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MECHANISTIC INSIGHT INTO SONOPORATION WITH ULTRASOUND-STIMULATED POLYMER MICROBUBBLES

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Abstract

Sonoporation is emerging as a feasible, non-viral gene delivery platform for the treatment of cardiovascular disease and cancer. Despite promising results, this approach remains less efficient than viral methods. The objective of this work is to help substantiate the merit of polymeric microbubble sonoporation as a non-viral, localized cell permeation and payload delivery strategy by taking a ground-up approach to elucidating the fundamental mechanisms at play. In this study, we apply simultaneous microscopy of polymeric microbubble sonoporation over its intrinsic biophysical timescales—with sub-microsecond resolution to examine microbubble cavitation and millisecond resolution over several minutes to examine local macromolecule uptake through enhanced endothelial cell membrane permeability—bridging over six orders of magnitude in time. We quantified microbubble behavior and resulting sonoporation thresholds at transmit frequencies of 0.5, 1 and 2 MHz, and determined that sonic cracking is a necessary but insufficient condition to induce sonoporation. Further, sonoporation propensity increases with the extent of sonic cracking, namely, from partial to complete gas escape from the polymeric encapsulation. For the subset that exhibited complete gas escape from sonic cracking, a proportional relationship between the maximum projected gas area and resulting macromolecule uptake was observed. These results have revealed one aspect of polymeric bubble activity on the microsecond time scale that is

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SUPPLEMENTARY DATA

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associated with eliciting sonoporation in adjacent endothelial cells, and contributes toward an understanding of the physical rationale for sonoporation with polymer-encapsulated microbubble contrast agents.

Keywords

Sonoporation; Microbubbles; High-speed imaging; Endothelial cells; Sonic cracking

INTRODUCTION

Gene therapy is becoming an increasingly attractive therapeutic approach to modulating molecular determinants of disease pathogenesis, such as in cancer or cardiovascular disease. A range of different strategies have been applied to cancer gene therapy, including delivery of tumor suppressor genes (Roth 2006; Ventura et al. 2007), immunotherapy (Rosenberg et al. 2004) and gene-directed enzyme pro-drug therapy (Dachs et al. 2005). Cardiovascular molecular targets employed for therapeutic intervention include vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) to promote therapeutic angiogenesis in ischemia applications (Cao et al. 2003; Isner et al. 1996; Losordo et al. 1998), and genes responsible for calcium homeostasis (Jaski et al. 2009; Miyamoto et al. 2000). Despite these results, effective strategies to deliver nucleic acids intra-vascularly to the site of disease and traverse the endothelial cell membrane are limited. Viral vectors, although efficient, elicit specific inflammatory and antiviral immune responses (Otake et al. 1998; Verma and Somia 1997). Promising non-viral vectors, including liposomal (Audouy et al. 2002) and polymer-based nanoparticle (Leong et al. 1998; Salem et al. 2003) approaches, face the endothelial barrier and must escape early endosomes to deliver their payload to the cell. To address this, our group and others have explored using ultrasound-stimulated microbubbles to potentiate the delivery of therapeutics, either co-injected (Aryal et al. 2013; Jordao et al. 2010; Kinoshita and Hynynen 2005; Treat et al. 2007) or anchored to the bubble itself (Carson et al. 2012; Christiansen et al. 2003; Kopechek et al. 2015; Leong-Poi et al. 2007; Shohet et al. 2000; Taniyama et al. 2002). By applying focused ultrasound to the target tissue during microbubble microvascular transit, localized acoustic cavitation can cause transient increases in plasma membrane (Bao et al. 1997; van Wamel et al. 2006) and vasculature permeability (Price et al. 1998; Skyba et al. 1998), aiding in site-specific therapeutic delivery. The efficiency of this platform, currently less than that of viral methods (Li and Huang 2006; Mehier-Humbert and Guy 2005), can be improved by a more complete understanding of microbubble physics and how it affects neighboring cell membrane permeability over longer time scales. To this end, our previous work with phospholipid-encapsulated agent-coupled ultrafast microscopy (16 million frames per second [Mfps]) and epifluorescence microscopy (15 fps) and simultaneously gathered information on single microbubble–cell systems over the two biophysically intrinsic time scales associated with sonoporation—ultrasound cavitation on the sub-microsecond time scale and cellular response (including macromolecule uptake/diffusion *via* enhanced membrane permeability and plasma membrane reorganization) on the order of seconds to minutes (Helfield et al. 2016b). This work complements other sonoporation studies using lipid bubbles that examine biological consequences, including investigations into calcium signaling (Fan et al. 2010,

2012; Park et al. 2011), alterations in cell cycle (Chen et al. 2013a; Miller and Dou 2009; Zhong et al. 2011) and plasma membrane features (De Cock et al. 2015; Kudo et al. 2009; Leow et al. 2015; Schlicher et al. 2010; van Wamel et al. 2004).

As payload delivery vehicles, polymeric microbubbles can provide longer circulation times and increased drug/gene loading capacities within their encapsulation compared with lipid agents (Ferrara et al. 2007; Hernot and Klibanov 2008). Polymer-coated microbubbles, however, exhibit fundamentally different oscillation physics (Bloch et al. 2004) because of their relatively incompressible and rigid encapsulation. As opposed to lipid monolayer-based encapsulated agents, thick-shelled polymer agents can more easily withstand in-plane compression and can exhibit a globally non-spherical shape (Marmottant et al. 2011). Further, these microbubbles can undergo “sonic cracking,” whereby the encapsulation material cracks and results in anisotropic free gas release (Bouakaz et al. 2005; Postema et al. 2005). This phenomenon may have particular application in drug release strategies that incorporate a therapeutic within the bubble itself (*e.g.*, in oil phase [Kooiman et al. 2009; Unger et al. 2002]) as opposed to being chemically anchored along the shell surface, a strategy that may aid in payload shielding (Lentacker et al. 2006; Tinkov et al. 2009).

Previous work has employed ultrafast microscopy to relate microbubble dynamics to sonoporation, conducted as separate correlative experiments (Kudo et al. 2009; van Rooij et al. 2016). There have also been multiple insightful studies employing coupled microscopy approaches to observe microbubbles on kilohertz timescales (*e.g.*, Fan et al. 2014). Our coupled approach with an ultrafast camera provides us the unique opportunity to directly observe and relate microbubble cavitation physics (sub-microsecond time scales) to neighboring endothelial cell permeability (millisecond time scales).

The specific objective of this work was to gain insight into mechanisms responsible for sonoporation with polymeric microbubbles. In this manner, rather than simply describing microbubble and ultrasound parameters that are empirically effective in causing sonoporation, our work attempted to discover fundamental governing physical principles of sonoporation with polymeric microbubbles.

METHODS

Cell culture

Primary human umbilical vein endothelial cells (HUVECs; No. C2519A, Lonza, Verviers, Belgium) were cultured in medium (EGM-2, No. C3162, Lonza) and incubated at 37°C with 5% CO₂. HUVECs were plated in an Opticell chamber (Thermo Scientific, Rochester, NY, USA), consisting of two 50-cm² gas-permeable, polystyrene membranes approximately 75 μm in thickness and separated by 2 mm. Experiments were conducted on HUVEC monolayers characterized by an approximate confluency of 80% to 90%, and all experiments were performed on cells with passage numbers $p \leq 10$ (number of times these cells were subcultured and a surrogate measure for cell age).

Sonoporation marker

Propidium iodide (PI; P1304 MP, Molecular Probes, Eugene, OR, USA) was employed as a surrogate drug and sonoporation marker, and was diluted in the culture medium at a concentration of 120 μM . PI is a non-fluorescent, cell-impermeant intercalating agent, with a molecular weight of 668.4 Da. On entry within a cell through plasma membrane perforation, PI exhibits a dramatic increase in fluorescence as it binds to RNA/DNA complexes (maximum excitation and emission at 535/617 nm). As such, this reagent has been extensively employed for this purpose (Karshafian et al. 2009; Kooiman et al. 2011; Leow et al. 2015; van Wamel et al. 2006).

Microbubble contrast agent

Polymeric microbubbles (PB127) composed of a nitrogen gas core surrounded by a poly(DL-lactide) (PLA, MW = 40 kDa, Lakeshore Biomaterials, Birmingham, AL, USA) shell coated with crosslinked human serum albumin (HSA, Sigma-Aldrich, St. Louis, MO, USA) were fabricated using a solvent evaporation interfacial deposition technique previously described (Ottoboni et al. 2001). Briefly, isopropyl acetate solution (Sigma-Aldrich) containing 1% polymer and 13% cyclo-octane (Sigma-Aldrich) was emulsified into a 5% HSA aqueous solution by pumping the mixture solution through a metal filter with micron-sized pores. The resulting oil-in-water emulsion was poured into a 0.1% glutaraldehyde aqueous solution (Sigma-Aldrich) to crosslink and harden the emulsified microdroplets. The microbubbles were freeze-dried and sealed in nitrogen-containing vials with aluminum seals for storage at room temperature. Although this agent is interrogated on a single-bubble basis in the present study, for completion we present the size distribution of PB127 in Figure 1, in terms of both number and volume-weighted number density. Vials were reconstituted at room temperature in 2 mL of sterile water and agitated by hand ($n = 3$). Along with an 18G venting needle, 0.1 mL of agent was withdrawn with an 18G needle from a location just above the rubber stopper and placed within a microcentrifuge tube ($n = 3$). Agent was diluted in ISOTON II (1:1000) and measured using a Coulter Counter Multisizer 3 (Beckman Coulter, Brea, CA, USA) with a 30- μm aperture and 50- μL extraction volume (averaged in triplicate). The peak number-weighted diameter was determined to be $3.20 \pm 0.14 \mu\text{m}$, and the peak volume-weighted diameter was $3.95 \pm 0.08 \mu\text{m}$. The native concentration was $(7.65 \pm 0.18) \times 10^8$ bubbles/mL.

Experimental protocol

Simultaneous microscopy of ultrasound-stimulated microbubble behavior and subsequent cellular permeability dynamics were recorded as described previously (Helfield et al. 2016b). Briefly, a custom-made water tank held at 37°C housed any of three single-element transducers employed for this study ($f = 0.5 \text{ MHz}$, $f\# = 1.65$, Model A301 S-SU-F1.65-PTF; $f = 1 \text{ MHz}$, $f\# = 1.67$, Model A302 S-SU-F1.63-PTF; $f = 2.25 \text{ MHz}$, $f\# = 2$, Model V306-SU-PTF, Olympus NDT, Waltham, MA, USA) at an angle of 45° from below along with a port to house the liquid light guide used to deliver the flash lamp illumination (Fig. 2). Optical-acoustical co-alignment was performed with a pulse-echo approach (Chen et al. 2013b). Individual microbubbles adjacent to a HUVEC monolayer were insonicated in the presence of PI with a single, eight-cycle Tukey windowed ultrasound pulse ($\alpha = 0.5$) at 0.5,

1 or 2 MHz generated from an arbitrary function generator (AFG3252, Tektronix, Beaverton, OR, USA) and amplified by a gated power amplifier (Model 250 A250 AM8, Amplifier Research, Souderton, PA, USA). A single pulse of this duration was chosen such that the complete microbubble response (i) could be captured within the 128 frames of the ultrafast University of Pittsburgh Medical Center camera (UPMC-Cam) while sufficiently satisfying Nyquist sampling, and (ii) could be directly linked to the adjacent cellular permeability dynamics. The transmit pulses were sent at a peak negative pressure from 0.1 to 0.5 MPa, 0.5 to 1.5 MPa and 0.5 to 1.0 MPa for 0.5, 1 and 2 MHz, respectively, as measured in a separate water tank with a hydrophone (HGL-0200, Onda, Sunnyvale, CA, USA). With respect to the estimated pressures *in situ*, hydrophone measurements with Opticell membrane (75 μ m thick) in the beam path confirmed negligible transmission losses at these transmit frequencies. With the known transducer angle with respect to the optical axis (45°) and a known objective working distance (2 mm), we estimated the *in situ* peak negative pressure at the bubble location (primary wave and reflected wave) as approximately 1.6-, 1.06- and 1.05-fold the acoustic pressures as measured in the hydrophone tank at 0.5, 1 and 2 MHz, respectively, assuming constructive interference (worst-case scenario). Microbubble dynamics were extracted with custom edge-detection software (Helfield et al. 2012) and the epifluorescence microscopy recordings were analyzed with ImageJ (Schneider et al. 2012).

Scanning electron microscopy

Separate pilot experiments were performed to gain general intuition on the state of polymeric microbubbles subjected to ultrasound. After agent reconstitution in 2 mL of saline, 0.5 mL of native solution was diluted 1:1 in purified water and centrifuged for 3 min at 50g. On removal of the bottom 0.5 mL, this process of dilution and subsequent centrifugation was repeated four times to minimize the presence of saline crystals. The sample was then diluted 1:1000 in de-ionized water, and these populations of microbubbles were treated with ultrasound (0.5 MHz, 0.5 MPa, 8-cycle burst, 1-kHz pulse repetition frequency, pulse duration of 1 s) within a microcentrifuge tube placed at the transducer focus. A 2- μ L insonicated sample was then placed on a coverslip and allowed to dry for approximately 1 h. Samples were mounted on an aluminum stub and sputter coated with a 4.5-nm-thick coating of gold/palladium. Scanning electron microscopy (SEM) was performed with a JSM-6335 F system (Jeol USA, Peabody, MA, USA).

Statistics

For nominal variable outputs, Fisher's exact test was employed to determine dependence between relative proportions (significance at the $\alpha = 0.05$ level, compensated for multiple comparisons with the Bonferroni correction). Two separate linear discriminant analyses (LDAs) were performed to classify sonic cracking bubbles and sonoporation events in the transmit pressure-initial bubble size parameter space $\mathbf{x}$, namely, determining an optimal group-separating axis by assigning observations to set i if $P(\mathbf{x}|i)P(i) > P(\mathbf{x}|j)P(j)$ for $\forall j \neq i$, where the classes consist of either $i = \{\text{sonic cracking}\}$ or $i = \{\text{sonoporation positive}\}$, set j is the complement of set i , and $p(a|b)$ is the probability of observing event a given that event b is true (standard Bayesian probability notation). The strength of association between projected gas area and PI signal intensity was assessed with Pearson's correlation coefficient

(*i.e.*, linear regression model), and its significance determined in the usual manner (two-sided *t*-test). All statistics were performed in MATLAB (The MathWorks, Natick, MA, USA).

RESULTS AND DISCUSSION

Oscillation regimes

Polymer-encapsulated microbubbles exhibited unique, distinct regimes of oscillation (Bouakaz et al. 2005). These regimes consist of either (i) a limited oscillation response, defined as a radial oscillation amplitude less than 1% of its initial radius (Helfield et al. 2016a); (ii) compression-dominated oscillations (de Jong et al. 2007); or (iii) sonic cracking, consistent with the fracturing of the polymer encapsulation and at least a partial release of gas content (Fig. 3). The projected oscillating microbubble area is depicted as a function of both time and response frequency for the three types of oscillations to appreciate the associated vibrational asymmetries, consistent with large non-linear frequency components. A separate set of pilot experiments were performed to obtain SEM images of local nanometer-scale polymer depressions and punctures. It is perhaps important to note two points of clarification. First, because of the non-spherical nature of these oscillations, the projected area does not necessarily scale directly with bubble volume. Second, because of the anisotropy of the ejected gas within the imaging plane, the projected area may not entirely contain ultrasonically active gas (as opposed to portions of the projected area containing partially empty shell). It is for these two reasons that the quantification of projected area as a function of time is primarily for illustrative purposes.

Modeling work on polymer-encapsulated bubbles (Marmottant et al. 2011) predicts a threshold pressure (i) P_b above which buckling occurs (resulting in compression-dominated oscillations) and (ii) P_r above which shell rupture occurs (sonic cracking). These critical pressures are a function of shell thickness, collapsing to identical values for thicker shells (shell thickness-to-bubble radius ratio >4%–6% (Lensen et al. 2011)). Our data are consistent with modeling efforts in this regard, given the relatively thin encapsulation of PB127 (<1%–2% [Chitnis et al. 2013; Marmottant et al. 2011]) and the observably distinct buckled and sonic cracking phases.

Sonoporation

Direct correlation between polymer-encapsulated microbubble physics (microsecond time scale) and endothelial membrane permeability (seconds to minute time scale) is illustrated in Figure 4, which highlights an individual microbubble ($R_0 = 3.5 \mu\text{m}$) insonicated at 2 MHz and 900 kPa. This bubble exhibits sonic cracking at multiple locations, and the quantification of total projected area is shown. The simultaneous epifluorescence recording reveals immediate PI entry originating at the site of microbubble–endothelial cell contact (*white arrow*), further diffusing to the nucleus. The uptake of PI within the cytoplasm near the site of entry returns to a steady-state value after an initial increase, whereas PI delivery to the nucleus reaches a plateau by way of a monotonically increasing, exponential-like function. The exponential-like uptake curve in the nucleus is a consequence of its relative immobility compared with the free RNA/DNA complexes within the cytoplasm. The relative

time lag between the initiations of PI uptake within the nucleus versus within the cytoplasm is a consequence of the nucleus location (and PI/RNA/DNA diffusion time) with respect to the pore site.

A summary of the complete data set is illustrated in Figure 5 for each of the three transmit frequencies employed (Supplementary Table S1, online only, available at <http://dx.doi.org/10.1016/j.ultrasmedbio.2017.07.017>). Statistical classification was performed in transmit pressure–initial projected microbubble area space to threshold sonic cracking (*solid line*) and sonoporation (*dashed line*) events. It can be seen that sonic cracking generally occurs for large microbubbles at larger transmit pressures and that it is a necessary condition for sonoporation, albeit not a sufficient one for frequencies >0.5 MHz: only a subset of microbubbles undergoing sonic cracking result in sonoporation. The quantification of the overlap between these two data sets, that is, the intersection between sonic cracking and sonoporation events, reveals 100% overlap at 0.5 MHz (*e.g.*, every microbubble that exhibited sonic cracking resulted in sonoporation), 60% at 1 MHz and 50% at 2 MHz ($n = 39$, $n = 44$, $n = 27$, respectively). Sonoporation efficiency with this type of agent is directly related to sonic cracking, as has been previously postulated indirectly (Cochran and Wheatley 2013; Mehier-Humbert et al. 2007). The optimization of polymeric-based sonoporation strategies therefore lies in the ability to understand, predict and control sonic cracking and the subsequent free gas dynamics. To this end, ultrasound pressure thresholds above which polymer microbubble disruption occurs from single bursts have been investigated both acoustically (Bevan et al. 2007; Leong-Poi et al. 2002) and optically (Bouakaz et al. 2005; Lensen et al. 2011), resulting in rupture pressures between 400 and 1200 kPa depending on the frequency and polymer shell thickness (MI between 0.4 and 1.0, frequencies between 1 and 2 MHz).

Sonic cracking characteristics

In furtherance of understanding the relationship between sonic cracking and sonoporation beyond pressure thresholds, the extent of sonic cracking was further delineated into instances of (i) complete gas escape or (ii) gas re-entry—characterized by either a single or multiple geometrically confined regions of escape (presumably proportional to the number of cracks in the encapsulation). Complete gas escape (Fig. 6a, fourth frame; Fig. 6b, third frame) was defined by a measurable separation between the oscillating, escaped gas content and the non-oscillating polymeric material. Alternatively, gas re-entry in the compressional oscillation phase does not exhibit a distinct detachment from the original microbubble (Fig. 5b, fourth frame; Fig. 5c, third frame). There appears to be a clear relation between sonoporation efficiency and the extent of sonic cracking—the escaped gas content facilitating sonoporation (Fig. 5e). For the subset of sonic cracking microbubbles that resulted in complete gas escape, quantification of the free oscillating gas bubble can be meaningfully performed. Entry of PI within the cytoplasm near the site of entry (Fig. 6f) and within the nucleus (Fig. 6g) exhibit correlations with maximum escaped projected gas area ($r = 0.45$, $p = 0.04$, and $r = 0.54$, $p < 0.02$). Indeed, once sonic cracking is achieved, the extent of sonoporation appears to be dictated by the projected free gas area impinging on the cell. It is important to note here that the cytoplasmic PI entry exhibits a weak linear relation (Fig. 6f). A statistical test to assess the influence of individual data points on the regression

estimators (Cook's analysis) was performed and highlights two data points as potential outliers. Even with these removed, however, the slope and statistical significance remain similar.

For lipid-encapsulated microbubbles, our previous work suggests that endothelial membrane pores were generated as a consequence of the estimated microbubble-generated shear-stress (a function of radial excursion and oscillation frequency), resulting in a direct relation between permeability and maximum shear stress (Helfield et al. 2016b). Similar modeling of sonic cracking-induced free gas cavitation and the subsequent shear-stress profile is a non-trivial task because of the inherent asymmetries of gas release, notably for incomplete gas-escape conditions, and the large-magnitude oscillations ($A_{\max} > 50 \mu\text{m}^2$; see Fig. 6). Previous studies, however, examining asymmetric bubble collapse (both free gas bubbles [Brujan et al. 2001; Ohl and Ikink 2003; Ohl et al. 2006] and those released from polymer encapsulation [Postema et al. 2004] as in the present study) have indicated that this phenomenon can be accompanied by the formation of high-speed liquid jets. Fluid jets from bubble collapse have been reported to generate short-duration, large-magnitude shear stresses (~kilopascal range [Dijkink and Ohl 2008; Phares et al. 2000]) that may exceed thresholds for sonoporation. Although we were unable to optically resolve fluid jetting, future mechanistic studies employing high-speed microscopy from orthogonal imaging planes (Vos et al. 2011) to better capture the presence of jets generated from bubbles adjacent to endothelial cells, in conjunction with finite-element modeling of polymer rupture (Bergstrom and Hayman 2016) and anisotropic gas release behavior, should provide additional insight into the optimization of polymer-encapsulated agents for use as an ultrasound-stimulated gene delivery vehicle, and may extend the insights presented here.

Limitations

Although a direct link between the propensity of sonic cracking and enhanced plasma membrane permeability (*i.e.*, sonoporation) was observed, the scope of this study does not extend to the resulting cell viability. Assessment of cell viability post-ultrasound treatment has indeed been the subject of numerous investigations, in considering cells in suspension as well as cultured cell monolayers. Depending on the ultrasound parameters and microbubble concentrations employed, as well as the experimental arrangement (Kinoshita and Hynynen 2007), the proportion of cells that remain viable after having been sonoporated with pulse trains (pulse repetition frequencies ~ kilohertz) has been reported to be approximately ~20%–50% (Fan et al. 2014; Karshafian et al. 2009). Perhaps most directly comparable to the present study, Fan et al. (2013) estimated the proportion of PI-positive cells that remained viable immediately after treatment from an individual adjacent lipid microbubble exposed to a single 8- μs burst from 0.4 to 1.6 MPa at 1.25 MHz to be 55% to 80%. As a crude measure for cell viability, we compared the maximum PI staining within the nucleus exhibited by sonoporation to a positive control (*i.e.*, non-viable cells, 2% Triton-X) under the same experimental conditions. Under the assumption that a re-sealing membrane is indicative of post-sonoporation viability, from these results (Fig. 7) and given that our delivered ultrasound energy is low compared with most other studies (only a single, 8-cycle burst to a single microbubble adjacent to a cell), we anticipate that a significant percentage of the sonoporated cells in this study were viable post-treatment.

CONCLUSIONS

With the use of a simultaneous microscopy system, we have investigated relations between microsecond-scale polymeric microbubble cavitation physics and sonoporation. Our results suggest that the extent of sonoporation is directly related to the maximum projected area of the escaped, free gas. These data should help guide pulse design strategies for use of polymeric microbubbles in drug/gene delivery approaches. In summary, this work helps to substantiate the merit of sonoporation using polymeric microbubbles as a targeted, non-viral drug/gene delivery approach.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Aryal M, Vykhodtseva N, Zhang Y, Park J, McDannold N. Multiple treatments with liposomal doxorubicin and ultrasound-induced disruption of blood–tumor and blood–brain barriers improve outcomes in a rat glioma model. *J Control Release*. 2013; 169:103–111. [PubMed: 23603615]
- Audouy SA, Leij De LF, Hoekstra D, Molema G. In vivo characteristics of cationic liposomes as delivery vectors for gene therapy. *Pharm Res*. 2002; 19:1599–1605. [PubMed: 12458664]
- Bao S, Thrall BD, Miller DL. Transfection of a reporter plasmid into cultured cells by sonoporation in vitro. *Ultrasound Med Biol*. 1997; 23:953–959. [PubMed: 9300999]
- Bergstrom JS, Hayman D. An overview of mechanical properties and material modeling of polylactide (PLA) for medical applications. *Ann Biomed Eng*. 2016; 44:330–340. [PubMed: 26369638]
- Bevan PD, Tickner EG, Burns PN. Quantitative measurement of ultrasound disruption of polymer-shelled microbubbles. *Ultrasound Med Biol*. 2007; 33:1777–1786. [PubMed: 17656011]
- Bloch SH, Wan M, Dayton PA, Ferrara KW. Optical observation of lipid- and polymer-shelled ultrasound microbubble contrast agents. *Appl Phys Lett*. 2004; 84:631–633.
- Bouakaz A, Versluis M, de Jong N. High-speed optical observations of contrast agent destruction. *Ultrasound Med Biol*. 2005; 31:391–399. [PubMed: 15749563]
- Brujan EA, Nahen K, Schmidt P, Vogel A. Dynamics of laser-induced cavitation bubbles near elastic boundaries: Influence of the elastic modulus. *J Fluid Mech*. 2001; 44:283–314.
- Cao R, Brakenhielm E, Pawliuk R, Wariaro D, Post MJ, Wahlberh E, Leboulch P, Cao Y. Angiogenic synergism, vascular stability and improvement of hind-limb ischemia by a combination of PDGF-BB and FGF-2. *Nat Med*. 2003; 9:604–613. [PubMed: 12669032]
- Carson AR, McTiernan CF, Lavery L, Grata M, Leng X, Wang J, Chen X, Villanueva FS. Ultrasound-targeted microbubble destruction to deliver siRNA cancer therapy. *Cancer Res*. 2012; 72:6191–6199. [PubMed: 23010078]
- Chen X, Wan JM, Yu AC. Sonoporation as a cellular stress: Induction of morphological repression and developmental delays. *Ultrasound Med Biol*. 2013a; 39:1075–1086. [PubMed: 23499345]
- Chen X, Wang J, Versluis M, de Jong N, Villanueva FS. Ultra-fast bright field and fluorescence imaging of the dynamics of micrometer-sized objects. *Rev Sci Instrum*. 2013b; 84:63701.

- Chitnis PV, Koppolu S, Mamou J, Chlon C, Ketterling JA. Influence of shell properties on high-frequency ultrasound imaging and drug delivery using polymer-shelled microbubbles. *IEEE Trans Ultrason Ferroelectr Freq Control*. 2013; 60:53–64. [PubMed: 23287913]
- Christiansen JP, French BA, Klibanov AL, Kaul S, Lindner JR. Targeted tissue transfection with ultrasound destruction of plasmid-bearing cationic microbubbles. *Ultrasound Med Biol*. 2003; 29:1759–1767. [PubMed: 14698343]
- Cochran M, Wheatley MA. In vitro gene delivery with ultrasound-triggered polymer microbubbles. *Ultrasound Med Biol*. 2013; 39:1102–1119. [PubMed: 23562023]
- Dachs GU, Tupper J, Tozer GM. From bench to bedside for gene-directed enzyme prodrug therapy of cancer. *Anticancer Drugs*. 2005; 16:349–359. [PubMed: 15746571]
- De Cock I, Zagato E, Braeckmans K, Luan Y, de Jong N, De Smedt SC, Lentacker I. Ultrasound and microbubble mediated drug delivery: Acoustic pressure as determinant for uptake via membrane pores or endocytosis. *J Control Release*. 2015; 197:20–28. [PubMed: 25449801]
- de Jong N, Emmer M, Chin CT, Bouakaz A, Mastik F, Lohse D, Versluis M. “Compression-only” behavior of phospholipid-coated contrast bubbles. *Ultrasound Med Biol*. 2007; 33:653–656. [PubMed: 17320268]
- Dijkink R, Ohl CD. Measurement of cavitation induced wall shear stress. *Appl Phys Lett*. 2008; 93:3–6.
- Fan Z, Kumon RE, Park J, Deng CX. Intracellular delivery and calcium transients generated in sonoporation facilitated by microbubbles. *J Control Release*. 2010; 142:31–39. [PubMed: 19818371]
- Fan Z, Liu H, Mayer M, Deng CX. Spatiotemporally controlled single cell sonoporation. *Proc Natl Acad Sci U S A*. 2012; 109:16486–16491. [PubMed: 23012425]
- Fan Z, Chen D, Deng CX. Improving ultrasound gene transfection efficiency by controlling ultrasound excitation of microbubbles. *J Control Release*. 2013; 170:401–413. [PubMed: 23770009]
- Fan Z, Chen D, Deng CX. Characterization of the dynamic activities of a population of microbubbles driven by pulsed ultrasound exposure in sonoporation. *Ultrasound Med Biol*. 2014; 40:1260–1272. [PubMed: 24486236]
- Ferrara K, Pollard R, Borden M. Ultrasound microbubble contrast agents: Fundamentals and application to gene and drug delivery. *Annu Rev Biomed Eng*. 2007; 9:415–447. [PubMed: 17651012]
- Helfield BL, Cherin E, Foster FS, Goertz DE. Investigating the subharmonic response of individual phospholipid encapsulated microbubbles at high frequencies: A comparative study of five agents. *Ultrasound Med Biol*. 2012; 38:846–863. [PubMed: 22402024]
- Helfield B, Chen X, Qin B, Villanueva FS. Individual lipid encapsulated microbubble radial oscillations: Effects of fluid viscosity. *J Acoust Soc Am*. 2016a; 139:204–214. [PubMed: 26827018]
- Helfield B, Chen X, Watkins SC, Villanueva FS. Biophysical insight into mechanisms of sonoporation. *Proc Natl Acad Sci U S A*. 2016b; 113:9983–9988. [PubMed: 27551081]
- Hernot S, Klibanov AL. Microbubbles in ultrasound-triggered drug and gene delivery. *Adv Drug Deliv Rev*. 2008; 60:1153–1166. [PubMed: 18486268]
- Isner JM, Pieczek A, Schainfeld R, Blair R, Haley L, Asahara T, Rosenfield K, Razvi S, Walsh K, Symes JF. Clinical evidence of angiogenesis after arterial gene transfer of phVEGF 165 in patient with ischaemic limb. *Lancet*. 1996; 348:370–374. [PubMed: 8709735]
- Jaski BE, Jessup ML, Mancini DM, Cappola TP, Pauly DF, Greenberg B, Borow K, Dittrich H, Zsebo KM, Hajjar RJ. Calcium Upregulation by Percutaneous Administration of Gene Therapy in Cardiac Disease (CUPID trial), a first-in-human phase 1/2 clinical trial. *J Card Fail*. 2009; 15:171–181. [PubMed: 19327618]
- Jordao JF, Ayala-grosso CA, Markham K, Huang Y, Chopra R, McLaurin J, Hynynen K, Aubert I. Antibodies targeted to the brain with image-guided focused ultrasound reduces amyloid- β plaque load in the TgCRND8 mouse model of Alzheimer’s disease. *PLoS One*. 2010; 5:4–11.
- Karshafian R, Bevan PD, Williams R, Samac S, Burns PN. Sonoporation by ultrasound-activated microbubble contrast agents: Effect of acoustic exposure parameters on cell membrane permeability and cell viability. *Ultrasound Med Biol*. 2009; 35:847–860. [PubMed: 19110370]

- Kinoshita M, Hynynen K. A novel method for the intracellular delivery of siRNA using microbubble-enhanced focused ultrasound. *Biochem Biophys Res Commun*. 2005; 335:393–399. [PubMed: 16081042]
- Kinoshita M, Hynynen K. Key factors that affect sonoporation efficiency in in vitro settings: The importance of standing wave in sonoporation. *Biochem Biophys Res Commun*. 2007; 359:860–865. [PubMed: 17568561]
- Kooiman K, Böhmer MR, Emmer M, Vos HJ, Chlon C, Shi WT, Hall CS, de Winter SHPM, Schroën K, Versluis M, de Jong N, van Wamel A. Oil-filled polymer microcapsules for ultrasound-mediated delivery of lipophilic drugs. *J Control Release*. 2009; 133:109–118. [PubMed: 18951931]
- Kooiman K, Foppen-Harteveld M, van der Steen AFW, de Jong N. Sonoporation of endothelial cells by vibrating targeted microbubbles. *J Control Release*. 2011; 154:35–41. [PubMed: 21514333]
- Kopeček JA, Carson AR, Mctiernan CF, Chen X, Hasjim B, Sen M, Grandis JR, Villanueva FS. Ultrasound targeted microbubble destruction-mediated delivery of a transcription factor decoy inhibits STAT3 signaling and tumor growth. *Theranostics*. 2015; 5:1–10. [PubMed: 25553094]
- Kudo N, Okada K, Yamamoto K. Sonoporation by single-shot pulsed ultrasound with microbubbles adjacent to cells. *Biophys J*. 2009; 96:4866–4876. [PubMed: 19527645]
- Lensen D, Gelderblom EC, Vriezema DM, Marmottant P, Verdonschot N, Versluis M, Jong D, Van Hest JCM. Biodegradable polymeric microcapsules for selective ultrasound-triggered drug release. *Soft Matter*. 2011; 7:5417–5422.
- Lentacker I, Geest De BG, Vandenbroucke RE, Peeters L, Demeester J, De Smedt SC, Sanders NN. Ultrasound-responsive polymer-coated microbubbles that bind and protect DNA. *Langmuir*. 2006; 22:7273–7278. [PubMed: 16893226]
- Leong KW, Mao H, Roy K, Walsh SM, August JT. DNA-polycation nanospheres as non-viral gene delivery vehicles. *J Control Release*. 1998; 53:183–193. [PubMed: 9741926]
- Leong-Poi H, Song J, Rim S, Christiansen J, Kaul S, Lindner JR. Influence of microbubble shell properties on ultrasound signal: Implications for low-power perfusion imaging. *J Am Soc Echocardiogr*. 2002; 15:1269–1276. [PubMed: 12411916]
- Leong-Poi H, Kuliszewski MA, Lekas M, Sibbald M, Teichert-Kuliszewska K, Klibanov AL, Stewart DJ, Lindner JR. Therapeutic arteriogenesis by ultrasound-mediated VEGF165 plasmid gene delivery to chronically ischemic skeletal muscle. *Circ Res*. 2007; 101:295–303. [PubMed: 17585071]
- Leow RS, Wan JMF, Yu ACH. Membrane blebbing as a recovery manoeuvre in site-specific sonoporation mediated by targeted microbubbles. *J R Soc Interface*. 2015; 12:20150029. [PubMed: 25694544]
- Li S, Huang L. Gene therapy progress and prospects: Non-viral gene therapy by systemic delivery. *Gene Ther*. 2006; 13:1313–1319. [PubMed: 16953249]
- Losordo DW, Vale PR, Symes JF, Dunnington CH, Esakof DD, Maysky M, Ashare AB, Lathi K, Isner JM. Gene therapy for myocardial angiogenesis: Initial clinical results with direct myocardial injection of phVEGF165 as sole therapy for myocardial ischemia. *Circulation*. 1998; 98:2800–2805. [PubMed: 9860779]
- Marmottant P, Bouakaz A, de Jong N, Quilliet C. Buckling resistance of solid shell bubbles under ultrasound. *J Acoust Soc Am*. 2011; 129:1231–1239. [PubMed: 21428486]
- Mehier-Humbert S, Guy RH. Physical methods for gene transfer: Improving the kinetics of gene delivery into cells. *Adv Drug Deliv Rev*. 2005; 57:733–753. [PubMed: 15757758]
- Mehier-Humbert S, Yan F, Frinking P, Schneider M, Guy RH. Ultrasound-mediated gene delivery: Influence of contrast agent on transfection. *Bioconjug Chem*. 2007; 18:652–662. [PubMed: 17419583]
- Miller DL, Dou C. Induction of apoptosis in sonoporation and ultrasonic gene transfer. *Ultrasound Med Biol*. 2009; 35:144–154. [PubMed: 18723272]
- Miyamoto MI, Monte F, Schmidt U, Disalvo TS, Kang ZB, Matsui T, Guerrero JL, Gwathmey JK, Rosenzweig A, Hajjar RJ. Adenoviral gene transfer of SERCA2 improves left-ventricular function in aortic-banded rats in transition to heart failure. *Proc Natl Acad Sci U S A*. 2000; 97:793–798. [PubMed: 10639159]

- Ohl CD, Ikink R. Shock-wave-induced jetting of micron-size bubbles. *Phys Rev Lett*. 2003; 90:1–4.
- Ohl CD, Arora M, Ikink R, de Jong N, Versluis M, Delius M, Lohse D. Sonoporation from jetting cavitation bubbles. *Biophys J*. 2006; 91:4285–4295. [PubMed: 16950843]
- Otake K, Ennist DL, Harrod K, Trapnell BC. Nonspecific inflammation inhibits adenovirus-mediated pulmonary gene transfer and expression independent of specific acquired immune responses. *Hum Gene Ther*. 1998; 9:2207–2222. [PubMed: 9794205]
- Ottoboni, T., Short, R., Yamamoto, R. Microparticles useful as ultrasonic contrast agents and for delivery of drugs into the bloodstream. Patent WO1998048783A1. 2001.
- Park J, Fan Z, Deng CX. Effects of shear stress cultivation on cell membrane disruption and intracellular calcium concentration in sonoporation of endothelial cells. *J Biomech*. 2011; 44:164–169. [PubMed: 20863503]
- Phares DJ, Smedley GT, Flagan RC. The wall shear stress produced by the normal impingement of a jet on a flat surface. *J Fluid Mech*. 2000; 418:351–375.
- Postema M, van Wamel A, Lancée CT, de Jong N. Ultrasound-induced encapsulated microbubble phenomena. *Ultrasound Med Biol*. 2004; 30:827–840. [PubMed: 15219962]
- Postema M, Bouakaz A, Versluis M, de Jong N. Ultrasound-induced gas release from contrast agent microbubbles. *IEEE Trans Ultrason Ferroelectr Freq Control*. 2005; 52:1035–1041. [PubMed: 16118985]
- Price RJ, Skyba DM, Kaul S, Skalak TC. Delivery of colloidal particles and red blood cells to tissue through microvessel ruptures created by targeted microbubble destruction with ultrasound. *Circulation*. 1998; 98:1264–1267. [PubMed: 9751673]
- Rosenberg SA, Yang JC, Restifo NP. Cancer immunotherapy: Moving beyond current vaccines. *Nat Med*. 2004; 10:909–915. [PubMed: 15340416]
- Roth JA. Adenovirus p53 gene therapy. *Expert Opin Biol Ther*. 2006; 6:55–61. [PubMed: 16370914]
- Salem AK, Searson PC, Leong KW. Multifunctional nanorods for gene delivery. *Nat Mater*. 2003; 2:668–671. [PubMed: 12970757]
- Schlicher RK, Hutcheson JD, Radhakrishna H, Apkarian RP, Prausnitz MR. Changes in cell morphology due to plasma membrane wounding by acoustic cavitation. *Ultrasound Med Biol*. 2010; 36:677–692. [PubMed: 20350691]
- Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. *Nat Methods*. 2012; 9:671–675. [PubMed: 22930834]
- Shohet RV, Chen S, Zhou Y-T, Wang Z, Meidell RS, Unger RH, Grayburn PA. Echocardiographic destruction of albumin microbubbles directs gene delivery to the myocardium. *Circulation*. 2000; 101:2554–2556. [PubMed: 10840004]
- Skyba DM, Price RJ, Linka AZ, Skalak TC, Kaul S. Direct in vivo visualization of intravascular destruction of microbubbles by ultrasound and its local effects on tissue. *Circulation*. 1998; 98:290–293. [PubMed: 9711932]
- Taniyama Y, Tachibana K, Hiraoka K, Namba T, Yamasaki K, Hashiya N, Aoki M, Ogihara T, Yasufumi K, Morishita R. Local delivery of plasmid DNA into rat carotid artery using ultrasound. *Circulation*. 2002; 105:1233–1239. [PubMed: 11889019]
- Tinkov S, Bekeredjian R, Winter G, Coester C. Microbubbles as ultrasound triggered drug carriers. *J Pharm Sci*. 2009; 98:1935–1961. [PubMed: 18979536]
- Treat LH, Mcdannold N, Vykhodtseva N, Zhang Y, Tam K, Hynynen K. Targeted delivery of doxorubicin to the rat brain at therapeutic levels using MRI-guided focused ultrasound. *Int J Cancer*. 2007; 121:901–907. [PubMed: 17437269]
- Unger EC, Matsunaga TO, McCreery T, Schumann P, Sweitzer R, Quigley R. Therapeutic applications of microbubbles. *Eur J Radiol*. 2002; 42:160–168. [PubMed: 11976013]
- van Rooij T, Skachkov I, Beekers I, Lattwein KR, Voorneveld JD, Kokhuis TJA, Bera D, Luan Y, van der Steen AFW, de Jong N, Kooiman K. Viability of endothelial cells after ultrasound-mediated sonoporation: Influence of targeting, oscillation, and displacement of microbubbles. *J Control Release*. 2016; 238:197–211. [PubMed: 27469471]
- vanWamel A, Bouakaz A, Versluis M, de Jong N. Micromanipulation of endothelial cells: Ultrasound–microbubble–cell interaction. *Ultrasound Med Biol*. 2004; 30:1255–1258. [PubMed: 15550330]

- van Wamel A, Kooiman K, Harteveld M, Emmer M, ten Cate FJ, Versluis M, de Jong N. Vibrating microbubbles poking individual cells: Drug transfer into cells via sonoporation. *J Control Release*. 2006; 112:149–155. [PubMed: 16556469]
- Ventura A, Kirsch DG, McLaughlin ME, Tuveson DA, Grimm J, Lintault L, Newman J, Reczek EE, Weissleder R, Jacks T. Restoration of p53 function leads to tumour regression in vivo. *Nature*. 2007; 7128:661–665.
- Verma IM, Somia N. Gene therapy—Promises, problems and prospects. *Nature*. 1997; 389:239–242. [PubMed: 9305836]
- Vos HJ, Dollet B, Versluis M, de Jong N. Nonspherical shape oscillations of coated microbubbles in contact with a wall. *Ultrasound Med Biol*. 2011; 37:935–948. [PubMed: 21601137]
- Zhong W, Sit WH, Wan JMF, Yu ACH. Sonoporation induces apoptosis and cell cycle arrest in human promyelocytic leukemia cells. *Ultrasound Med Biol*. 2011; 37:2149–2159. [PubMed: 22033133]

Fig. 1. Size distribution of PB127 polymer microbubbles: number and volume-weighted distributions are presented. These measurements were performed with a Coulter counter using a 30-μm aperture in triplicate. See text for agent handling details.

Fig. 2. Simultaneous microscopy bridging up to six orders of magnitude in time. (a) Schematic cross section of the custom-designed water tank, housing a single-element transducer and a coil heater. (b) Synchronous triggering between the ultrasound transducer and ultrafast microscopy system was achieved to collect ultrafast recordings of microbubble dynamics and a fluorescence recording of the corresponding PI uptake before, during and after ultrasound transmission ($\approx 2\text{--}3$ min). HUVEC = human umbilical vein endothelial cell; UPMC-Cam = University of Pittsburgh Medical Center camera.

Fig. 3.

Polymer-encapsulated microbubbles can exhibit three distinct oscillation behaviors. Three separate ultrafast-frame-rate recordings (10.8 Mfps) highlighting three types of oscillation regimes exhibited by polymer microbubbles insonicated at 1 MHz, including (a) no measurable oscillation (0.5 MPa) (Supplementary Video S1), (b) “compression-only” oscillations (0.8 MPa) (Supplementary Video S2) and (c) shell cracking (1 MPa) (Supplementary Video S3), with the clearly observable tethered release of gas (*white arrows*) (Supplementary Videos S1–S3 are available online only at <http://dx.doi.org/10.1016/j.ultrasmedbio.2017.07.017>). Scale bar = 5 μm . (d, e) Quantification of the projected microbubble area for the microbubbles enumerated in the previous panels. (f) Scanning electron microscopy (SEM) images revealing (top) a control microbubble and ultrasound-treated microbubbles resulting in (middle) a local shell depression and (bottom) nanometer-sized hole within a local depression. Note that although these images are consistent with the morphology depicted *via* light microscopy, the SEM images depict only possible outcomes and are for intuitive purposes only. Scale bar = 1 μm .

Fig. 4.

Sonoporation is achieved by cracking polymer-encapsulated microbubbles. (a) An individual microbubble ($R_0 = 3.5 \mu\text{m}$) insonicated at 2 MHz and 900 kPa (16 Mfps). *White arrowheads* denote escaped gas content. Scale bar is $5 \mu\text{m}$. (b, c) Quantification of the projected microbubble area in both the time and frequency domains, respectively. (d) Corresponding epifluorescence microscopy recording, with the endothelial cell borders highlighted in *yellow*, revealing propidium iodide (PI) entry (*i.e.*, sonoporation) within the cell starting at the point of bubble–cell contact (*white arrow*—observable with bright-field imaging [not shown here]) and diffusing through the cytoplasm to the nucleus. Scale bar = $10 \mu\text{m}$. (e) Quantification of the average PI intensity as a function of time within the nucleus (*red, solid*) and cytoplasm at the site of entry (*blue, dashed*).

Fig. 5.

Sonic cracking is necessary, but not entirely sufficient to induce sonoporation in endothelial cells. The first three panels (a–c) illustrate microbubble behavior as a function of initial projected microbubble area (x-axis) and acoustic, free-field transmit pressure (y-axis) for each of the transmit frequencies employed ($n = 39$, $n = 44$, $n = 27$). Oscillation regimes, as defined in Fig. 2, are denoted by symbols (*triangles* for limited response, *squares* for compression-dominated oscillations and *circles* for sonic cracking). *Closed symbols* indicate sonoporation (*i.e.*, propidium iodide [PI] uptake, see Fig. 3) in the adjacent cell, whereas *open symbols* denote no resulting sonoporation. Linear discriminant analysis in the microbubble area–transmit pressure parameter space was performed to classify the data sets responsible for (*solid line*) sonic cracking and (*dashed line*) sonoporation. Note that at 0.5 MHz, these regions are identical; this is further illustrated in (d), where the intersection between sonic cracking and PI-positive data sets is quantified (* denotes statistical significance at $p < 0.01$).

Fig. 6.

Sonic cracking can consist of either complete gas escape from the polymer encapsulation or gas re-entry in the compressional oscillation phase. (a–c) These panels highlight ultrahigh-speed bright-field microscopy recordings illustrating different types of sonic cracking behavior, selected frames of which overlay an example of the microbubble quantification border detection algorithm (shown in *red*). Complete gas escape is characterized by a measurable separation between oscillating gas body and the original polymer shell (panel a, fourth frame, and panel b, third frame). Sonic cracking followed by subsequent gas re-entry can be the product of a single (panel b, fourth frame) or multiple (panel c, third frame) cracks. See Supplementary Videos S4–S6 (online only, available at <http://dx.doi.org/10.1016/j.ultrasmedbio.2017.07.017>). The observed sampling of these sonic cracking behaviors and their relationship to sonoporation propensity are observed in (d) and (e), respectively ($p < 0.015$). Quantification of propidium iodide entry as a function of the

maximum projected area of the escaped gas pocket (for the subset of bubbles that exhibited complete gas escape) reveals a correlation ($n = 21$, $r = 0.45$, $p = 0.04$; $n = 20$, $r = 0.54$, $p < 0.02$) in both the cytoplasm near the site of entry (f) and the nucleus (g). PI = propidium iodide.

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Fig. 7.

The majority of sonoporated cells exhibit lower magnitudes of propidium iodide (PI) entry compared with non-viable cells. From the results of the present study, the grouped maximum PI intensity within the nucleus at 3 min post-ultrasound (all PI positive cells, regardless of microbubble oscillation regime, for all frequencies and pressures) is significantly lower compared with that of a positive control (*i.e.*, cell death) *via* 2% Triton-X treatment ($p < 0.001$) under the same light exposure ($n = 28$ and $n = 9$, respectively). Note that sonoporated cells that exhibited no PI within the nucleus ($n = 8$) were necessarily excluded from this analysis. Under the assumption that viable cells reseal their membranes and therefore cease PI entry, these data suggest that the majority of sonoporated cells investigated in the present work remain viable after sonoporation treatment.