transmission electron microscopy image and
Methods for synthesis) with Lipo–PPIX–TTX at a 1:2 (Lipo–DMED-to-Lipo–PPIX–TTX) mass ratio. DMED, an α2-adrenergic agonist, prolongs the local anaesthetic effects of TTX29. Lipo–PPIX–TTX + Lipo–DMED caused an initial nerve block of 34.5 ± 5.0 h (Fig. 5 and Supplementary Table 2). Repeated insonation (3 W cm–2, 1 MHz, 10 min) after a return to ≤ 4 s latency triggered three separate consecutive nerve blocks with durations of 1.8 ± 1.2 h, 0.9 ± 0.3 h and 0.5 ± 0.3 h (Fig. 5a and Supplementary Table 2). The fourth ultrasound application induced an increase in hindpaw thermal latency to a mean of 5.3 ± 1.6 s. These results demonstrate that the co-administration of Lipo–DMED and Lipo–PPIX–TTX enhanced the repeatability and duration of nerve blocks compared with Lipo–PPIX–TTX alone. Administration of free TTX and DMED caused an initial nerve block of 9.27 ± 2.86 h. Insonation after the thermal latency returned to 4 s did not cause an increase in thermal latency (Supplementary Fig. 9). These results demonstrate that the residual free drug was not sufficient to induce a nerve block upon insonation. A corollary to this is that ultrasound was not able to change the effect of the drugs so as to achieve a nerve block.

The intensity and duration of the ultrasound-triggered nerve blocks could be controlled by varying the intensity and duration of the applied insonation after the initial nerve block caused by the administration of Lipo–DMED + Lipo–PPIX–TTX had worn off (Fig. 5b–d and Supplementary Table 3). Insonation pulses of 2, 5 and 10 min induced nerve blocks with mean durations of 0.2 ± 0.2 h, 0.5 ± 0.2 h and 2.3 ± 0.9 h, respectively (Fig. 5b). Up to five separate consecutive ultrasound-triggerable nerve blocks were achieved by insonation of 5 min (Supplementary Table 3). The mean peak thermal latency was 12 s for 10 min insonations, 9.9 ± 2.5 s for 5 min insonations and 7.3 ± 1.4 s for 2 min insonations. The survival rate
for all rats injected with Lipo–PPIX–TTX (with or without Lipo–
DMED; 20 animals in total) was 100% and no increase in contra-
lateral latency was observed, suggesting no clinically significant
systemic distribution of TTX. The duration of the ultrasound-
triggered nerve block increased as the duration of insonation
increased, with a plateau at 10 min (Fig. 5c).

To examine the effect of insonation intensity on ultrasound-
triggered nerve blocks, insonations of different ultrasound intensi-
ties (10 min, 1–3 W cm−2) were applied after the initial nerve block
had worn off (Fig. 5d). The intensity and duration of the nerve
blocks increased with the intensity of insonation (Fig. 5d and
Supplementary Table 4). No effective nerve block occurred below
insonation intensities of approximately 1 W cm−2, above which the
duration of the nerve block increased with ultrasound intensity.

**Tissue reaction.** In all animals that underwent neurobehavioural
testing, the sciatic nerve and surrounding tissues were collected four
days after the last ultrasound application. Animals administered
PPIX-loaded liposomes had reddish-brown (the colour of PPIX)
liposome deposits surrounding the sciatic nerve (Supplementary
Fig. 10), demonstrating accurate liposome injection at the tar-
get site. Collected tissues were processed into haematoxylin and
eosin-stained slides. All animals injected with liposomes showed
mild inflammation at the injection site, which was consistent with
previous reports of perineural microparticle injection30, but mini-
mal inflammation was seen in the adjacent muscle. Foamy macro-
phages were observed at the injection site, showing particle uptake
(Supplementary Fig. 11). All slides were scored for inflammation
(0–4) and myotoxicity (0–6; see Methods). All groups had median
inflammation scores of 1 (Supplementary Fig. 11; see Supplementary
Table 5 for detailed scores). The median myotoxicity score was 0
in all groups (Supplementary Fig. 11 and Supplementary Table 5).
Insonation itself (in the absence of liposome administration) caused
no significant inflammation (P = 0.4 compared with normal tissue),
myotoxicity (P = 0.4 compared with normal tissue) or signs of other
tissue injury (Supplementary Fig. 12) 1 h after application. These
data indicate that the ultrasound conditions used in this work do
not induce tissue injury either immediately after application or in
the following four days.

Sciatic nerves were stained with toluidine blue and sectioned. No significant neurotoxicity was observed in any animals
(Supplementary Fig. 13).

**Ultrasound-guided injection and sonography of liposomes.**
Ultrasound-guidance has become a standard of practice for periph-
eral nerve blockade31. It would be advantageous if ultrasound could
be used both for procedural imaging and subsequent triggering.
We assessed whether Lipo–PPIX could be visualized by ultrasound
(Fig. 6). PPIX-loaded liposomes (Lipo–PPIX) were placed within
ultrasonic gel in vitro and imaged using high-frequency ultrasound
(40 MHz, spatial peak temporal average intensity: < 0.08 W cm−2).
The liposome layer was densely echogenic while the same volume
of water was not (Fig. 6a). Under the same experimental condi-
tions, the echogenicity of Lipo was similar to that of Lipo–PPIX
(Supplementary Fig. 14). To show that Lipo–PPIX could be imaged
at clinical imaging frequencies (2–20 MHz), Lipo–PPIX was suc-
cessfully imaged with a 20 MHz transducer (Supplementary Fig. 16).

In anaesthetized animals, the sciatic nerve was identified under
sonography at a tissue depth of approximately 7 mm (Supplementary
Fig. 15 and Fig. 6b). A 23G needle was then advanced beside the
depending on the clinical context and anatomical site. The need for opioid prescription. Further developments of this pain management. It is possible that such a system would mitigate of on-demand nerve blockade, enabling personalized narcotic-free perioperative contexts. It would then allow an additional half-day continuous initial nerve block—a duration very suitable for many peripheral nerve blockade24,43.

As ultrasound is commonly used in clinics and hand-held therapeutic ultrasound devices are commercially available, controlling patent relief with ultrasound could be a readily translatable method for on-demand local anaesthesia. Ultrasound has been used safely for therapy at tissue depths of 2.3 to 5 cm (ref. 32). The device used in this study was a commercial ultrasound employed in physiotherapy under conditions approved for clinical use. No tissue injury was observed.

Ultrasound is a versatile energy source as it allows image-guided injection, identification of the injectate in situ, confirmation of the appropriate site and triggering of drug release. It is therefore possible to envision a single ultrasound device for pain treatments that uses a diagnostic ultrasound setup (higher frequency and lower power) during injection, followed by formulation imaging and then a therapeutic setup (lower frequency and higher power) to trigger further anaesthetic events post-injection.

The hypothesis that sonochemistry and the production of ROS play a major role in ultrasound-triggered cargo release is supported by the observation that liposomes loaded with PPIX produced a higher amount of ROS compared with blank liposomes (Fig. 1), as well as the fact that the incorporation of PPIX was necessary for triggered release of the cargo (Figs. 1, 3 and 4). The production of ROS upon insonation in the presence of sonosensitizers could be due to several mechanisms18,33. Ultrasound-induced cavitation can produce light (sonoluminescence)34, which could then activate the photosensitizer PPIX to produce ROS. Implosion of ultrasound-induced cavitation bubbles could also induce the formation of sonosensitizer-derived free radicals that generate ROS18. These effects are dependent on inertial cavitation upon insonation15, which could be induced by the ultrasound conditions used here (3 W cm−2, 1 MHz, continuous wave)16.

Another possible mechanism for the ultrasound-triggered cargo release may be the mechanical effect of ultrasound. Liposome disruption caused by the mechanical effects of ultrasound is known to be more efficient at frequencies much lower than those used in this study18,33. Here, we found that PPIX-loaded liposomes (Lipo–PPIX) were more mechanically stable (that is, they had a higher elastic modulus) than liposomes without PPIX (Lipo). This is in agreement with previous studies, in which the incorporation of photosensitizers reduced the fluidity (raised the elastic modulus) of lipid membranes35, perhaps by enhancing hydrophobic interactions in the lipid bilayer, thereby stabilizing the liposomes36. The higher mechanical stability of PPIX-loaded liposomes would make it more difficult for them to be activated by ultrasound-induced mechanical effects. These data suggest that sonochemical effects, rather than mechanical effects, may be the major mechanism for the ultrasound-triggered release of TTX.

The difference between the elastic moduli of Lipo and Lipo–PPIX may also explain the difference between their release kinetics, as shown in Fig. 3b,c. A decrease in the fluidity of the lipid bilayer would decrease the rate of cargo release. This is consistent with our observation in Fig. 3, where Lipo was found to release its cargo more rapidly than Lipo–PPIX due to the higher fluidity of its lipid bilayers.

The ability of ultrasound to trigger PPIX-loaded liposomes increased with PPIX loading at low PPIX concentrations, then decreased above a loading capacity of 0.3% (Fig. 1). This observation is consistent with previous reports37, in which it was attributed to an increase in liposomal stability due to the hydrophobicity of the PPIX included in the lipid bilayer. It is also possible that above a certain concentration of PPIX there is a self-quenching effect that diminishes the efficacy of PPIX in generating ROS (as has been described for aluminium and zinc phthalocyanines40,41). The formulation that produced the greatest amount of ROS was the same formulation that had the greatest ultrasound-triggered dye release. These results indicate that the decrease in the ability of ultrasound to trigger formulations with PPIX loadings higher than the optimal PPIX loading may be due to the increase in the PPIX-induced liposomal bilayer stability and the decrease in ROS generation of the formulation due to the self-quenching effect of sonosensitizers.

TTX and other compounds27 that block the sodium channel at a site (site 1) different from that targeted by conventional local anaesthetics have generally been used as scientific tools. However, their potent local anaesthetic properties have long been recognized42, as has the fact that their systemic toxicity can be curtailed with other drugs25 or via sustained release1. Recently, site 1 sodium channel blockers including TTX and neosaxitoxin have undergone clinical trials; in particular, neosaxitoxin has undergone clinical trials for peripheral nerve blockade26,43.

Particles containing local anaesthetics—including those described here—generally serve as a depot, with drugs diffusing from the site of injection to adjacent nerves44. The present system could be applied in the same general way as most local anaesthetics (that is, throughout the body, either as an injection throughout the tissue planes (infiltration anaesthesia) or along specific nerves or groups of nerves (regional anaesthesia)). In regional anaesthesia,

**Fig. 6 | Ulasonography of Lipo–PPIX.** a. In vitro comparison of the echogenic contrasts of Lipo–PPIX and water in ultrasound gel (see Methods). b. Sonograms before and after ultrasound-guided perisciatic injections of Lipo–PPIX. Sonograms were taken with a 40 MHz ultrasound transducer used for small animal imaging. Scale bars, 1 mm.
the particle depot is remote from the painful site; analgesia is provided by interruption of the nerve signal proceeding to the brain.

We hypothesized that ultrasound-induced TTX release was the main mechanism of ultrasound-triggered nerve blockade. Modulation of peripheral nerve activity by ultrasound (‘neuro-modulation’) was another potential contributing factor. In our experiments, insonation of rats injected with PPIX-loaded liposomes without TTX did not induce an increase in thermal latency (Supplementary Fig. 5), and neither did insonation of uninjected animals (Supplementary Fig. 6). These experiments suggest that ultrasound itself does not cause nerve blocks in the absence of TTX. Ultrasound did not cause nerve blocks after the recovery of analgesia from TTX + DMED solution (Supplementary Fig. 9), although DMED solution (0.375 mg ml–1 PBS; Abcam) or SRho solution (10 mg ml–1 PBS; Aldrich) caused by the liposomes or insonation (the ultrasound parameters used in this study are used for therapeutic ultrasound). TTX has caused by the liposomes or insonation (the ultrasound parameters used in this study are used for therapeutic ultrasound). TTX has caused by the liposomes or insonation (the ultrasound parameters used in this study are used for therapeutic ultrasound).

The eventual lack of effect after multiple triggering of PPIX-loaded liposomes in vitro could be attributable to a number of factors. Ultrasound-triggered peroxidation is irreversible, limiting the number of triggerable (peroxidation-dependent) events. Another potential contributing factor is the depletion of drug remaining in the liposomal reservoir due to basal release and repeated triggering. In vivo, it is possible that degradation and/or the removal of particles could also play a role.

We sought to improve the performance of the system by increasing the effect of each packet of TTX released. We did so by co-delivering DMED, an α4-adrenergic agonist that prolongs the therapeutic effects of local anaesthetics. Since the DMED was contained in separate liposomes from the TTX, the effect of DMED was likely pharmacological, rather than being due to increased TTX release. DMED increased the duration of the initial nerve block, as well as the duration and number of triggered nerve blocks (Figs. 4 and 5 and Supplementary Table 2). One potential mechanism of such enhancement is DMED’s inhibition of hyperpolarization-activated cation current. Another contributing factor is local α4-adrenergic receptor mediated vasoconstriction, which inhibits redistribution of the local anaesthetic, maintaining a high local concentration at the target tissue.

Many local anaesthetic drug-delivery systems continue to release the drug after the local effect has ended. This was seen here in the marked prolongation of the initial block due to the addition of DMED to the formulation. All released drugs eventually enter the systemic circulation and are eliminated—renally in the case of TTX (ref. 93). Of note, the second drug can also be beneficial in mitigating the systemic toxicity of TTX, particularly if it is a local vasoconstrictor, as are α2-adrenergic agonists such as DMED.

The mild inflammation caused by liposome injections is generally considered to be safe, and we saw no significant tissue toxicity caused by the liposomes or insonation (the ultrasound parameters used in this study are used for therapeutic ultrasound). TTX has also been shown to have minimal myo- and neurotoxicity. PPX is the active agent of the Food and Drug Administration-approved drug 5-aminovaleric acid. The major ROS that are involved in sonosensitization are hydroxyl radicals and singlet oxygen. Hydroxyl radicals have a half-life of 10−8 s and a diffusion distance of 0.06 nm, while singlet oxygen has a half-life of 10−8 s and a diffusion distance of 268 nm (ref. 99). These distances are small compared with the length scales of tissue, suggesting a low likelihood of toxicity, which is consistent with the results of this study.

Sustained release local anaesthetic systems act by continuously releasing doses high enough to be effective over an extended period of time. Consequently, those that can achieve very long durations of effect commonly contain substantial quantities of drug. Sustained release also prevents systemic toxicity; the fact that the drug payload is contained in a vast number of separate particulate compartments is one safeguard against catastrophic dumping of a drug with resulting toxicity. Another important safety consideration when translating such systems for use in humans lies in the difference in size between humans and rats. The dose required for a given local anaesthetic endpoint tracks weakly with animal size. In contrast, local anaesthetic toxicity tracks fairly linearly with animal size, in proportion to the increased volume of distribution. Therefore, the therapeutic index would be expected to increase in larger animals. This was seen in humans who were able to achieve safe prolonged local anaesthesia with neosaxitoxin—a compound that acts by a mechanism similar to that of tetrodotoxin. The doses used in those humans would have been uniformly fatal in rats.

Liposomes may have intrinsic echogenic properties that are dependent on their lipid composition, concentration and vesicle structure. Oligo- and multilamellar liposomes, such as the ones used in this study, are more echogenic than unilamellar liposomes, so they can be more readily imaged using ultrasound. Here, 40 MHz was used to provide higher-resolution imaging to better target the rat sciatic nerve, which is much smaller than that of humans. This imaging frequency is often used in small animal imaging. In the clinic, ultrasound frequencies of 2–20 MHz are used for sonography. Our liposomes can be imaged at these frequencies (Supplementary Fig. 16).

We have developed an ultrasound-triggerable, on-demand nerve-block system that allows for the effective control of nerve-block duration and intensity simply through the duration and intensity of insonation. Diagnostic ultrasound allowed the guided injection and imaging of the formulation, whereas therapeutic ultrasound induced adjustable and repeatable nerve blocks.

Methods

Liposome preparation. The liposomes were prepared using the thin-film hydration method, as reported in brief, the lipid formulation (OMPC (Avanti Polar Lipids), DLPC (Avanti Polar Lipids), DSPG (Genzyme) and cholesterol (Sigma) at a molar ratio of 3:3:2:3), along with the indicated amount of the sonosensitizer PPIX, was dissolved in a solution of chloroform and methanol (ratio: 9:1). The solvent was evaporated under reduced pressure, and the lipid was redissolved in tert-butanol, followed by freeze drying. The lipid cake was hydrated with PBS, TTX solution (0.175 mg ml−1 PBS; Abcam) or SRho solution (10 mg ml−1 PBS; Aldrich). After ten freeze–thaw cycles, the solution was dialysed against PBS for 48 h in a dialysis tube with a molecular mass cut-off of 1,000 Da. The dialysis media were changed with fresh PBS at least twice a day. Lipo–DMED was made following the same procedure, but with PPIX not included in the formulation and with hydration in 1 mg ml−1 of DMED in PBS solution.

Liposome characterization. The liposome size was determined using a Beckman Coulter Multisizer 3 (Beckman Coulter). The liposomal SRho content was determined using UV–vis absorption (λmax = 565 nm) after disrupting the liposomes with octyl β-D-glucopyranoside (Sigma–Aldrich). The liposomal PPIX content was determined using UV–vis absorption at 402 nm after disrupting the liposomes in ethanol. The liposomal TTX content was determined using an enzyme-linked immunosorbent assay (Reagen) after removing the lipid fraction via the Bligh and Dyer method.

ROS detection. Carboxy-H2DCFDA (Molecular Probes) is a non-fluorescent reagent commercially available for the detection of ROS. Upon exposure to ROS, it undergoes oxidation and forms the highly fluorescent 2′,7′-dichlorofluorescin, which can be detected using fluorescence measurements (Supplementary Scheme 2). Carboxy-H2DCFDA was dissolved in ethanol and diluted 100-fold in PBS. This was then added to liposome suspension to produce a final concentration of 10 μM. The fluorescence emission at 527 nm (excitation: 493 nm) was monitored after applying ultrasound to 1 ml of the solution at 3 W cm−2 for 10 min.
In vitro release of fluorescent dye. Self-quenching Srho was used as a hydrophilic model dye. To measure ultrasound or 400 nm light-triggered release, Srho liposomes were diluted 200-fold in PBS. For the ultrasound-triggered experiments, 1 ml of the suspension was placed in a 20 ml glass vial, which was sealed with a latex membrane. Ultrasonic gel was then placed between the latex membrane and the ultrasonic source. Ultrasound was applied at the reported power and duration. For the light-triggered experiments, the diluted liposome solution was directed against 400 nm light source for 10 min at 5 mW cm⁻². The fluorescence intensity (excitation: 580 nm; emission: 580 nm) was recorded. The release of dye from the liposomes upon ultrasound or light exposure was quantified according to the following equation:

\[ \text{Cumulative release} \% = \frac{F - F_{\text{break}}}{F_{\text{break}} - F_0} \times 100 \]

where \( F \) is the fluorescence of the solution upon ultrasound exposure, \( F_0 \) is the fluorescence of the liposome solution before ultrasound exposure and \( F_{\text{break}} \) is the fluorescence of the surfactant (octyl-\( \beta \)-glucopyranoside)-disrupted liposome solution.

AFM. Silicon wafer (Ted Pella) was functionalized with 3-triethoxysilylpropylamine (APTES; Sigma–Aldrich) using a previously reported method. Liposomes were placed onto the wafer and left set for 1 h at room temperature. A DNP-10 AFM tip (Bruker Nano) was used. The AFM machine model was MFP-3D Bio (Asylum Research). Force curves were measured and analysed using the computational program Igor Pro 6.3.7.2 (WaveMetrics). The Hertzian model was applied to obtain the elastic modulus as previously reported.

In vivo imaging with dye-loaded liposomes (Lipo–PPIX–SRho). Lipo–PPIX–SRho (150 μl) was injected subcutaneously and the fluorescence intensity of the released fluorophore was detected and quantified using an in vivo imaging system (IVIS Spectrum; Caliper Life Sciences) before and after ultrasound application (3 W cm⁻², 10 min, 1 MHz) at excitation and emission wavelengths of 335 nm and 580 nm respectively.

In vitro TTX release. TTX release experiments were performed by placing 150 μl of TTX-loaded liposomes into a Slide-A-Lyzer MINI dialysis device (Thermo Scientific) with a 20,000 molecular weight cut-off. The sample was dialysed against 14 ml PBS and incubated at 37 °C on a platform shaker at 150 r.p.m. (New Brunswick Innova 40; Eppendorf). At predetermined intervals, the dialysis solution was exchanged with fresh PBS. To measure the ability of ultrasound to trigger the liposomes, ultrasound was applied (3 W cm⁻², 1 MHz) for 10 min at the 5th time point. The TTX concentration was determined using an enzyme-linked immunosorbent assay (Reagen LLC).

Animal studies. Animal studies were performed according to protocols approved by the Boston Children's Hospital Animal Care and Use Committee following the guidelines of the International Association for the Study of Pain. Male Sprague–Dawley rats (Charles River Laboratories) weighing 300–400 g were housed in groups under a cycle of 12 h of light followed by 12 h of dark with lights on at 07:00. The rats were randomly assigned to each group. Under brief isoflurane–oxygen anaesthesia, the rats were injected with 200 μl of liposome or water on top of a layer of ultrasonic gel and covering it with another layer of gel. The top layer of gel was in contact with the transducer. Sprague–Dawley rats of 300–400 g were anaesthetized using isoflurane–oxygen and positioned under the ultrasound transducer. Ultrasound gel was applied between the transducer and the rat. A 23G needle was used to inject 200 μl of Lipo–PPIX. The needle was placed beside the sciatic nerve as shown by the real-time sonogram before liposome administration (Supplementary Fig. 6).

Statistical analysis. Statistical comparisons were performed using the Student's t-test (one-sided) unless stated otherwise. The variance between the groups that were statistically compared was similar. Thermal latency, inflammation and myotoxicity scores were reported as medians and quartiles due to their ordinal or non-Gaussian character. All other data were described as means and s.d.

Data availability. The data that support the findings of this study are available within the paper and its Supplementary Information.

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Author contributions
A.Y.R., J.L.P., W.W. and D.S.K. designed the experiments. A.Y.R., J.L.P., B.W. and C.D.A. performed the experiments. A.Y.R., J.L.P., W.W., M.V.-R., R.L. and D.S.K. analysed the data. A.Y.R., J.L.P., R.L. and D.S.K. wrote the paper.

Competing interests
Two provisional patent applications (U.S.S.N. 62/329,721) have been filed concerning the technology presented in this work.

Additional information
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