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Achieving Spatial and Molecular Specificity with Ultrasound-Targeted Biomolecular Nanotherapeutics

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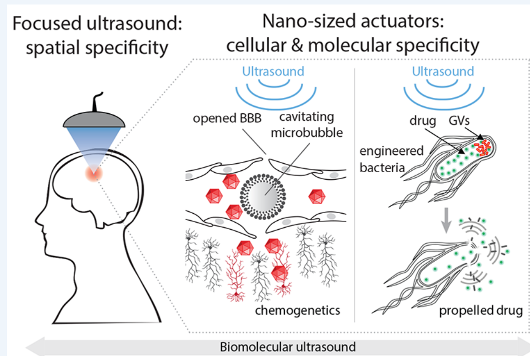

CONSPECTUS: The precise targeting of cells in deep tissues is one of the primary goals of nanomedicine. However, targeting a specific cellular population within an entire organism is challenging due to off-target effects and the need for deep tissue delivery. Focused ultrasound can reduce off-targeted effects by spatially restricting the delivery or action of molecular constructs to specific anatomical sites. Ultrasound can also increase the efficiency of nanotherapeutic delivery into deep tissues by enhancing the permeability of tissue boundaries, promoting convection, or depositing energy to actuate cellular activity. In this review we focus on the interface between biomolecular engineering and focused ultrasound and describe the applications of this intersection in neuroscience, oncology, and synthetic biology. Ultrasound can be used to trigger the transport of therapeutic payloads into a range of tissues, including specific regions of the brain, where it can be targeted with millimeter precision through intact skull. Locally delivered molecular constructs can then control specific cells and molecular pathways within the targeted region. When combined with viral vectors and engineered neural receptors, this technique enables noninvasive control of specific circuits and behaviors. The penetrant energy of ultrasound can also be used to more directly actuate micro- and nanotherapeutic constructs, including microbubbles, vaporizable nanodroplets, and polymeric nanocups, which nucleate cavitation upon ultrasound exposure, leading to local mechanical effects. In addition, it was recently discovered that a unique class of acoustic biomolecules—genetically encodable nanoscale protein structures called gas vesicles—can be acoustically "detonated" as sources of inertial cavitation. This enables the targeted disruption of selected cells within the area of insonation by gas vesicles that are engineered to bind cell surface receptors. It also facilitates ultrasound-triggered release of molecular payloads from engineered therapeutic cells heterologously expressing intracellular gas vesicles. Finally, focused ultrasound energy can be used to locally elevate tissue temperature and activate temperature-sensitive proteins and pathways. The elevation of temperature allows noninvasive control of gene expression *in vivo* in cells engineered to express thermal bioswitches. Overall, the intersection of biomolecular engineering, nanomaterials and focused ultrasound can provide unparalleled specificity in controlling, modulating, and treating physiological processes in deep tissues.

One of the key goals of nanomedicine is to enable more selective treatment of diseased cells without invasive surgery. Attempts to achieve such selectivity often rely on targeting therapeutics to molecular markers over-represented on the target cell population or taking advantage of tissue accumulation mechanisms such as enhanced permeability and retention in tumors. However, the complexity of living organisms makes it difficult to achieve perfect specificity and avoid off-target effects. In particular, molecular targeting is often insufficient to direct systemically administered nanomedicines to desired anatomical locations such as tumors or specific regions of the brain. In this perspective, we discuss how anatomical specificity can be improved by combining nanomedicines with ultrasound—a versatile form of physical energy that can be applied and focused at depth in a variety of tissues with millimeter precision. Ultrasound enables the spatial targeting of therapy through diverse mechanisms that include localized ultrasound-enhanced transport, the activation of local mechanical events such as inertial cavitation, the elevation of temperature at the ultrasound focus, and direct interactions with mechanosensitive components of tissue.[1,2]

On its own, focused ultrasound is already a clinical tool used to treat diseases raging from prostate cancer to essential tremor, owing to the ability of modern ultrasound instruments to focus high intensity sound waves on millimeter-sized regions of tissue and deliver ablative heat, often under the guidance of magnetic resonance imaging (MRI). In the approaches described in this perspective, the same ultrasound technology is used with pulse parameters that do not on their own result in tissue ablation. The goal of these approaches is to combine nanoscale and genetically encoded materials with focused ultrasound to enable more selective biological perturbation and disease treatment.

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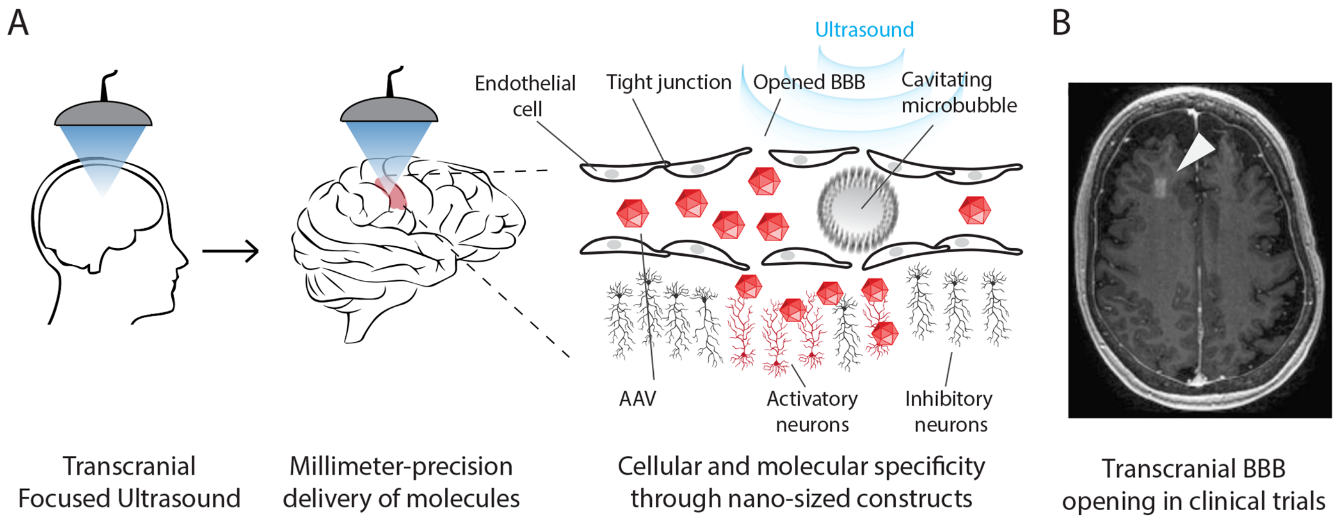

Figure 1. Ultrasound enhanced and triggered transport into the brain. (A) The human body contains thousands of types of molecules in different tissues. Restricting the region of delivery to a small subset of cells by focused ultrasound-enhanced delivery reduces off-target effects in nontargeted tissues. By combining ultrasound specificity with molecular engineering it is possible to both target the specific sites within the body and specific cells within the targeted site. Such specificity can be achieved by localized delivery of molecular constructs (AAV viral vectors, nanoparticles, proteins, small molecules) through the BBB into the brain. When microbubbles are injected into the bloodstream and insonated, they begin to oscillate (cavitate) and loosen tight junctions in the BBB, transiently, locally, and safely, improving transport from blood into the brain tissue. (B) Example of ultrasound-enhanced molecule delivery to the brain. The arrowhead points to the area of the BBB opened with ultrasound to allow the passage of a small molecule MRI contrast agent. (Reproduced with permission from ref 25. Copyright 2018. Nature-Springer.)

■ ULTRASOUND ENHANCED AND TRIGGERED TRANSPORT INTO THE CENTRAL NERVOUS SYSTEM

The use of ultrasound to enhance or target the delivery of nanosized therapeutic compounds into tissues relies on its ability to open biological barriers, trigger physical changes in nanoscale drug delivery vehicles, or propel materials via convective transport. These capabilities have been used for site-specific delivery of small molecules, nanoparticles, and viral vectors to tissues such as tumors,[3−5] the gastrointestinal tract,[6] the eye,[7] muscle,[8] and brain.[9] Several recent reviews have covered ultrasound-enhanced delivery to these areas of the body.[10,11] In this review, we focus specifically on delivery to the brain as an example target.

Brain delivery and targeting represent a particularly challenging problem. The brain is composed of anatomically defined regions containing a multitude of different cell types, including neurons, that cannot be easily distinguished by their molecular markers but perform vastly different functions. For example, nearly identical neuron types can control movement, register sensory inputs, or support complex reasoning depending on where they are in the brain. In addition, the entry of most molecules into the central nervous system (CNS) is restricted by a specialized endothelial structure called the blood brain barrier (BBB), making it difficult to deliver nanomaterials to the brain by systemic administration. Even if the BBB can be crossed at a specific anatomical site, additional selectivity is needed at that site to target the correct subset of the multiple cell types present at that location.[12−14]

These challenges can be addressed by combining nanomaterials with focused ultrasound BBB opening (FUS-BBBO). In this combination, low intensity focused ultrasound interacts with systemically administered, intravascular microbubble contrast agents—micron-scale bubbles of gas typically stabilized by a lipid shell—which are also used for clinical diagnostic ultrasound. When insonated, the microbubbles oscillate in size and exert mechanical forces on the endothelium, resulting in the temporary opening of the tight junctions comprising the BBB. FUS-BBBO allows for the delivery of small molecules,[9] proteins,[15] viral vectors,[16] and nanoparticles[17−19] to brain sites defined by the ultrasound focus (Figure 1A). Larger molecules typically require higher pressures of ultrasound for efficient delivery.[20] Typically, after several hours the BBB closes[21] leaving little to no damage at the site of insonation.[22] The use of FUS-BBBO is safe even after multiple exposures[22,23] and has been successfully used in humans[24,25] (Figure 1B). Pioneering applications of this technology include the treatment of brain cancer[24] and neurodegenerative diseases.[25−27]

To combine the spatial precision of ultrasound with the molecular, cell type, and temporal control provided by genetically engineered therapeutics, we recently developed an approach to noninvasive control of neural circuits called acoustically targeted chemogenetics, or ATAC[28] (Figure 2A, B). This technology uses FUS-BBBO to deliver adeno-associated viral (AAV) vectors into specific brain regions (Figure 2C). These vectors transfect neurons and cause genetically defined neuronal subtypes to express engineered receptors, which provide control over the activity of these neurons using an otherwise inert brain-permeable drug.[29] With dimensions of approximately 20 nm, AAVs are small enough to efficiently enter the brain after nondamaging FUS-BBBO and can be delivered in sufficient dose to achieve more than 50% transfection in certain brain regions (Figure 2D). Cellular specificity is achieved by engineering the DNA contained inside the AAV to express the desired protein under a promoter that is only active in select cell types,[30] for example, excitatory neurons or neurons that use the neurotransmitter dopamine. In this case, the protein payload comprises a "chemogenetic" G-protein coupled receptor that has been engineered to no longer respond to endogenous neurotransmitters and instead becomes activated

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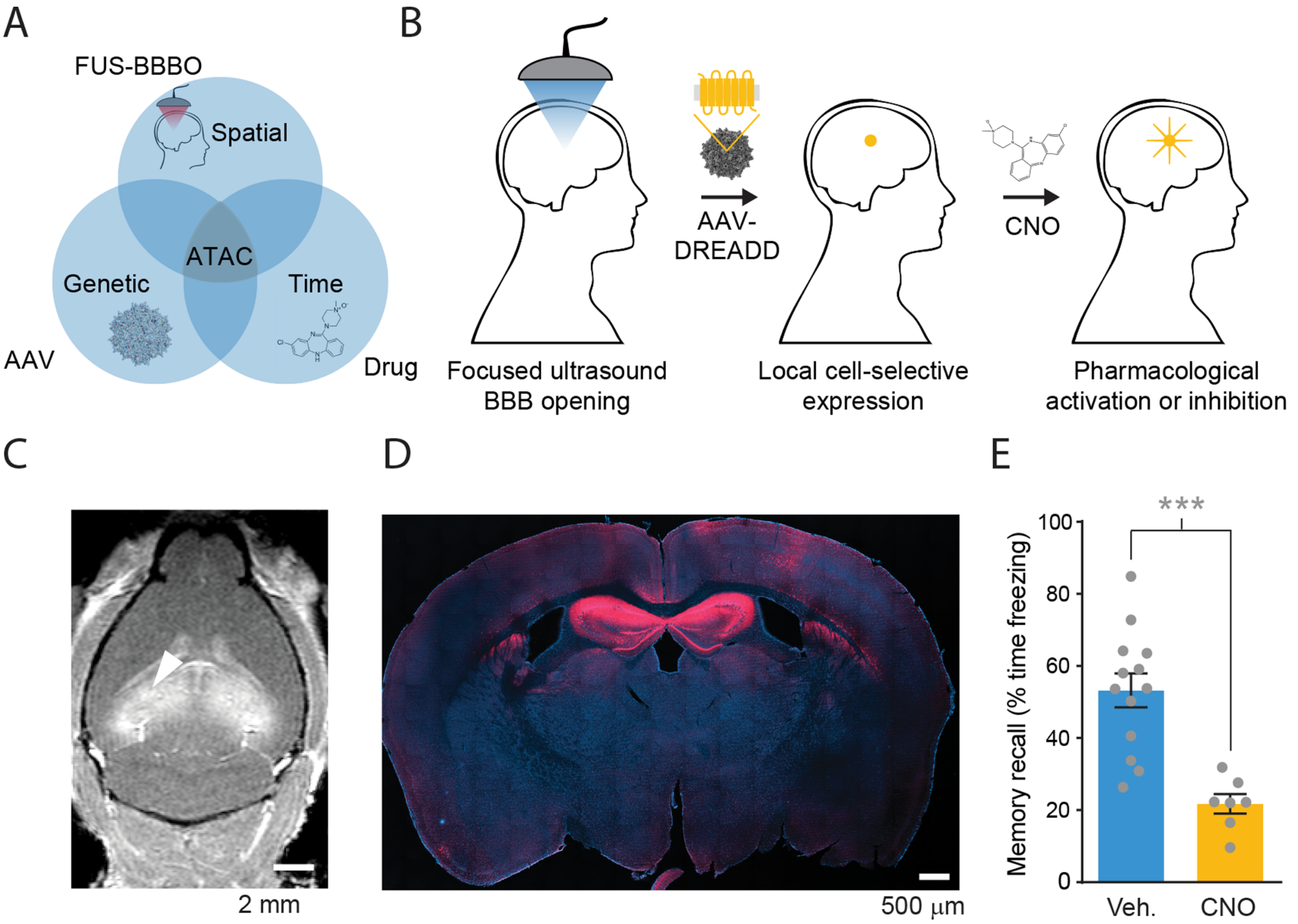

Figure 2. Acoustically targeted chemogenetics (ATAC). (A) ATAC combines FUS-BBBO, viral vector gene delivery, and chemogenetics to achieve fully noninvasive spatially, genetically, and temporally specific control cells in the brain. (B) In the ATAC process MRI-guided focused ultrasound reversibly opens the BBB to deliver viral vectors carrying chemogenetic receptors that can be activated specifically by a BBB-permeable ligand. (C) Safe and noninvasive opening of the BBB with FUS in hippocampus which was used to deliver viral vectors carrying DNA with a cell specific promoter and a chemogenetic receptor. The BBB opening is visualized by extravasation of gadolinium contrast agent in a T_1-weighted MRI. (D) Gene expression of engineered chemogenetic receptors that respond to a specific BBB-permeable drug, as visualized by immunostaining (red). (E) The expression of engineered receptors allows subsequent pharmacological control of specific neurons and resulting behavior, such as memory recall. (Adapted with permission from ref 28. Copyright 2018. Nature-Springer).

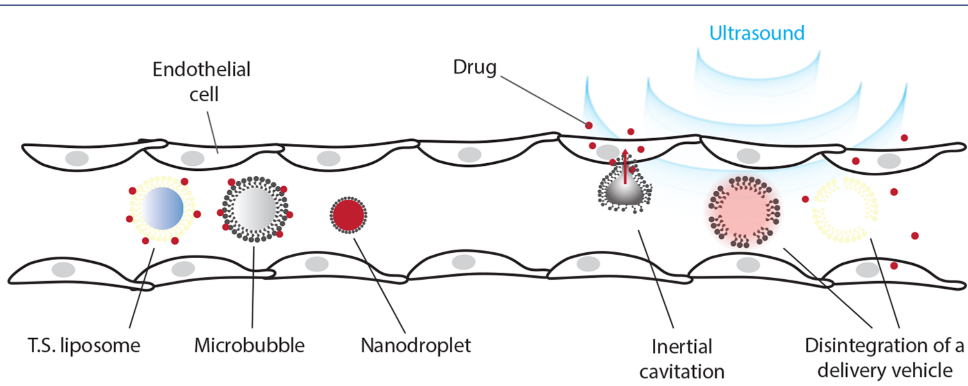

Figure 3. Site-specific delivery of drugs for control of cellular functions. Site-specific molecular delivery is enabled through transcranially focused ultrasound, which can target brain regions with millimeter precision. Multiple approaches can be used to control cellular activity in the brain, including focused-ultrasound BBB opening with intravenous coadministration of viral vectors or nanoparticles. Delivery of molecules to all sites within the body can be achieved with ultrasound-responsive delivery vectors, such as nanodroplets, microbubbles, and temperature-sensitive liposomes (T.S. liposomes). Molecules can be incorporated within the core or shell of these delivery vehicles or can be attached to the exterior. Upon insonation these vehicles cavitate (in the case of gas-containing bubbles) and/or disintegrate, releasing their cargo.

by an otherwise inert, systemically bioavailable drug. Several such receptor−drug combinations are available, allowing metabotropic or ionotropic excitation or inhibition of neurons.[29] ATAC comprises a brief, noninvasive FUS-BBBO

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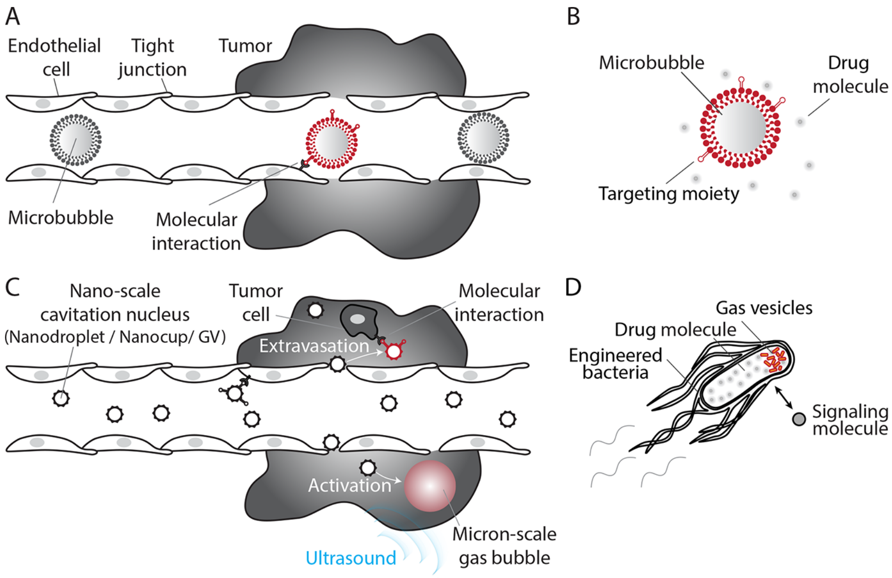

Figure 4. Nanoscale and genetically encodable nuclei for molecularly targeted cavitation. (A) Due to their micron scale, microbubbles cannot exit the vasculature through leaky tumor blood vessels and reach the cancerous tissue. (B) Therefore, they primarily engage in molecular interactions with endothelial cells. (C) Nanoscale cavitation nuclei can exit the vasculature and interact with cells and other targets in the tissue in addition to endovascular targets. Their activation inside the tumor microenvironment enables selective generation of strong mechanical forces within the tumor core. (D) Engineered cells expressing gas vesicles can be triggered with ultrasound to nucleate cavitation, producing potent mechanical effects and releasing therapeutics they produce in situ.

treatment and a several-week period to attain robust expression of the chemogenetic receptor that lasts for at least several months.[28] Subsequently, the transfected neurons are controlled on-demand via the simple systemic administration of the chemogenetic ligand.

In a proof-of-concept study,[28] we used ATAC to non-invasively inactivate the mouse hippocampus (Figure 2D) and inhibit the formation of associative memories (Figure 2E). The effect was highly specific; we did not observe off-target effects on untargeted neurons or untargeted behaviors. The unprecedented combination of targeting based on spatial focusing, genetic specificity, and the molecular precision of chemogenetics creates opportunities for more specific therapies and neuroscience experiments. Importantly, all three components of ATAC—FUS-BBBO, viral vectors, and chemogenetics—have either been used in clinical trials[25,31] or in nonhuman primates,[32] increasing the feasibility of clinical translation.

In some scenarios, a shorter-term approach to neuromodulation not involving gene therapy is beneficial, for example in clinical research studies and in piloting potential site-specific interventions in patients. In these cases, direct delivery of therapeutics would be beneficial (Figure 3). One such approach is based on nanodroplets that are superheated liquid droplets with typical diameters of 200−300 nm[33] made of perfluorocarbons or halocarbons, covered by a stabilizing shell made of albumin or lipids.[34] In their liquid state, nanodroplets can circulate in the blood for several hours.[35] Once delivered to the desired location, nanodroplets can be activated using ultrasound to phase-transition into gas bubbles in a process called acoustic droplet vaporization. This results in unstable gas bubbles that are 3−5.5 times larger and can be used as either contrast agents for imaging or mechanical actuators.[35] Recently, a method of transient localized delivery of neuroactive therapeutics was developed by loading the local anesthetic Propofol into perfluorocarbon nanodroplets, then activating the local release of this drug from the droplets in the neurovasculature using focused ultrasound.[36] While the mechanism by which the drug is released is still uncertain in this particular case, the flow rate of the relevant neurovasculature and the kinetics of Propofol uptake into the brain parenchyma allow the inhibition of neural activity to be localized within the insonated region.[37] This approach is conceptually related to previous and ongoing work on the localized delivery of therapeutics to various organs of the body.[38] For example, doxorubicin has been delivered to tumors using temperature-sensitive liposomes, which release their drug contents at the ultrasound focus as a result of temperature elevation above their phase transition threshold,[39,40] typically below 42 °C. Another application is the delivery of tissue-plasminogen activator using echogenic liposomes.[41,42]

■ ULTRASOUND-ACTUATED NANOMECHANICAL THERAPEUTICS

In addition to facilitating the delivery of nanoscale and molecular therapeutics into targeted regions of the body, focused ultrasound can produce more immediate mechanical and thermal effects in the target tissue.[2] For example, low frequency ultrasound in combination with cavitation nuclei can produce a range of mechanical effects via stable and inertial bubble cavitation. The latter phenomenon, occurring at relatively high acoustic pressure, involves the unstable expansion and violent collapse of bubbles.[43] In comparison to the gentle opening of tight junctions in the BBB and microstreaming achieved by stable cavitation, inertial cavitation can lyse cells or enhance drug delivery by creating pores in their membranes.[43]

Targeted cavitation treatments are often facilitated by the use of untargeted or, less frequently, targeted micron-sized bubbles as cavitation nuclei.[40] However, microbubbles have several characteristics limiting their biological specificity. First, bubbles are fundamentally unstable, making it difficult for them to undergo extended wash-in and clearance protocols to achieve specific biological targeting. Second, micron-sized bubbles cannot exit the vascular system and, thus, cannot interact with

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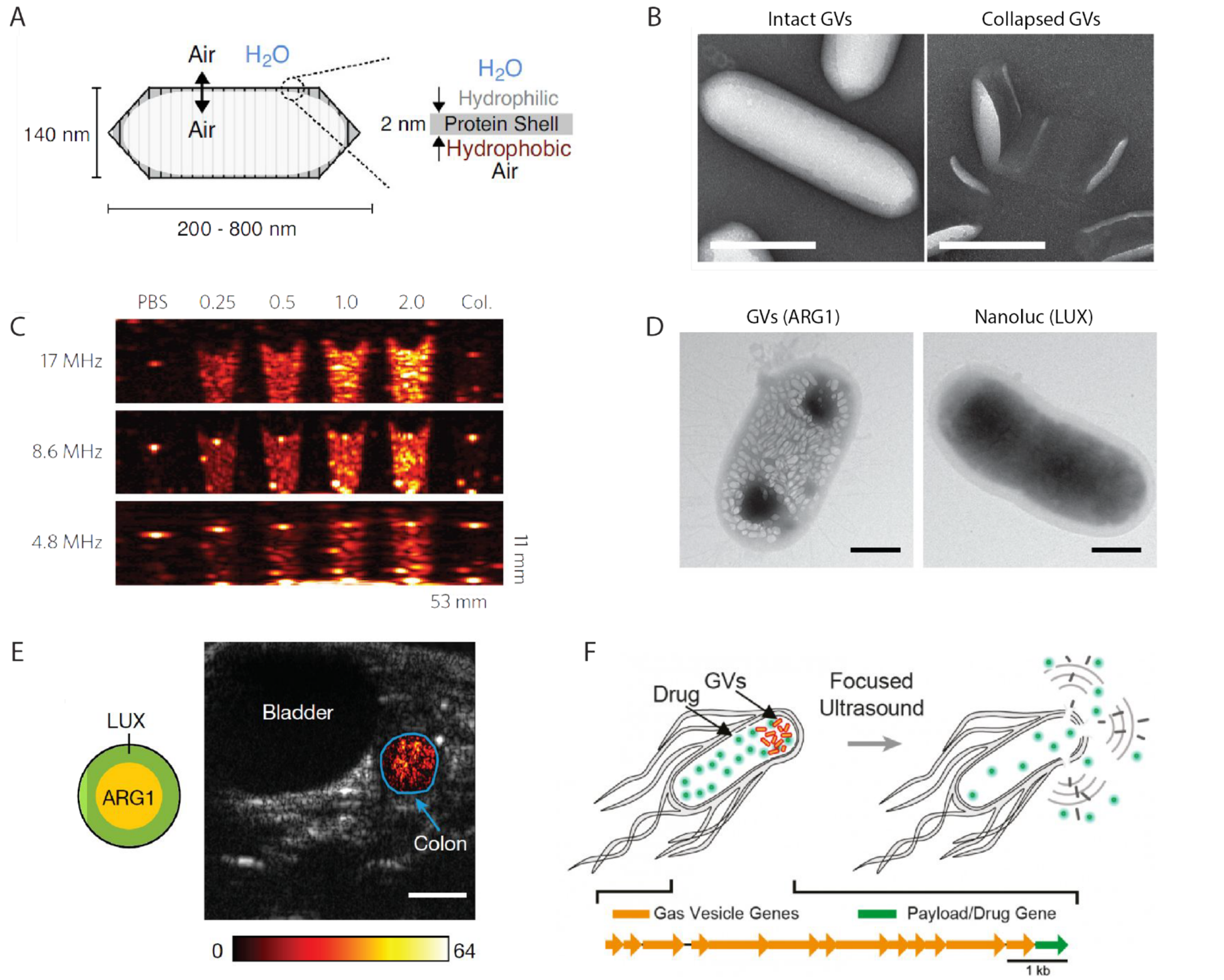

Figure 5. Gas vesicles as genetically encoded nuclei for imaging and therapy. (A) Illustration of a gas vesicle (GV) structure. (Reproduced with permission from ref 62. Copyright 2018. Elsevier). (B) Transmission electron micrographs of purified GVs from Anabaena flos-aquae. GVs can be collapsed either hydrostatically or acoustically, releasing the encapsulated air bubble. Scale bars, 200 nm. (C) Purified gas vesicles produce robust acoustic contrast with concentration-dependent signal. (B and C reproduced with permission from ref 46. Copyright 2014. Nature-Springer). (D) Transmission electron micrographs of an *E. coli* cell transformed with the acoustic reporter gene (ARG1), chemically induced to produce GVs. *E. coli* cell expressing nanoluc luciferase, presented for comparison. Scale bars, 500 nm. (E) Ultrasound abdominal scan of a mouse showing ARG1-expressing *E. coli* cells arranged in the colon as indicated in the diagram. Functional GV contrast is overlaid in color on top of a grayscale anatomical scan. (D and E reproduced with permission from ref 47. Copyright 2018. Nature-Springer). (F) GVs used as genetically encoded cavitation nuclei that can lyse the host cell and release a coexpressed payload.

the extracellular matrix or cells located deep within tissues of interest[44] (Figure 4A, B). For ultrasound-activated therapeutics to reach such targets, the cavitation nuclei will either need to become nanoscale to enable extravasation, or they will need to be genetically encoded within the cells themselves. To overcome these limitations, several acoustically active nanomaterials have recently been developed.

Among synthetic materials, perfluorocabon nanodroplets were among the first nanoscale cavitation nuclei shown to extravasate through leaky tumor vessels (Figure 4C), which then can facilitate drug delivery into tumors through an ultrasound-triggered phase transition.[4] In addition to liquid droplets, another recent approach has focused on seeding bubble nucleation using polymeric "nanocups".[45] These polymeric structures with diameter of 480 ± 24 nm[44] have a cup shape that holds an air nanobubble stabilized by an inner hydrophobic cavity. Upon insonation, the nanobubble grows and then detaches from the nanocup to nucleate free bubble cavitation activity. In vivo studies showed that the resulting cavitation can propel drug models deeper into cancerous tissue.[44] Moreover, the stable hydrophobic cavity can assist in nucleating additional cavitation even after the release of the initial bubble.

To expand the potential of ultrasound-targeted therapy to specific cells and biomolecular targets, we recently introduced the first genetically encodable acoustic biomolecules (Figure 4D). Gas vesicles (GVs) are genetically encoded all-protein nanostructures that in nature are used by photosynthetic bacteria to regulate their flotation.[46] GVs are composed of a gas-filled protein shell with a hydrophobic interior and hydrophilic exterior. Thus, while enabling the free exchange of gas, GVs exclude water from their interior and are instead filled with gas that partitions into them from surrounding media (Figure 5A). Wild type GVs have dimensions on the order of 45−800 nm (depending on their genotype) and indefinite

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physical stability. In the past few years, GVs have been shown to produce robust contrast (Figure 5C) in ultrasound,[46−49] MRI,[50,51] and optical imaging.[52] Furthermore, gene cassettes have been engineered for heterologous expression of GVs as acoustic reporter genes in bacteria[47] and mammalian cells,[53] enabling deep tissue imaging of cell location and activity (Figure 5D, E). The GV shell can be collapsed hydrostatically or acoustically[54] (Figure 5B), releasing the enclosed air and producing backgroundless differential images.[47,51]

The ability to collapse GVs and break their protein shell also provides a new mechanism for noninvasively producing local mechanical forces.[55] While typical diagnostic ultrasound frequencies in the range of several MHz can be safely used to visualize GVs, we found that low-frequency ultrasound pulses can drive the growth and cavitation of air nanobubbles released following GV collapse.[55] The ability of purified GVs to nucleate cavitation was demonstrated using passive cavitation recording, which detects the acoustic signature of cavitation, and using ultrahigh frame rate optical microscopy, which provided direct images of GV collapse and subsequent formation of cavitating bubbles. Based on the insights obtained from these in vitro experiments, in vivo GVs cavitation inside disease-relevant tissue was shown with a subcutaneous tumor model.[55]

In addition to being genetically encodable, GVs have a range of unique characteristics that make them exceptional contrast agents and actuators. In comparison to microbubbles, GVs' nanoscale size is compatible with their assembly inside bacterial[47] and mammalian cells,[53] and potentially with passing through leaky tumor vessels. In addition, the constituent proteins comprising GVs can be engineered to provide new acoustic, structural, surface, and functional properties.[54] These changes can enable highly specific ultrasound imaging of GVs based on nonlinear acoustic output,[48,49] tailored collapse pressure, selective attachment of GV to particular cells, and fluorescent GVs.[54] For example, the fusion of GVs' external shell protein GvpC with a C-terminal RGD peptide enables targeting to $\alpha_V\beta_3$ integrin receptors, which are overexpressed in certain tumors.[54] When insonated with focused ultrasound waves, GVs attached to U87 glioblastoma tumor cells nucleated cavitation activity, opening the membrane of these cells.[55] This sonoporation resulted in an influx of a cell-impermeable dye, functioning as a drug model.[55] Cavitation of GVs attached to U87 cells was also documented using high frame rate microscopy.[55]

An even wider range of therapeutic effects can be achieved by expressing GVs in engineered cells. In previous studies, engineered bacteria were shown to selectively home to and colonize tumors, monitor the microenvironment, and produce antitumor therapy in situ.[56,57] GV cavitation complements these capabilities by providing a new mechanism to deliver mechanical effects and externally trigger the release of intracellular therapeutic proteins with high spatial and temporal precision. In our recent study, GV cavitation was shown to facilitate ultrasound-triggered lysis of engineered bacteria and selective release of coexpressed luminescent protein Nanoluc, which served as a payload model[55] (Figure 5F). In addition, GV cavitation provides these engineered cells with a mechanism for producing strong mechanical forces that can potentially be used to propel drugs deeper into adjacent tissue. The recent mammalian expression of GVs[53] could extend these capabilities to a broader range of therapeutic cell types.

The ability of GVs to serve as imaging as well as therapeutic agents is expected to enable their use in theranostics—an emerging therapeutic paradigm in which molecular imaging modalities are used to guide and control targeted therapeutic activity. In particular, it is possible to use nondestructive imaging modes to visualize GV biodistribution in vivo before applying focused ultrasound pulses to collapse and cavitate the GVs and resulting bubbles for therapeutic purposes, then using imaging to confirm that GVs at the targeted location have been destroyed.

■ ULTRASOUND-ACTUATED THERMAL BIOSWITCHES

In addition to the mechanical effects mediating the ultrasound uses described above, focused ultrasound can also be used to locally elevate temperature. This can be performed under real-time MRI guidance, allowing the delivered temperature to be within a desired target range. Focused ultrasound heating to ablative temperatures is used clinically for focal ablation. However, it can also be used in combination with temperature-sensitive biomolecules to achieve control over cellular functions such as gene expression using thermal pulses within the well-tolerated range of 37−42 °C. This has been accomplished in mammalian cells using their endogenous heat shock promoter machinery, allowing FUS to drive the expression of genes driven by a heat shock promoter (HSP) genes.[58−60] In bacteria, endogenous HSPs were found to provide poor switching responses within the temperature range compatible with mammalian physiology, prompting us to develop new classes of orthogonal transcriptional bioswitches with tunable temperature setpoints.[61] These bioswitches enable gene expression in engineered microbes to be controlled with more than 300-fold switching induction.

■ SUMMARY

The combination of molecular engineering and ultrasound allows for more specific targeting of cell populations in deep tissues. The idea of a silver bullet—a single molecule that specifically binds to a single target—is challenging to achieve in vivo due to the sheer number of molecular interactions throughout the body. However, restricting the area of activity to the sites specified by an ultrasound beam will simplify the problem of specific targeting. The millimeter-sized volume of a tissue exposed to focused ultrasound has fewer cells and fewer off-target binding partners than the entirety of a human body. Thus, the simple use of ultrasound and its application to nanomedicine provides an added layer of specificity that would be difficult to achieve with molecular engineering alone.

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Notes

The authors declare no competing financial interest.

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Engineering from MIT in 2009, where he worked on engineering protein contrast agents for MRI in the laboratories of Alan Jasanoff and Robert Langer. He then received his Ph.D. in Bioengineering from Caltech in 2015 for his work on programmable therapeutics for modulating gene expression in animal models of cancer in the laboratory of Peter Dervan. As a postdoctoral fellow in the Shapiro laboratory at Caltech, he developed the acoustically targeted chemogenetics technology for noninvasive control of neural circuits. More information about the Laboratory for Noninvasive Neuroengineering can be found online at szablowskilab.org.

Avinoam Bar-Zion is a Marie Skłodowska-Curie postdoctoral fellow at the Shapiro laboratory at Caltech. He received his B.Sc. degree Summa Cum Laude in biomedical engineering from Technion in 2010. His Ph.D., also received from Technion in 2016, was focused on imaging of tumor angiogenesis using contrast-enhanced ultrasound. During his Ph.D. work, he also completed a year of research at the University of Toronto, as a part of a collaboration between Technion and with the Medical Biophysics Department, University of Toronto. His research interests include contrast-enhanced ultrasound imaging and therapy, medical signal and image processing, synthetic biology, and computer-aided diagnosis.

Mikhail G. Shapiro is a Professor of Chemical Engineering and an Investigator of the Heritage Medical Research Institute at the California Institute of Technology. The Shapiro laboratory develops biomolecular technologies allowing cells to be imaged and controlled inside the body using sound waves and magnetic fields to enable the study of biological function in vivo and the development of cell-based diagnostic and therapeutic agents. Mikhail received his Ph.D. in Biological Engineering from MIT and his B.Sc. in Neuroscience from Brown University and conducted postdoctoral research at the University of Chicago and the University of California, Berkeley, where he was a Miller Fellow. More information about the Shapiro Lab can be found online at shapirolab.caltech.edu.

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