Manipulating Cellular Activities Using an Ultrasound–Chemical Hybrid Tool

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Supporting Information

ABSTRACT: We developed an ultrasound–chemical hybrid tool to precisely manipulate cellular activities. A focused ultrasound coupled with gas-filled microbubbles was used to rapidly trigger the influx of membrane-impermeable chemical dimerizers into living cells to regulate protein dimerization and location without inducing noticeable toxicity. With this system, we demonstrated the successful modulation of phospholipid metabolism triggered by a short pulse of ultrasound exposure. Our technique offers a powerful and versatile tool for using ultrasound to spatiotemporally manipulate the cellular physiology in living cells.

KEYWORDS: chemically inducible dimerization, focused ultrasound, microbubble, mammalian cells

A focused ultrasound (FUS) can be used to deliver acoustic waves with a great penetration depth in a focused manner.1 These unique characteristics make the ultrasound a powerful and versatile tool for diagnosis and noninvasive surgery. When coupled with gas-filled microbubbles (MBs), which are oscillated upon the ultrasound excitation, FUS pulses can transiently increase the membrane permeability of MB-bound cells, which is an approach termed sonoporation.2 Several substances have been shown to have the feasibility of enhancing the delivery of materials, including genes, nanoparticles, membrane-impermeable reagents, proteins, and peptides, into cells via sonoporation.3–7 Most of these studies mainly focused on the increase in the gene transfection rate and the elimination of tumor cells in deep tissues. Very few of the existing systems can precisely modulate cell physiology, except for a newly emerging approach, sonogenetics, which modulates neuronal activities by the mechanical force derived from ultrasound wave.8 However, it is challenging to apply these systems to other aspects of cell physiology in non-neuronal cells, which greatly limits the applications of FUS.

To overcome these technical limitations, we aimed to develop a new approach using ultrasound to manipulate a wide range of cellular activities in different types of cells. The basis of our approach is a chemically inducible dimerization (CID) system which has been used to spatiotemporally control various cellular events, including cell signaling, gene transcription, and protein functions.9,10 Typically, a small chemical dimerizer, such as rapamycin (Rapa), rapidly induces the dimerization of two protein binding partners: FK506 binding protein (FKBP) and the FKBP12-Rapa binding protein (FRB). When FRB and FKBP-fused protein of interest (POI) are prelocalized in the plasma membrane and cytosol, respectively, the Rapa-induced dimerization rapidly traps the cytosolic FKBP-POI onto the plasma membrane. The local accumulation of the POI spatiotemporally triggers its downstream signaling and biological effects. The entire process can be induced rapidly in vitro and in vivo.9–12

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To test the possibility of using FUS to spatiotemporally control cellular activities, here, we introduce an ultrasound—chemical hybrid system using sonoporation to induce the influx of membrane-impermeable chemical dimerizers and, in turn, trigger the CID system in living cells. We named this system the SonoCID (sonoporation triggered CID system (Figure 1b)).

To design a cellular impermeable Rapa analogue, we conjugated a bulky moiety onto Rapa to afford a blocked analogue. Biotin was then chosen as the blocker moiety since it appears to bind to streptavidin tightly and does not permeate across the cell membrane once bound with streptavidin. Rapa conjugated a bulky moiety onto a previously established method.13 In brief, the hydroxyl group of plasma membrane (as indicated by "ON") locally activates the POI-dependent signaling in the membrane-anchoring D1 is located. The accumulation of the POI on the membrane locally activates the POI-dependent signaling in the plasma membrane (as indicated by "ON").

Figure 1. Schematic representation of the system. (a) Structure of the two-membrane-impermeable dimerizers (MID) used in this study, Rapamycin (Rapa)-PEG₆-Biotin (RPB) and GA₃. (b) Excitation of the focused ultrasound (FUS) on microbubble (MB)-bound cells transiently increases the membrane permeability and, thus, induces the influx of the impermeable dimerizers into the cells. RPB triggers the dimerization between two binding partners (D1 and D2), thus promoting the translocation of the cytosolic D2-tagged protein of interest (POI) onto the plasma membrane (PM), which is where membrane-anchoring D1 is located. The accumulation of the POI on the membrane locally activates the POI-dependent signaling in the plasma membrane (as indicated by “ON”).

To determine whether RP3 and RPB of various sizes could permeate across the plasma membrane and induce protein dimerization inside cells, we transfected COS7 cells with DNA constructs encoding membrane-tethering FRB (Lyn-CFP-FRB) and cytosolic YFP-FKBP (Supporting Information, Figure S1). Using the system we have previously shown, the addition of Rapa induces a rapid dimerization between FRB and FKBP, promoting the translocation of YFP-FKBP from the cytosol to the plasma membrane, which is accompanied by an elevation of the FRET signal (Supporting Information, Figure S1a,b).11 The kinetics of the RP3-induced protein dimerization is slightly slower than Rapa as confirmed by the slow elevation of the FRET signal (Figure S1b,c). Unexpectedly, the RPB treatment did not induce protein dimerization, which suggests that RPB, even without streptavidin binding, is inefficient in permeating the plasma membrane to trigger the CID system inside cells (Figure S1b,c, Movie S1). Conjugating bulky moieties on Rapa may provide a steric hindrance to prevent the dimerization.14

To verify whether RPB is a functional chemical dimerizer, the FRB/FKBP dimerization on the cell surface upon RPB treatment was evaluated. We tagged FRB and FKBP with cell surface VAMP2 protein and transfected the resulting constructs into COS7 cells (VAMP2-CFP-FRB and VAMP2-YFP-FKBP, Figure S2).15 The addition of the purified RPB rapidly induced the dimerization of FRB and FKBP on the cell surface as confirmed by the elevation of the FRET signal with kinetics that was comparable to that with Rapa (Figure S2, Movie S2). These results confirmed that RPB serves as a functional chemical dimerizer with an inefficient membrane permeability.

To trigger the influx of RPB into the cells by sonoporation, the MBs were coated with folate molecules (folate-coated microbubbles, FMBs; Figure S3)16 and incubated with HeLa epithelial cells endogenously expressing folate receptors. The three-dimensional live-cell imaging showed that the FMBs efficiently bind to the surface of the HeLa cells. These membrane-bound FMBs are disrupted to lipid debris after a short pulse of a low-frequency ultrasound (1-MHz, 0.5 MPa, 500 cycles, 0.1 Hz PRF, 5 s sonication duration; Figure S4, Movie S3). Ultrafast live-cell imaging showed the microbubbles rapidly collapse upon ultrasound excitation (less than 4.4 μs; Figure S5, Movie S4). To evaluate the sonoporation in real-time, live-cell imaging in the presence of the membrane-impermeant dye propidium iodide (PI) was conducted in HeLa cells with membrane-bound FMBs upon the ultrasound excitation. The excitation by the ultrasound rapidly disrupted the FMBs, which was accompanied by an elevation in the PI fluorescence in the cells (Figure S6, Movie S5). This sonoporation does not affect the cell morphology, the phospholipid composition in the cell membrane, or the cell viability as determined by live-cell imaging of the cell membrane (Figure S4, Movie S3), membrane phospholipid biosensors (Figure S7, Movie S6) and an MTT assay (Figure S8), respectively.

Next, we investigated whether the FUS-induced sonoporation could trigger the RPB-dependent CID system. HeLa cells expressing Lyn-CFP-FRB and YFP-FKBP were preincubated with RPB and FMBs. The excitation by the FUS rapidly disrupted the cell-attached FMBs and subsequently triggered the translocation of cytosolic YFP-FKBP to the plasma membrane, where Lyn-CFP-FRB is localized, which was accompanied by an elevation in FRET signal (Figure 2, Movie S7). Sonoporation in the absence of RPB did not affect the cytosolic level of YFP-FKBP, suggesting that the cytosolic components, at least our engineered molecular actuators, do not leak to the extracellular space (Figure 2b). These results confirmed that the excitation by the ultrasound can trigger the RPB-dependent CID system via sonoporation.

To assess whether the SonoCID could be utilized to manipulate cellular signaling, we attempted to control phosphoinositide metabolism. Sonoporation-induced influx of RPB was used to recruit FKBP-tagged Inp54p, which is a yeast
inositol polyphosphate 5-phosphatase that specifically cleaves the 5-phosphate on PtdIns(4,5)P$_2$ to the plasma membrane.$^9,10$ The level of PtdIns(4,5)P$_2$ in the plasma membrane was monitored in real-time by a PHPLCd biosensor fused to a green–yellow fluorescence protein, Neon (Figure 3).$^{17}$ The excitation of the cells by ultrasound in the presence of RPB quickly induced the translocation of mCherry-FKBP-Inp54p from the cytosol to the plasma membrane as confirmed by a decline and increase of mCherry-FKBP-Inp54p in the cytosol and plasma membrane, respectively (Figure 3a,b, Movie S8). A line scan analysis showed that the recruitment of Inp54p to the plasma membrane results in an instantaneous release of PHPLCd in the cytosol, indicating that PtdIns(4,5)P$_2$ was significantly depleted (Figure 3a,c,d). These results clearly demonstrate that the SonoCID can manipulate phosphoinositide metabolism in living cells.

To further extend the toolkit of the SonoCID, we then attempted to trigger the Rapa orthogonal CID system by FUS.$^{11,17}$ Theoretically, our strategy is capable of triggering any CID system by introducing membrane-impermeable dimerizers into targeted cells. To test this hypothesis, we then verified whether sonoporation could trigger the influx of a membrane-impermeable dimerizer, Gibberellin acid 3 (GA$_3$), and the dimerization of its binding partner, mGID1 (mammalian optimized Gibberellin insensitive dwarf1) and GAI proteins (Gibberellin insensitive).$^{11,17}$ Previous studies have reported that GA$_3$ is a membrane-impermeable dimerizer, and the addition of an AM ester group to GA$_3$ (GA$_3$-AM) largely improves its membrane permeability.$^{11,18}$ To confirm the membrane permeability of GA$_3$ and GA$_3$-AM in our system,

Figure 2. Sonoporation triggers the RPB-dependent CID system. (a) HeLa cells expressing membrane-anchored Lyn-CFP-FRB and cytosolic YFP-FKBP were incubated with FMBs (arrowheads) in the presence of RPB (50 nM). The excitation by ultrasound rapidly disrupted the red FMBs and induced the translocation of YFP-FKBP from the cytosol to the plasma membrane, which was accompanied by an elevation in FRET signal. Scale bar: 10 μm. In addition, see Movie S7. (b) The FRET/CFP ratio in each cell was normalized by dividing the averaged FRET/CFP value before the ultrasound excitation. Arrow indicates time point of RPB treatment and/or ultrasound excitation. Error bars represent the SEM ($n = 13$ cells from three independent experiments). Note that strong signal of red FMBs can be also observed with the YFP channel due to the fluorophore bleed-through of Dil dye to the YFP channel.

Figure 3. Sonoporation triggers the RPB-dependent CID system for manipulating phosphoinositide metabolism. (a) HeLa cells expressing Lyn-CFP-FRB, mCherry-FKBP-Inp54p, and Neon-PHPLCd were incubated with FMBs in the presence of RPB (50 nM). The excitation by the ultrasound induced the translocation of the mCherry-FKBP-Inp54p to the plasma membrane, which was accompanied by a reduction in Neon-PHPLCd in the plasma membrane. Scale bar: 10 μm. In addition, see Movie S8. (b) The normalized fluorescence intensity of the mCherry-FKBP-Inp54p in the cytosol and plasma membrane was measured at the indicated time points. Error bars represent the SEM ($n > 5$ cells). (c) The linescan analysis of Neon-PHPLCd (a) before and after the ultrasound excitation for 27 min (blue and orange dot arrows, respectively, drawn in panel a). (d) The normalized intensity of Neon-PHPLCd on the plasma membrane before and after FUS was measured. The double asterisks (**) represent $P < 0.01$. Error bars represent the SEM ($n = 6$ cells from three independent experiments).
HeLa cells expressing membrane tethering Lyn-CFP-GAIs and cytosolic YFP-mGID1 were treated with GA$_3$ and GA$_3$-AM in tandem. The addition of GA$_3$ did not induce the GAIs-mGID1 dimerization inside the cells as confirmed by no alteration in the FRET signal. The subsequent addition of GA$_3$-AM rapidly induces a robust increase in the FRET signal (Supporting Information, Figure S9, Movie S9). These results suggest that GA$_3$-AM, but not GA$_3$, is able to permeate across the plasma membrane and induce dimerization events inside cells. To assess whether sonoporation would trigger the GA$_3$-dependent CID system, HeLa cells expressing Lyn-CFP-GAIs and YFP-mGID1 were preincubated with GA$_3$ and FMBs. The excitation by FUS rapidly disrupted the FMBs and induced a dimerization between the GAIs and mGID1 as confirmed by an elevation in the FRET signal (Figure 4, Movie S10). The excitation by FUS in the absence of GA$_3$ does not alter the FRET signal, suggesting that our molecular actuators do not leak upon sonoporation. To determine whether sonoporation can trigger the multiple orthogonal CID systems simultaneously RPB and GA$_3$. Ultrasound stimulation triggered the translocation of mCherry-mGID1 and YFP-FKBP onto Tom20-CFP-GAIs-labeled mitochondria and Lyn-CFP-FRB-labeled plasma membrane, respectively (Figure 5 and Movie S11). These results validated that sonoporation triggers multiple orthogonal CID systems simultaneously (Figure S10, Movie S12) and C2BBe1 intestinal epithelial cells (Figure S11, Movie S13) bound by FMBs, indicating SonoCID is not cell-type specific.

In conclusion, we demonstrated the possibility of using FUS to precisely control cellular activities. This was achieved by our developed technique, SonoCID, which triggers the CID system by the sonoporation-mediated influx of membrane-impermeable chemical dimers. Using this system, we have successfully manipulated phospholipid metabolism in living cells. The SonoCID is versatile and capable of manipulating other cellular activities in specific subcellular sites by using other dimerization actuators and targeting sequences that have already been developed by us and others.$^{9-11,18}$ Moreover, since CID has been utilized in various model organisms including mice, yeast, and so on,$^{19-22}$ it is expected to apply sonoCID to different biological systems. The versatility and flexibility of the present approach are further expanded by coating distinct molecules on MBs and spatiotemporally controlling the US wave.$^{16,24,25}$ The FUS has been widely used in the clinic to enhance the delivery of anti-inflammatory and anesthetic agents into local tissues.$^{26,27}$ Most likely, due to its slow pharmacokinetics, it often relies on the continuous exposure of FUS for minutes or even hours, which may be accompanied by a thermal or destructive effect on the tissues. The ultrahigh sensitivity of the CID system allows for the triggering of biological effects with rather short pulses of FUS. Our promising system is potentially valuable for extending therapeutic applications.

### METHODS

**Cell Culture and Transfection.** HeLa, COS-7, C6, and C2BBe1 cells were maintained at 37 °C in 5% CO$_2$ in DMEM (Dulbecco’s modified Eagle’s medium; Gibco), supplemented with 10% fetal bovine serum (HyClone Laboratories), penicillin (Sigma–Aldrich), and streptomycin (Invitrogen) in a humidiﬁed 5% CO$_2$ incubator with 5% CO$_2$ at 37 °C. The DNA plasmid transfection was performed with LT-1 (Mirus) or Viafect (Promega) and then incubated at 37 °C in a CO$_2$ incubator for 18–24 h before the harvest.

**DNA Plasmid Construction.** The TRizol reagent (Life Technologies) was used to extract the total RNA from primary cortical neurons of Sprague–Dawley rats according to the manufacturer’s instructions. The total RNA was then subjected to RT-PCR using a reverse transcription kit (Applied Biosystems). VAMP2 (NM_012663.2) cDNA was ampliﬁed by PCR using primers (Forward: 5′AAA GGA TCC CCA CCA TGT CGG CTA CGG CGA TCG CCA CCG 3′ and Reverse: 5′ TCC ACC GGT CCC GCC GCT TCC GCC GCT AGT GCT GAA GTA AAC GAT GAT 3′). The PCR product was digested with Nhel and AgeI and inserted in-frame into the CFP-FRB and YFP-FKBP vectors. Both plasmids, VAMP2-CFP-FRB and VAMP2-YFP-FKBP, were sequenced to confirm the correct construction.

**Preparation of Folate-Conjugated, Fluorescent-Labeling Microbubbles (FMB).** 1,2-Distearyl-sodium-glycero-3-phosphoethanolamine-Ν-[carboxy(polyethylene glycol)-2000] (DSPE-PEG 2K, Avanti Polar Lipids), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC, Avanti Polar Lipids, AL,
USA), and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (DSPE-NBD, Avanti Polar Lipids) (molar ratio of 1.8:1:1.5) were dissolved in chloroform. The chloroform was then removed via an evaporator (R-210, Büchi Labortechnik AG, Flawil, Switzerland). The 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000]-folate (Avanti Polar Lipids) was synthesized by 1-ethyl-3-(3-(dimethylamino)propyl) carbodiimide in dimethyl sulfoxide/phosphate-buffered saline. The morphology of the FMBs could be imaged via bright field microscope images, while the green fluorescence images of the FMBs were observed by embedding DSPE-NBD into the lipid shell of the FMBs. The lipid film was then mixed with glycerol-PBS (5 μL/mL) and C3F8 gas. The solution was shaken in an agitator for 45 s to form the FMBs. Then, the unreacted lipids were removed from the FMBs via centrifugation (2 min, 6000 rpm). The red fluorescence FMBs were prepared using the same method as was used for the green fluorescence labeled FMBs, but NBD was replaced with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI).

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The size and concentration of the FMBs were measured by a coulter counter (Multisizer 3, Beckman Coulter Inc., CA, USA). The efficiency of conjugating the folates onto the FMBs was measured by a spectrophotometer (Cary 50, Agilent, CA, USA) with UV absorption at 280 nm.

Figure 5. Sonoporation simultaneously triggers two orthogonal CID systems. (a) Schematic representation of the two orthogonal CID systems, rapamycin and GA3-dependent systems, are triggered by sonoporation. (b) HeLa cells expressing Lyn-CFP-FRB, Tom20-CFP-GA3s, YFP-FKBP, and mCherry-mGID1 were incubated with FMBs in the presence of GA3 (1 μM) and RPB (50 nM). The excitation by the ultrasound induced the translocation of YFP-FKBP and mCherry-mGID1 to plasma membrane and mitochondria, respectively. Scale bar: 10 μm. In addition, see Movie S11.

**Setup of Focused Ultrasound Sonication.** The FUS exposure (acoustic pressure = 300–700 kPa; cycle number (CN) = 500; pulse repetition frequency (PRF) = 10 Hz; exposure time = 5 s) was applied with a 1-MHz FUS probe (V302, Panametrics) driven by a power amplifier (32SLA, Electronics & Innovation, Ltd.) and a function generator (33120A, Agilent Technologies). A removable water cone was mounted between the probe and the cell dish to maximize the transmission of the FUS between the transducer and the cell. The focal zone of 1-MHz FUS had a length and diameter of 26 and 3 mm, respectively. The acoustic pressures used in this study were measured with a polyvinylidene difluoride-type hydrophone (model HGL-0085, ONDA, Sunnyvale) in distilled and degassed water at 25 °C.

Prior to the FUS sonication, the targeting procedure with FMBs in the cells was performed. Because of the buoyancy of the MBs, the dishes were inverted to allow the cells to contact the FMBs. Following 10 min of FUS treatment at room temperature, the dishes were then washed with DPBS (100 μL) twice to remove the untargeted MBs.

**Live Cell Imaging.** Cells were transfected with the indicated constructs and incubated for 24 h. Before the imaging, the transfected cells were incubated with folate-conjugated, fluorescent-labeling microbubbles for 10 min and then washed with PBS. After the addition of RBP (50 nM) or...
GAβ (1 μM), the cells were treated with FUS. Live-cell imaging was performed with a 60× or 100× oil objective mounted on a Nikon inverted TiE microscope equipped with a motorized stage. Fluorescence images were collected by a Q2 camera every 1 min for 20 min to 1 h. The 3D cell images were processed with Huygens Deconvolution (Scientific Volume Imaging). The maximum intensity projections of images and image analysis were mainly conducted by Nikon elements AR software (Nikon). FRET images were obtained under essentially the same condition of live cell imaging as above. After background subtraction, FRET/CFP ratio images were created with the ratio/FRET module of Nikon elements AR software (Nikon).

The imaging of the FMBs collapse was performed by an acousto-optical system, which allowed for concurrent FUS sonication and high-speed bright-field microscopy imaging. A high-speed camera (225,000 fps, model FASTCAMSA4, Photron Ltd.) was integrated onto a microscope (model IX71, Olympus) with a water tank. During the experiment, the tank was filled with deionized and degassed water at 37 °C. A sonication system consisting of 1 MHz FUS transducer (V302, Panametrics) was confocally positioned with a 63× objective immersed in the water tank. A waveform generator (33120A, Agilent Technologies) created a signal which was amplified with the power amplifier (325LA, Electronics & Innovation, Ltd.) to drive the transducer [(acoustic pressure = 500 kPa; cycle number = 500; pulse repetition frequency = 10 Hz; exposure time = 5 s).

**Cytotoxicity Measurements.** Cell proliferation and viability were determined by an Alamar Blue (AbD Serotec) indicator. Cells were grown in 96-well tissue culture-treated plates (96-well Microtest Plate, BD Falcon) at a density of 1 × 10⁴ cells/well in 100 μL of the culture medium. All cells were incubated overnight and maintained in a humidified atmosphere at 5% CO₂ and 37 °C. Cells were treated the following day. Each sample was adjusted to the same concentration, containing the same amounts of the drug. FUS was then applied. Wells without the drug treatments served as controls. The medium (containing drugs) was removed after a 2 h incubation, and the cells were washed twice with DPBS and resuspended in the culture medium. Following 24 h of incubation time, 10 μL of Alamar Blue with 90 μL of the FBS and penicillin–streptomycin-free DMEM/F12 medium was added to the cells, which were further incubated for 2 h. The absorbance of each plate was measured at a wavelength of 570 nm, and a wavelength of 600 nm served as a reference. The absorbance of each plate was measured at a wavelength of 600 nm. The absorbance of the positive growth control well − OX is the molar extinction coefficient of Alamar BlueTM oxidized form (blue), A is the absorbance of experimental wells, and A⁰ is the absorbance of the positive growth control well (cells with Alamar Blue but without test agent).

**Statistical Analysis.** The results are presented as the mean and standard deviation of the mean of at least three independent samples, and the standard deviations are displayed as error bars in the MTT results. All statistical evaluations were carried out with unpaired two-tailed Student’s t tests. P-values greater than 0.05 and less than 0.01 were considered not significantly different and highly significant, respectively.

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.7b00162.

**REFERENCES**


