Building Blood Vessels and Beyond Using Bubbles

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Introduction

The axolotl (*Ambystoma mexicanum*), a type of salamander (see tinyurl.com/59u26vdm), has an incredible ability to regenerate entire limbs and other body parts that become damaged. Although humans are unable to match the axolotl, the capability of the human body to repair wounds is ultimately critical for our survival. Wound healing is a complex process driven by cells initially present at the wound site as well as cells that migrate into the wound environment. In general, cell behavior is guided by biochemical and biophysical "cues" in the local environment. Biochemical cues (e.g., proteins) are molecular in nature, whereas biophysical cues (e.g., stiffness) are mechanical and/or structural characteristics of the environment surrounding a cell. In the human body, the extracellular matrix is the environment surrounding each cell within solid tissue and contains large molecules like proteins and carbohydrates.

These biochemical and biophysical cues, which are regulated in both space and time by intricate pathways, cause a cell to undergo processes that directly or indirectly facilitate wound healing. Many wounds like minor cuts, scrapes, and bruises heal without a visit to a doctor's office. Other wounds may necessitate medical treatments like stitches or an orthopedic cast in addition to pharmaceuticals for the wound to properly heal. Surgical reconstruction and/or organ transplantation is needed when tissues or organs are severely damaged by trauma (e.g., car accident) or disease (e.g., cardiovascular, cancer). These higher risk and more invasive interventions are required when the damaged tissues or organs have a very limited ability to regain their structure and function via the body's normal wound-healing mechanisms. Unfortunately, there is a practical limit to the types of defects that can be surgically reconstructed. Moreover, some patients do not qualify for surgery because of other medical issues. Complicating matters further, the demand for organ transplantation exceeds the supply of donated organs and organ recipients often require long-term immunosuppressive drugs, both of which impact morbidity and quality of life.

Tissue Engineering and Regenerative Medicine

The field of tissue engineering and regenerative medicine (TERM) seeks to overcome limitations associated with conventional, medical interventions like surgery and organ transplantation. The goal of TERM is the development of biological constructs that facilitate restoration, maintenance, or regeneration of impaired or injured tissues/organs. A common strategy within TERM is the preprogrammed design of a construct consisting of a biocompatible scaffold loaded with cells and/or biochemical cues (**Figure 1A**). The scaffold provides a three-dimensional (3D) microenvironment for cells, thereby mimicking the function of the extracellular matrix.

The scaffold component of the construct often consists of a hydrogel, which is a porous, water-laden matrix consisting of natural (e.g., fibrin, collagen) or synthetic (e.g., dextran, polyethylene glycol) polymers. Hydrogels are made by cross-linking solutions of polymers to yield a solid-like material. Many commonly used hydrogels in TERM are biodegradable, which can assist with regenerative processes because they stay as long as needed and then disappear over time.

Constructs are implanted inside a living organism to assist with tissue regeneration. However, a problem with preprogramming the design of a construct is that it involves manipulating the physiochemical properties of the scaffold and its precursor components before the construct is implanted. This manipulation yields constructs with predefined patterns of biochemical and biophysical cues. For example, with hydrogels, the composition of the

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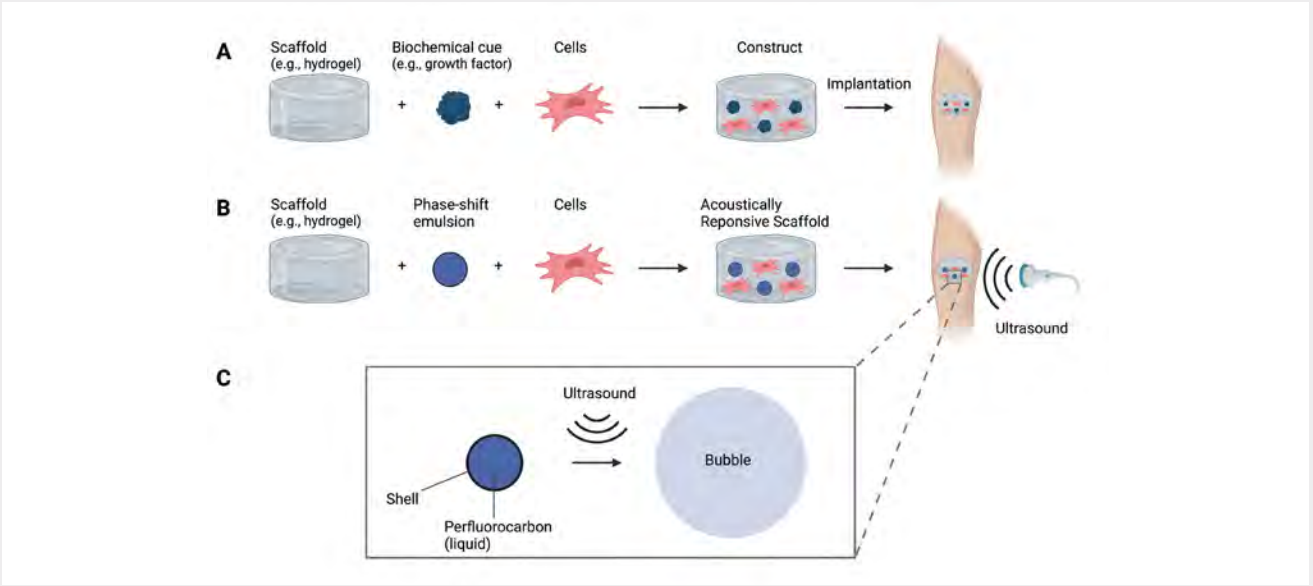


Figure 1. ***A:*** *constructs used in tissue engineering and regenerative medicine consist of a scaffold loaded with biochemical cues and/or cells. The construct is implanted within the body to facilitate regeneration at a particular location. A limitation of this common, preprogrammed paradigm is the inability to actively modulate biochemical and/or biophysical cues within the construct after implantation.* ***B:*** *an acoustically responsive scaffold (ARS) can be noninvasively controlled using ultrasound, thereby enabling spatiotemporal modulation of cues after implantation. The phase-shift emulsion within the ARS is responsive to ultrasound.* ***C:*** *acoustic droplet vaporization (ADV) is the process by which a phase-shift emulsion in the ARS is converted into gas bubbles using ultrasound. ADV enables modulation of biochemical and biophysical cues within the ARS.*

polymer network and cross-linking conditions impact the rate at which biochemical cues are released from the hydrogel as well as its stiffness. But the preprogrammed design may not be best for the specific site of implantation. Or the design could be well suited at the time of implantation, but then its suitability decreases over time.

After implantation, the ability to dynamically modulate cues within a preprogrammed construct, and hence tissue regeneration, in an on-demand manner defined by a physician or even a patient is extremely limited. From a basic science perspective, the reliance on a preprogrammed design has hampered elucidating the roles of fundamental, biochemical, and biophysical cues in situ. In fact, this points to the need for a better understanding of these cues to help drive the development of new, regenerative therapies. Indeed, from an applied perspective, the preprogrammed design hinders real-time personalization of regenerative therapy for the simple reason that there is no way to easily adjust the performance of a preprogrammed construct in situ. These shortcomings have led to the development of constructs in which biochemical and/or biophysical cues can be externally controlled using light, heat, electricity, and magnetic fields. However, these stimuli are limited by factors such as a superficial depth of penetration, the need for invasive procedures, and/or poor spatial localization.

The Sound of Healing: Ultrasound and Regeneration

Ultrasound has been exploited in many regenerative applications because it can noninvasively produce desired thermal and mechanical bioeffects in a spatiotemporally regulated manner. These bioeffects can be produced at depths of up to 10 centimeters within the human body. Low-intensity ultrasound (LIUS) is one of the most studied ultrasound techniques and can be used to induce a myriad of biological responses including blood vessel growth and bone repair. The exact mechanisms underpinning the actions of LIUS in regeneration are being actively investigated. Studies highlight the involvement of mechanotransduction, whereby mechanical forces generated by LIUS activate mechanically sensitive receptors in cells, which leads to biochemical signaling (Sato et al., 2014).

Pulsed, focused ultrasound with a higher intensity than LIUS has been shown to transiently increase levels of signaling proteins within tissue. This, in turn, can locally

attract cells that are intravenously injected, which can help revascularize ischemic tissue (Tebebi et al., 2017). Acoustic shock waves, which in addition to LIUS have clinically approved uses, promote healing of bone and soft tissues (Simplicio et al., 2020). Ultrasound can also pattern cells within hydrogels, which assist with the growth of blood vessel-like structures (Garvin et al., 2011). Therefore, as seen with these examples, ultrasound can help drive regeneration in many ways.

The (Sort of) New Kid on the Block

A new, ultrasound-based approach for controlling biochemical and biophysical cues in tissue regeneration involves phase-shift emulsions: shell-stabilized liquid droplets that can be converted into gas bubbles in situ using ultrasound. Phase-shift emulsions use perfluorocarbon (PFC) liquids because they have favorable thermodynamic properties as well as a high biocompatibility. They also have vapor pressures that are an order of magnitude higher than that of water. The volatility of a PFC liquid provides a thermodynamic driving force for the liquid to phase transition into a gas.

The liquid-to-gas transition requires a certain amount of thermal energy or tensile stress (i.e., negative pressure). Ultrasound can trigger a phase transition in a PFC liquid without the generation of heat. Specifically, the negative component of the ultrasound wave reduces the local pressure below the vapor pressure of the PFC liquid, thereby making vaporization thermodynamically favorable.

A liquid can exist in a metastable state (i.e., below its saturated vapor pressure) while experiencing a negative pressure. Ultimately, as the magnitude of the negative pressure increases, vapor bubbles spontaneously form within the liquid. These bubbles grow until their internal pressure reaches the equilibrium pressure of the liquid (Fisher, 1948). The same concept is employed in phase-shift emulsions where the application of ultrasound induces bubble formation in a process known as acoustic droplet vaporization (ADV).

Acoustic Droplet Vaporization

The concept of ADV can be traced back to the 1950s and the late Donald Glaser, who was awarded the Nobel Prize in Physics for developing bubble chambers. These chambers contained a superheated liquid and enabled detection of atomic particles that left a path of bubbles as they traversed the liquid. Building on this work, in 1979, the late Robert Apfel (see bit.ly/AT-Apfel), former president of the Acoustical Society of America and recipient of the Society's Gold Medal, developed a radiation dosimeter in which the superheated liquid was fractionated into droplets. Apfel (1998) patented this technology, envisioning that ultrasound, in addition to radiation, could vaporize the droplets (i.e., phase-shift emulsion), which could be used in biomedical applications. The first experimental results on ADV were published by Kripfgans et al. (2000). Currently, many groups around the world are actively investigating phase-shift emulsions, ADV, and their biomedical applications, as seen in earlier articles in *Acoustics Today* (Burgess and Porter, 2015; Gray et al., 2019).

ADV is a threshold phenomenon, with the minimum acoustic pressure required to generate ADV termed the ADV threshold. The ADV threshold depends significantly on the physical properties of the phase-shift emulsion (e.g., diameter, molecular weight of the PFC species) as well as acoustic parameters (e.g., frequency) (Schad and Hynynen, 2010). For ultrasound frequencies of 1-10 MHz, ADV thresholds are in the megapascal range (i.e., peak negative pressure).

Phase-shift emulsions possess some distinct advantages compared with the microbubbles that are used diagnostically as ultrasound contrast agents to visualize blood flow and therapeutically to enhance drug delivery. One such advantage is that emulsions exhibit greater stability than microbubbles because of their liquid cores. Compared with microbubbles that only persist for minutes after injection into the body, emulsions can persist for much longer (e.g., hours to days). Another advantage is that emulsions have a greater drug-loading capacity. Drugs can be loaded into the liquid core of the emulsion compared with microbubbles where drugs are loaded into the shell.

Phase-shift emulsions for tissue regeneration are not directly injected into the bloodstream, which is typically how the emulsions are used in many other biomedical applications. Rather, the emulsions are incorporated into hydrogels to yield an acoustically responsive scaffold (ARS) that can be implanted into the body (**Figure 1**, **B** and **C**). This administration method also enables the use of larger diameter emulsions (e.g., >6 μm), which can be formulated more easily in uniform sizes compared

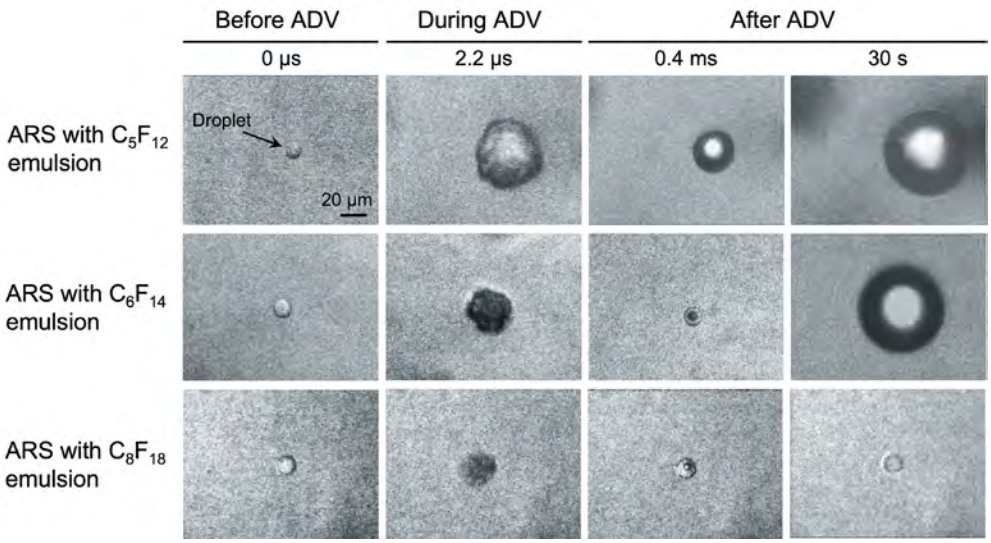


Figure 2. *In an ARS, bubble dynamics during and after ADV were dependent on the perfluorocarbon (PFC) liquid in the phase-shift emulsion. Longitudinal images, which were taken using ultra-high-speed microscopy, are shown for ARSs with emulsions containing perfluoropentane (C_5F_{12}; **top**), perfluorohexane (C_6F_{14}; **center**), or perfluorooctane (C_8F_{18}; **bottom**). In each series of images, a single droplet is shown during three stages: before (**left**), during (**center**), and after (**right**) ADV. During ADV, ultrasound caused formation of vapor in the PFC phase. Note the differences in bubble dynamics once the ultrasound is turned off (i.e., after ADV). A stable bubble was formed with C_5F_{12} and C_6F_{14}. With C_8F_{18}, the generated bubble recondensed.*

with smaller emulsions. Additionally, unlike other applications that often utilize lower molecular weight (and hence higher volatility) PFCs like perfluorobutane (i.e., C_4F_{10}) (Sheeran et al., 2016) or perfluoropentane (i.e., C_5F_{12}) (Mercado-Shekhar et al., 2019), emulsions in ARSs are typically formulated with higher boiling point PFCs like perfluorohexane (i.e., C_6F_{14}) or perfluorooctane (i.e., C_8F_{18}). These higher molecular weight PFCs offer better thermal stability by eliminating the potential for spontaneous bubble formation. Higher molecular weight PFCs also yield interesting bubble dynamics during and after ADV that can be utilized for specific applications. For example, emulsions with lower molecular weight PFCs undergo irreversible vaporization, where a stable bubble is formed (**Figure 2**); this yields a complete release of a drug loaded within the emulsion. Comparatively, with higher molecular weight PFCs, the generated bubble recondenses; this yields partial release of a drug.

Building Blood Vessels Using Bubbles

Conventional treatments for ischemic cardiovascular disease, which is characterized by insufficient blood flow, include bypass surgery and endovascular procedures. With the former, a healthy blood vessel is harvested from the patient's body and surgically connected to circumvent a blocked blood vessel. With the latter, a catheter is used to remove the occluding material (e.g., atherosclerotic plaque) in the blocked vessel, and removal is sometimes followed by the installation of a stent in the vessel. However, current treatments for cardiovascular disease are insufficient. For example, with critical limb ischemia, an advanced stage of peripheral artery disease characterized by poor blood flow in the leg, 25% of patients are ineligible for current treatments because of other medical issues and 29% of patients will either die or undergo a major amputation within one year of diagnosis.

Due to issues associated with standard interventions, alternative treatments are constantly being sought. One approach being investigated is the use of proteins to stimulate blood vessel growth. However, despite success in animal models, clinical translation has remained a challenge for multiple reasons. One critical limitation is that simply injecting the proteins into the body, in either the bloodstream or muscle, is ineffective and can cause serious side effects. Incorporating

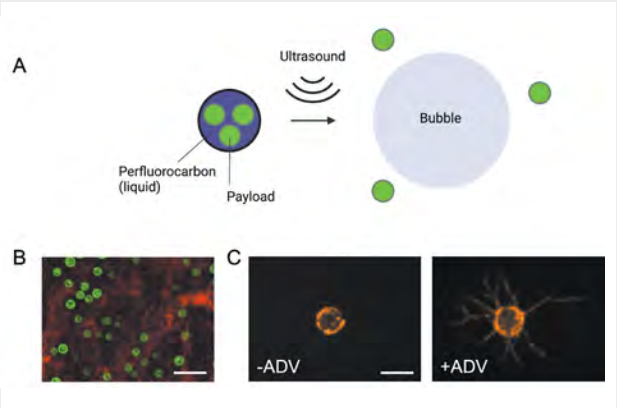


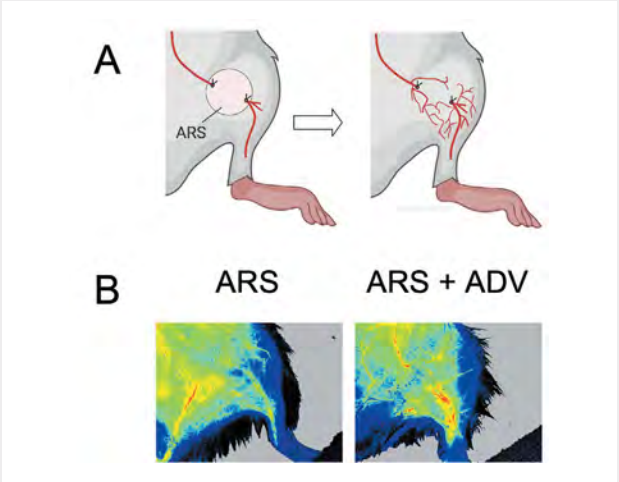
Figure 3. ***A:*** *payloads are encapsulated within a phase-shift emulsion using a double-emulsion approach. The payload is contained within tiny water droplets that are surrounded by liquid PFC. The encapsulated payload is released during ADV when the generated bubble disrupts the morphology of the double emulsion.* ***B:*** *the microstructure of an ARS was visualized using fluorescence microscopy, with the fibrin hydrogel matrix (**red**) and phase-shift emulsion (**green**) shown. Scale bar, 10 μm.* ***C:*** *ADV was used to release basic fibroblast growth factor, which controlled the growth of an in vitro model of blood vessels. The model consisted of microbeads coated with endothelial cells (**orange**), and fibroblasts. In this model, endothelial cells form tubes that are similar to blood vessels in the presence of appropriate biochemical cues. Note the presence of tubules emanating from the microbead for the +ADV condition (**right**), whereas no tubules were seen in the –ADV condition (**left**). Scale bar, 200 μm.*

the protein into a hydrogel is a more biocompatible approach, as discussed in **Tissue Engineering and Regenerative Medicine**, but there is a relatively limited control afforded by this method. Furthermore, the optimal delivery parameters for these potent proteins are still being determined (Briquez et al., 2016).

To attempt to solve some of these issues, ADV has been used to spatiotemporally control the release of proteins from ARSs using the following approach. A therapeutic payload like basic fibroblast growth factor (bFGF), a protein that stimulates blood vessel growth, is encapsulated in a phase-shift emulsion using a double-emulsion technique (**Figure 3A**). In this technique, bFGF is contained within tiny water droplets surrounded by a liquid PFC. Due to its hydrophobicity, the liquid PFC inhibits the release of bFGF from the emulsion. Ultrasound is then applied to the ARS to generate ADV and this results in the release of bFGF because the emulsion morphology is disrupted by bubble formation. Controlled stimulation of blood vessel growth has been demonstrated in both in vitro (**Figure 3B**) and in vivo studies with ARSs (Moncion et al., 2017; Dong et al., 2019). Human studies have not yet been conducted.

In a recent study utilizing a mouse model of critical limb ischemia (Jin et al., 2021), mice that received ARSs with bFGF in conjunction with periodic applications of ADV displayed significantly better therapeutic outcomes (e.g., increased blood vessel growth, increased perfusion, decreased tissue necrosis, decreased fibrosis) compared with all other experimental groups (**Figure 4**). In another study, focused ultrasound was used to spatially pattern ADV and, hence, the release of bFGF within ARSs. This led to spatially defined patterns of blood vessel formation and host cell migration (Huang et al., 2021). Overall,

Figure 4. *Blood vessel growth and perfusion were stimulated when ADV released basic fibroblast growth factor from an ARS.* ***A:*** *an ARS was implanted in a mouse model of peripheral artery disease. The model involved surgically removing a segment of artery in the leg, thereby causing a dramatic decrease in perfusion. Subsequently, an ARS was placed at the site of vessel removal.* ***B:*** *using a laser-based technique, perfusion in the leg was measured and is displayed as a colormap. Greater perfusion, as seen with the presence of the warmer colors (e.g., **yellow**, **orange**, and **red**), was observed for the ARS+ADV group compared with the group receiving only an ARS, as seen with the presence of the cooler colors (e.g., **blue** and **green**). Reprinted from Jin et al. (2021), with permission from Elsevier.*



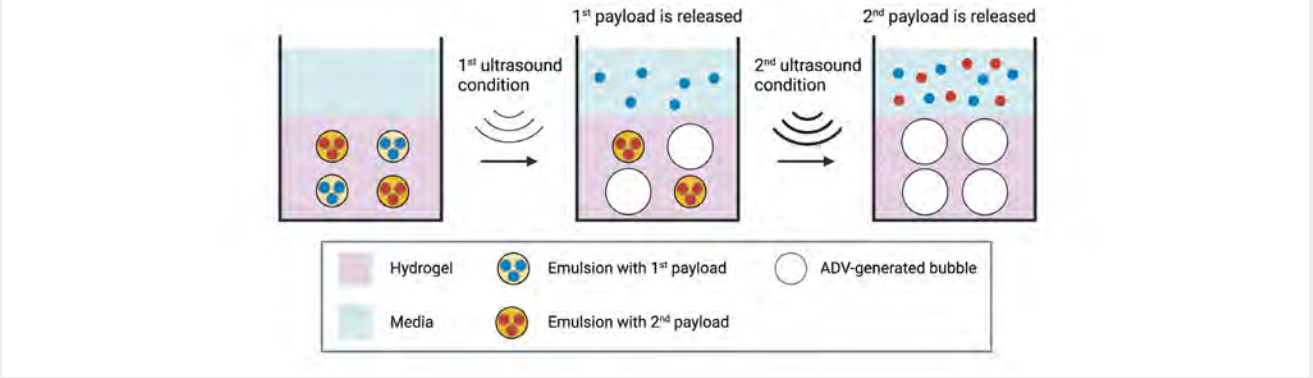


Figure 5. *Two payloads can be sequentially released from an ARS. Each payload is encapsulated within a separate phase-shift emulsion. The first payload is encapsulated in an emulsion with a lower ADV threshold than the second payload. Sequential ultrasound applications of lower and higher amplitudes release the first and second payloads, respectively. Adapted from Moncion et al. (2018), with permission from Elsevier.*

these studies highlight the exciting potential of using ADV and ARSs for stimulating blood vessel growth and in developing new treatments for cardiovascular disease.

Two Can Be Better Than One: Sequential Release Using Ultrasound

Complex, regenerative processes like blood vessel or bone growth require multiple signaling proteins. In addition to their spatial presentation, the temporal sequence of these proteins is critical. For example, bFGF and platelet-derived growth factor BB (PDGF-BB) are both involved in the growth of new blood vessels. bFGF stimulates the initial growth of the blood vessel, particularly the sprouting of endothelial cells that form the inner lining (i.e., lumen) of the vessel. PDGF-BB stimulates other cells to stabilize the outer lining of the vessel, thereby rendering a mature vessel. However, if bFGF and PDGF-BB are present simultaneously, the proteins will inhibit each other, thereby disrupting blood vessel formation (Tengood et al., 2011).

By exploiting the properties of the ADV threshold, ARSs can be designed to enable sequential release of two therapeutic payloads. This involves encapsulating each payload within separate emulsions. The ARS is then exposed to ultrasound at acoustic conditions that will selectively release the first payload without causing release of the second payload. At a later time point, the ARS is exposed to acoustic conditions that release the second payload. This concept has been demonstrated using two strategies: (1) ADV at a single ultrasound frequency (e.g., 2.5 MHz) using two peak rarefactional pressures (e.g., 2 MPa followed by 8 MPa) (**Figure 5**) (Moncion et al., 2018) and (2) ADV at two different ultrasound frequencies (e.g., 8.6 MHz followed by 2.5 MHz) (Aliabouzar et al., 2021). An ultrasound standing wave field has also been used for the sequential release from bilayer ARSs, in which each layer contains a different payload-carrying emulsion (Aliabouzar et al., 2020a).

Use the Force: Control of Biophysical Cues

A cell can sense biophysical cues via receptors linking structural proteins within the cell to the microenvironment surrounding the cell. Cell behavior is significantly impacted by these biophysical cues. For example, mesenchymal stromal cells (MSCs) are cells that can change into more specialized types of cells. When grown on hydrogels, MSCs change into different types of specialized cells based on the stiffness of the hydrogels (Engler et al., 2006). Beyond stiffness, other parameters that impact cellular processes include elasticity, porosity, fiber density, surface roughness, and surface curvature.

ADV enables the spatiotemporal modulation of biophysical properties in ARSs. During ADV, the liquid PFC phase within the phase-shift emulsion undergoes a dramatic increase in volume (up to 125-fold) as it is converted into a gas. Stable bubbles grow further in size due to inward diffusion of dissolved gases from the surrounding environment. In an ARS, stable bubbles remain trapped in the hydrogel matrix, thereby locally impacting both the

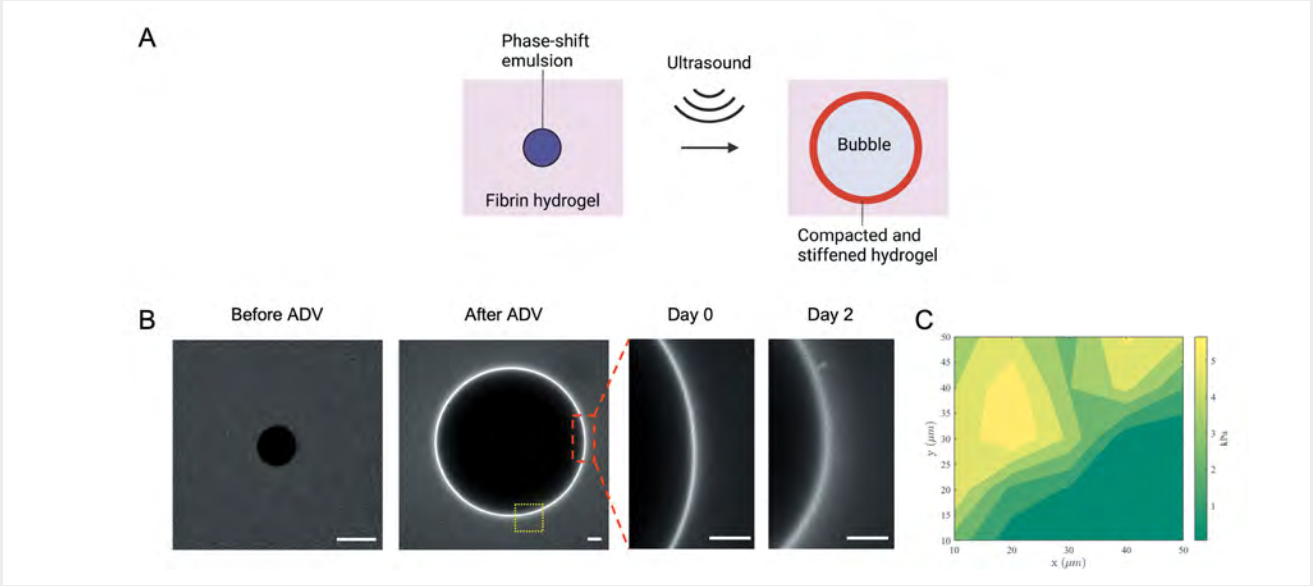


Figure 6. ***A:*** *in a strain-stiffening material like fibrin, ADV locally compacts and stiffens the fibrin matrix surrounding the bubble.* ***B:*** *the fluorescently labeled fibrin matrix was visualized with confocal microscopy. After ADV, the bubble compacted the matrix, causing an increase in fluorescence intensity that persisted over the course of days. Scale bar, 20 μm. Reprinted from Humphries et al. (2022), with permission from Wiley.* ***C:*** *Young's modulus was mapped adjacent to the bubble in a location denoted by* ***yellow box*** *in* ***B****. Note the higher moduli proximal to the bubble (****yellow****) versus the lower moduli distal to the bubble (****green****). Reprinted from Farrell et al. (2022), with permission from Elsevier.*

structural and mechanical properties of the hydrogel (Fabiilli et al., 2013; Aliabouzar et al., 2020b). In ARSs made with fibrin, bubbles radially compacted the fibrin matrix surrounding them while simultaneously increasing its stiffness and decreasing its porosity (**Figure 6**). Fibrin is a protein found in blood clots and is an incredibly biocompatible hydrogel for cells. As bubbles grew in size, there was additional compaction and stiffening of the matrix. In fibrin, matrix compaction leads to an increase in matrix stiffness, a behavior known as strain stiffening.

ADV-induced stiffening can have broad biomedical applications. A recent study investigated the ability to change fibroblasts into myofibroblasts in ARSs (Farrell et al., 2022). Fibroblasts are a common cell type found in connective tissue that can change into myofibroblasts when in a stiffened environment. Cells in stiffened regions of fibrin adjacent to bubbles exhibited more characteristics of myofibroblasts compared with cells in less stiffened regions further away from bubbles. Myofibroblasts play a key role in the repair of connective tissues. Thus, ADV could assist with understanding fibrosis, a disease characterized by the sustained presence of myofibroblasts, as well as developing therapies for chronic wounds that contain insufficient numbers of myofibroblasts. In another study, cellular signaling in a cancer model was modulated in ARSs using ADV (Humphries et al., 2022). Therefore, ADV could help elucidate how biophysical changes to the extracellular matrix impact tumor biology, which could lead to novel treatment approaches.

In contrast to bubbles that grow over time, ADV can also generate liquid-filled pores within ARSs. These pores are generated based on the collapse of the ADV-generated bubble, which causes localized erosion of the hydrogel matrix in the ARS. It has been shown that generation of pores within an ARS, in combination with bFGF release, increased migration of host cells into the implant (Lu et al., 2020). These host cells were cells that were initially surrounding the ARS on implantation. Comparatively, stable bubbles hindered host cell migration into an ARS. Thus, ADV can modulate cell migration, which can assist in directing regenerative processes.

The Next Generation: Three-Dimensional Bioprinting of Acoustically Responsive Scaffolds

The shape of an ARS is dictated by the shape of the container that holds the polymer solution as it is cross-linked into a solid material. Thus, there is a practical limit to what geometries can be achieved, and there is a limited ability to generate complex patterns of emulsions and hydrogel matrices. ARSs with patient-specific geometry (e.g., to fit in a wound area) as well as precise, spatial patterning of the hydrogel matrix and multiple phase-shift emulsions can further advance their applications in TERM. However, developing reproducible ARSs with the above-mentioned features requires advanced fabrication methods beyond conventional bulk polymerization techniques. To do this, 3D bioprinting is used in the development of such ARSs through precise layer-by-layer deposition of the hydrogel component of the ARS or phase-shift emulsions within the ARS based on user-defined computer-aided design.

Using an extrusion-based bioprinting technique, ARSs with spatially patterned phase-shift emulsions were fabricated (**Figure 7**) (Aliabouzar et al., 2022). ADV can be generated at significantly higher spatial resolutions in bioprinted ARSs compared with conventional ARSs. This implies that the ADV-induced modulation of biochemical and biophysical cues could be spatially patterned at higher resolutions with 3D bioprinting. Additionally, bioprinting enabled micropatterning of both phase-shift emulsions and cells in distinct patterns in ARSs.

Bioprinting offers another advantage of fabricating ARSs with different mechanical properties within each layer, which can provide a platform to tune the response of ADV-generated bubbles and, in turn, the associated biophysical and biochemical effects. Overall, integrating ADV with 3D bioprinting, which is incredibly underdeveloped, can open new opportunities in regenerative medicine.

Final Thoughts

Phase-shift emulsions, ARSs, and ADV are tools that can help unravel the complexities of tissue regeneration as well as drive the development of new, regenerative therapies via the modulation of biochemical and biophysical cues. The ability to noninvasively modulate an ARS using ADV in an on-demand, spatiotemporally controlled manner is a dramatic paradigm shift compared with conventional hydrogels widely used within TERM. A better understanding of acoustically driven interactions in the ARS, particularly with cells, will help spur their translational advancement, both within TERM and in other applications.

Acknowledgments

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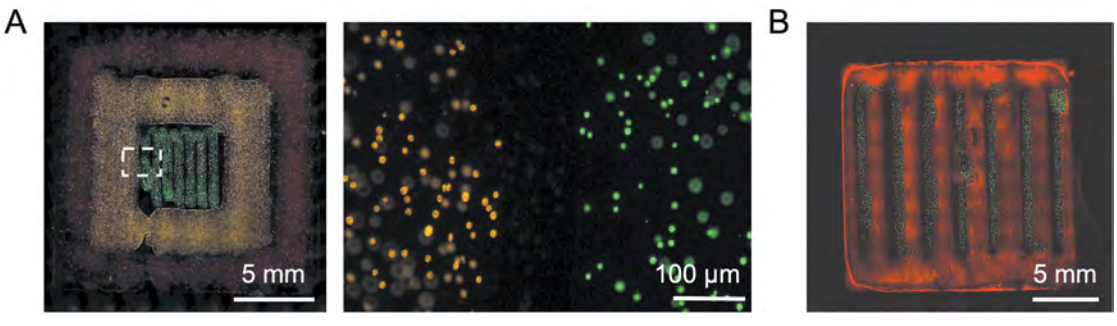
Figure 7. *Three-dimensional (3D) bioprinting generated ARSs with complex structures.* ***A:*** *three phase-shift emulsions with different fluorescent payloads (i.e.,* ***red****,* ***yellow****, and* ***green dextran****) were printed in a hydrogel matrix consisting of alginate and hyaluronic acid (****left****).* ***Right:*** *zoomed-in region of* ***white box*** *on* ***left****.* ***B:*** *reservoirs of emulsion (****green****) were printed in a matrix of fibrin and hyaluronic acid (****red****). Reprinted from Aliabouzar et al. (2022), with permission from Elsevier.*



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