Annals of Biomedical Engineering, Vol. 43, No. 3, March 2015 *(© 2014)* pp. 747–761
DOI: 10.1007/s10439-014-1158-6

Ultrasound Technologies for Biomaterials Fabrication and Imaging

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(Received 8 July 2014; accepted 9 October 2014; published online 18 October 2014)

Associate Editor Rosemarie Hunziker oversaw the review of this article.

Abstract—Ultrasound is emerging as a powerful tool for developing biomaterials for regenerative medicine. Ultrasound technologies are finding wide-ranging, innovative applications for controlling the fabrication of bioengineered scaffolds, as well as for imaging and quantitatively monitoring the properties of engineered constructs both during fabrication processes and post-implantation. This review provides an overview of the biomedical applications of ultrasound for imaging and therapy, a tutorial of the physical mechanisms through which ultrasound can interact with biomaterials, and examples of how ultrasound technologies are being developed and applied for biomaterials fabrication processes, non-invasive imaging, and quantitative characterization of bioengineered scaffolds *in vitro* and *in vivo*.

Keywords—Ultrasound, Imaging, Biomaterials, Scaffold, Tissue engineering, Regenerative medicine.

INTRODUCTION

Acoustic fields at frequencies greater than those audible to humans (i.e., ~20 kHz) are termed ultrasound fields. Biomedical ultrasound is currently used widely in medicine for both imaging and therapy. Ultrasound is most commonly associated with real-time, non-invasive diagnostic imaging. However, ultrasound also provides unique capabilities for a variety of non-invasive therapies, such as physiotherapy, lithotripsy, and bone fracture healing. Although still in developmental stages, new therapeutic applications of ultrasound in the areas of drug delivery, gene therapy, thrombolysis, and non-invasive surgery are now on the horizon.

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Analogous to the diagnostic imaging and therapeutic applications of ultrasound in medicine, ultrasound technologies are finding two new avenues for applications in scaffold fabrication and regenerative medicine (Fig. 1). In one avenue, the ability of ultrasound to generate localized heating and/or mechanical forces is exploited to non-invasively control the structural, mechanical, and biological properties of biomaterials during fabrication. In a second avenue, ultrasound-based imaging and materials characterization techniques provide great potential as unique, non-invasive tools for visualizing the structure and quantitatively characterizing the material properties of engineered tissues and scaffolds. These emerging ultrasound technologies will (i) provide important quantitative tools for monitoring the functionality of biomaterials and engineered constructs in real-time, (ii) offer rapid feedback for optimizing scaffold fabrication and design parameters, (iii) circumvent destructive testing of scaffold and biomaterial products, and (iv) deliver new solutions to current biomaterials fabrication and tissue engineering challenges, such as spatial patterning, drug delivery, and vascularization strategies. Moreover, ultrasound technologies are readily adaptable to both *in vitro* fabrication environments and *in vivo* applications. This review is aimed at providing a broad, multidisciplinary audience with an overview of the existing and potential applications of ultrasound technologies for biomaterials fabrication and imaging.

BIOMEDICAL ULTRASOUND

Ultrasound for Imaging and Therapy

Ultrasound is now an indispensable tool for non-invasive clinical diagnostic imaging. Conventional ultrasound

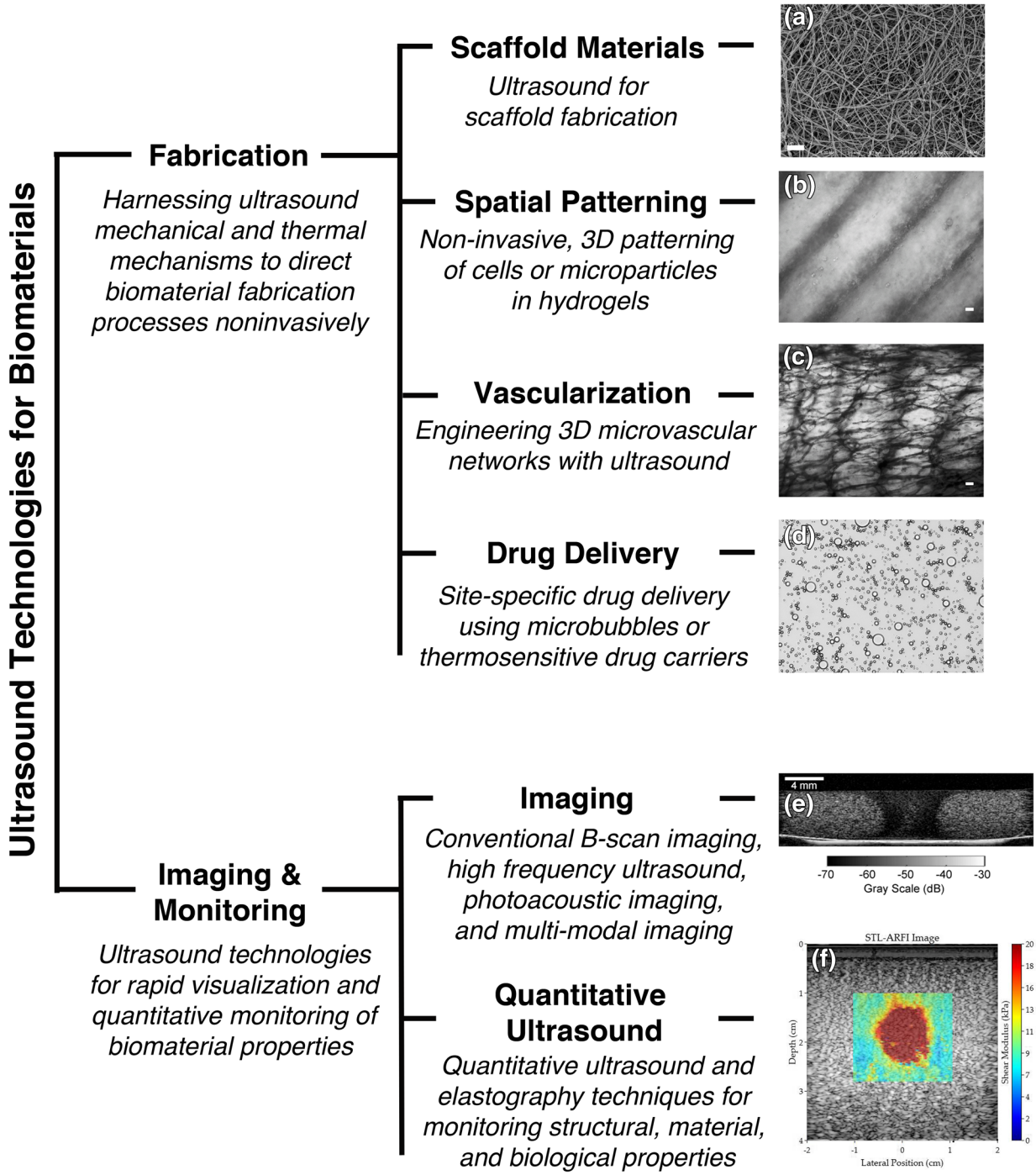

FIGURE 1. Overview of Ultrasound Technologies for Biomaterials Fabrication, Imaging and Monitoring. Illustrated examples include: (a) using ultrasound to produce local changes in collagen fibril structure within 3D hydrogels,[40] as observed using scanning electron microscopy (scale bar = 1 μm), (b) non-invasive patterning of poly(lactide-co-glycolide) (PLGA) microparticles into discretely spaced planar bands using acoustic standing wave fields (scale bar = 100 μm), (c) spatially patterning endothelial cells embedded within collagen hydrogels using ultrasound standing wave fields leads to the formation of 3D microvascular networks[38] (scale bar = 100 μm), (d) targeted and non-targeted microbubble contrast agents can be used to deliver drugs or genetic material with temporal and spatial control, (e) B-scan imaging of collagen hydrogels embedded with cells provides rapid, non-destructive visualization of cell density (scale bar = 4 mm), and (f) regional differences in the shear modulus of soft biomaterials may be quantified and monitored over time using acoustic radiation force imaging.

imaging modalities provide rapid visualization of tissue and biomaterial structures. B-scan imaging is the most widely used ultrasound imaging modality. As ultrasound propagates through a material, reflections arise from discontinuities in acoustic impedance within the material. In B-scan imaging, pulse-echo ultrasound is used to create a

two-dimensional (2-D) gray-scale image of internal structure. Three-dimensional ultrasound (3-D) images can be generated from a series of 2-D B-scan images acquired over a volume. In addition, functional information can be obtained using Doppler ultrasound techniques that allow for the visualization of fluid flow and assessment of tissue perfusion. Conventional ultrasound imaging techniques use frequencies nominally in the 1–20 MHz frequency range. High-frequency ultrasound (typically > 20 MHz) imaging provides enhanced resolution. Acoustic microscopy describes imaging techniques that employ ultrasound at frequencies near or exceeding 100 MHz. Although higher frequencies offer greater spatial resolution, this comes at the expense of a decrease in effective imaging depth because the absorption of ultrasound increases with increasing frequency. Thus, high-frequency ultrasound techniques are best applied for imaging either relatively thin or weakly attenuating samples. Beyond the use of ultrasound imaging for qualitative visualization of structure, ultrasound also offers unique capabilities for non-destructive characterization of biomaterials. Quantitative ultrasound tissue characterization describes a variety of techniques that provide quantitative metrics of the structural, mechanical, and biological properties of biomaterials. Elastography techniques are under rapid development and provide non-invasive, non-destructive estimations of material properties, such as the elastic modulus.[90] The development of sophisticated new imaging modalities continues to enhance the utility of ultrasound as an essential diagnostic imaging tool, and the combination of ultrasound with other imaging modalities offers further unique visualization capabilities.

Interactions of ultrasound with native tissue are also the basis for a variety of non-invasive therapies. As examples, ultrasound is commonly used in physiotherapy, and lithotripsy procedures employ high-amplitude focused acoustic shock waves to fragment kidney stones. High-intensity focused ultrasound (HIFU) can selectively heat and destroy tissue non-invasively and has promising applications for the non-surgical treatment of tumors. These therapeutic applications of ultrasound rely on the ability of ultrasound to focus within tissues and produce a physical effect non-invasively. It is well documented that ultrasound can produce a wide variety of other effects in native tissues, and the acoustic mechanisms responsible for some of these bioeffects have been identified.[20,82] Ultrasound-induced bioeffects *in vitro* and *in vivo* can result from heating or mechanical forces associated with ultrasound propagation.[20,82] The thermal and mechanical mechanisms by which ultrasound interacts with native tissues and biomaterials are discussed in more detail in a later section. Harnessing the ability of ultrasound to exert thermal effects and mechanical forces non-invasively and site-specifically provides exciting opportunities for the development of new ultrasound technologies for biomaterials and scaffold fabrication processes for tissue engineering.

Microbubble Contrast Agents

Ultrasound contrast agents can enhance the capabilities of ultrasound imaging and are also implemented for therapeutic applications of ultrasound.[34,91] Ultrasound contrast agents are gas-filled microbubbles on the order of 1–10 μm in diameter that are stabilized from dissolution through a protein, polymer, or lipid shell. The microbubbles provide effective backscatter to enhance diagnostic ultrasound images. Modern microbubble contrast agents contain gases with low solubility (e.g., perfluorocarbons) and are fabricated with flexible shells.[7,30] Numerous theoretical models have been developed to accurately simulate the dynamic response of microbubble contrast agents to ultrasound exposure.[30,91] Innovative imaging modalities that take advantage of the characteristics of the interaction of the sound field with microbubbles have been developed, including harmonic and subharmonic imaging, coded excitation, destruction-reperfusion imaging, phase inversion imaging, power Doppler, and others.[34,91,110] Targeted microbubbles, produced by attaching ligands to the bubble surface, can provide site-specific localization of contrast agents to cells, proteins, or biochemicals. These agents are being developed for applications in molecular imaging and therapy.[34,42] The interaction of ultrasound with a microbubble contrast agent can produce oscillations of the bubble surface or microbubble rupture. A large body of literature has demonstrated that exposure of microbubbles to ultrasound can produce a variety of effects on cells and tissues *in vitro* and *in vivo*.[21,79,86] Thus, microbubble contrast agents are also finding therapeutic applications in areas including thrombolysis, and localized delivery of drugs or genetic material.[11,31,34,102] Development of novel diagnostic imaging techniques using contrast agents, combined with the emergence of new types of contrast agents tuned for imaging or therapy, promise to expand the utility of contrast agents. Microbubble ultrasound contrast agents are also finding new applications in biomaterials fabrication and imaging processes. Some examples are provided in later sections of this review.

Advantages of Ultrasound for Biomaterials Fabrication and Imaging

A variety of technologies are available for imaging biomaterial scaffolds, including light microscopy, multiphoton microscopy, second harmonic generation microscopy, scanning electron microscopy, confocal reflectance microscopy, magnetic resonance imaging,

optical coherence tomography, and computed tomography. Although each of these imaging technologies provides unique capabilities, there are distinct limitations that can restrict their utility in tissue engineering applications. Microscopic histological analyses and electron microscopy require time-consuming and destructive processing procedures and evaluate only small sections of samples. Many techniques have penetration depths of less than 1 mm and narrow fields of view limiting their ability to image large scaffold volumes. Further, most of these imaging technologies are expensive, often with limited availability.

In comparison, ultrasound has distinct advantages for applications in tissue and scaffold engineering. Ultrasound offers low-cost, portable, real-time imaging capabilities, and it is non-invasive, non-destructive, and non-ionizing. In addition to providing soft tissue contrast, ultrasound also offers the capability for volumetric imaging, visualization of flow and perfusion, and the potential for molecular imaging using targeted contrast agents. Quantitative ultrasound techniques provide non-destructive quantitative characterization of structural, mechanical and biological properties of engineered tissues and scaffolds. Furthermore, ultrasound can be used to monitor engineered constructs during the fabrication process, and can continue to image and quantitatively assess constructs post-implantation.

Similarly, ultrasound-based technologies offer unique advantages to non-invasively direct biomaterial fabrication processes. Ultrasound can exert non-invasive mechanical forces and site-specific heating, thereby providing both thermal and mechanical avenues to control the fabrication of engineered constructs. Ultrasound fields are highly controllable, thus allowing design of optimized exposure scenarios. Importantly, ultrasound can be implemented in sterile environments, incorporated into bioreactor designs, and adapted to large-scale commercial fabrication processes.

ACOUSTIC MECHANISMS AND ULTRASOUND FIELDS

Acoustic Mechanisms of Interaction with Biomaterials

Knowledge of acoustic mechanisms for the interaction of ultrasound with biomaterials is essential to facilitate the design and optimization of new applications of ultrasound for scaffold fabrication, biomaterials imaging and characterization, and tissue engineering. Ultrasound fields can interact with biomaterials and native tissues through either thermal or non-thermal physical mechanisms.[20,82,86] Ultrasound exposures used for diagnostic imaging are designed to minimize the potential for adverse effects on tissues and biomaterials. In contrast, applications of ultrasound for therapy or for control of biomaterials fabrication depend upon direct thermal and/or mechanical interactions of the sound field with the biomaterial to produce the desired effect. Importantly, the ultrasound exposure parameters used for imaging are typically dramatically different from those used for therapeutic or biomaterials fabrication applications.

Absorption of ultrasound in the medium of propagation results in the conversion of ultrasound energy to heat. Ultrasound-induced heating is dependent on both ultrasound exposure parameters and the material properties of the medium of propagation. Ultrasound exposure parameters include frequency, pressure amplitude, intensity, pulse duration, pulse repetition frequency, exposure duration, and beam and scanning configurations. Ultrasound-induced temperature rises are directly proportional to the temporal average ultrasound intensity. Thus, the generation of heat in biomaterials can typically be controlled non-invasively and site-specifically through proper design of acoustic exposure parameters and beam geometry. For example, imaging applications typically employ short pulses (few microseconds) of ultrasound at temporal average intensities less than 720 mW/cm^2. In comparison, applications where significant heating is the desired endpoint, such as high intensity focused ultrasound, may employ continuous wave exposures at intensities in excess of 1000 W/cm^2.

Material parameters are also relevant to the resultant ultrasound-induced temperature rise and include the absorption coefficient, density, specific heat, and perfusion of the biomaterial. The absorption coefficient is a property of the material, and increases with increasing ultrasound frequency. Bone has one of the highest absorption coefficients of native tissues, while the absorption of ultrasound in water is comparatively minimal. As such, for a given ultrasound exposure scenario, ultrasound heating in water-based hydrogels will typically be lower compared to that in other scaffold materials such as ceramics and polymers. Thus, the ability of ultrasound to heat non-invasively can be exploited to control thermosensitive processes site-specifically during biomaterials fabrication. When cells are present, ultrasound exposures must be designed to minimize heat generation in order to avoid adverse or confounding effects on cells.

Acoustic cavitation describes the interaction of a sound field with a gas bubble. Gas nuclei are typically present in fluid media during hydrogel fabrication processes, or can be intentionally introduced into biomaterials by seeding with stabilized microbubble contrast agents. When exposed to an ultrasound field, a gas bubble will oscillate radially around an equilib-

rium radius. Various theoretical models can be employed to describe the dynamic response of a gas bubble to a sound field.[30] Bubble dynamics can be nonlinear and depend on variables including the acoustic pressure amplitude, frequency, initial bubble size, and damping through viscous dissipation, sound radiation, and thermal conduction.[30] The maximum response of a bubble to an ultrasound field occurs when it is exposed at its resonance frequency. The resonance frequency depends on the initial size of the bubble, as well as properties of the gas and surrounding media. At ultrasound imaging frequencies, the radius of resonant bubbles is on the order of a few microns.[30,36,91]

Acoustic cavitation is often categorized as either noninertial or inertial cavitation. Noninertial cavitation describes the repetitive oscillation of a bubble over many acoustic cycles, where the maximum expansion radius does not exceed twice the equilibrium radius over any single acoustic cycle.[82,86] These acoustically driven bubble oscillations can lead to localized heat production, microstreaming of fluid near the bubble, localized shear stresses, and bubble coalescence or attraction of nearby particles or cells to an oscillating bubble.[82,86] Under appropriate exposure conditions, a microbubble may expand to a maximum radius greater than twice its original radius and then collapse to a fraction of the initial radius.[36] This highly nonlinear process is termed inertial cavitation because the collapse dynamics of the bubble are dominated by the inertia of the surrounding liquid.[36] Various physical phenomena can be associated with inertial cavitation. Inertial collapse of a microbubble can produce extremely high temperatures and pressures at the minimum radius of the bubble, and generate acoustic shock waves and free radicals.[30,82,86] Microbubbles near solid boundaries can collapse asymmetrically leading to the formation of high-speed liquid microjets capable of pitting solids, cells, and biomaterials. These phenomena associated with inertial cavitation are spatially localized near the microbubble and temporally limited to the duration of the bubble collapse. Sonochemistry employs inertial cavitation to generate chemical species, or enhance rates of chemical reactions.[3,99] Sonoluminescence[18] describes the generation of light from inertial cavitation activity. In general, the extent of cavitation activity increases with higher pressure amplitudes, longer pulse durations, lower acoustic frequencies, and higher concentrations of microbubbles.

Non-thermal mechanisms for the interaction of ultrasound with biomaterials that are not dependent on the presence of gas bubbles may be grouped as purely mechanical mechanisms.[20,82] Propagation of ultrasound in biomaterials produces localized cyclic variations in pressure, particle displacements, velocities, and accelerations. These first-order forces associated with particle oscillations occur at the fundamental frequency of the sound field. Additionally, compressive, tensile, and shear forces can also be generated by second-order acoustic radiation forces. Acoustic radiation force is a time-averaged force resulting from the transfer of momentum from the sound field to the medium of propagation.[93] In a traveling wave field, radiation forces are in the direction of sound propagation and cycle at the pulse repetition frequency.[93] Radiation forces associated with a standing wave field are more complex and can result in the movement of particles or cells to nodes in the standing wave field.[46–48] Radiation force underlies phenomena such as acoustic streaming, radiation torque, and acoustic levitation.[82] Radiation forces generated from propagation of ultrasound in a fluid medium can produce bulk fluid flow termed acoustic streaming.[85,97] The streaming velocity is dependent on parameters including the absorption coefficient, speed of sound, viscosity, sound intensity, beam geometry, and nonlinear propagation. Radiation torque can produce rotation or spinning of symmetrical particles or the preferential orientation of asymmetrical particles in a sound field.[82] Acoustic radiation forces acting on microbubbles present in an ultrasound field can produce translational displacement of the microbubbles along the axis of sound propagation.[22,23]

Measurement and Characterization of Ultrasound Fields

Design, control, and measurement of acoustic fields are critical for developing and advancing new ultrasound technologies for scaffold biomaterial engineering. Ultrasound exposure parameters and outcomes to measure and report include acoustic frequency, acoustic pressure amplitude, intensity, pulse duration, pulse repetition frequency, exposure duration, spatial distributions of pressure, exposure location in the field, ultrasound-induced temperature rise, and total acoustic output. An excellent review providing guidance on the measurement and reporting of ultrasound exposure parameters is provided by ter Haar *et al.*[101] Poorly characterized or reported acoustic exposures often limit interpretation of results, and may contribute to conflicting reports in the literature. Furthermore, investigations into how ultrasound exposure parameters influence effects on biomaterials often lend insight into understanding the underlying acoustic mechanisms, thereby facilitating optimization of ultrasound fields for biomaterials fabrication.

The environment for biomaterials fabrication (i.e., sample holder material and geometry, presence of liquid media, acoustically reflecting interfaces, *etc.*) can

significantly influence the ultrasound field. Effects of ultrasound that occur *in vitro* cannot be assumed to occur *in vivo*, and vice versa, because the potential for ultrasound-induced heating, cavitation, and radiation forces can be vastly different within *in vitro* environments compared to *in vivo*. For *in vitro* investigations, it is prudent to assume that gas nuclei capable of initiating acoustic cavitation are present in liquid media unless specific efforts have been taken to degas the liquid. Furthermore, acoustic streaming in culture media or other fluids can introduce bulk fluid flow, stirring, and shear stresses that may affect cells, particles, or materials that are responsive or sensitive to shear. The container used to hold the sample during biomaterials fabrication with ultrasound must also be evaluated for its influence on acoustic mechanisms. Holders made of plastics, polymers, or metals, can be heated by ultrasound exposure due to the relatively high absorption coefficients of these materials. Thus, if the holder is within the ultrasound beam width, heat conduction from the holder to the biomaterial sample can produce significant temperature rises and temperature gradients, even within weakly absorbing hydrogel samples. Acoustic reflections from geometric boundaries of sample holders or air interfaces can produce complex acoustic fields within the sample volume. Such reflections can result in the generation of an acoustic standing wave field within the sample. A standing wave field will have regions of maximum pressure amplitude (anti-nodes) and regions of minimum or zero pressure amplitude (nodes), as well as complex acoustic radiation force distributions.

The typical polystyrene well tissue culture plate presents a prime example of how an exposure environment can introduce significant confounding effects on the acoustic exposure.[53,64] A common exposure scenario reported in the literature is the use of an ultrasound transducer coupled directly to the plastic bottom of a well in a tissue culture plate containing some combination of media, cells, hydrogel, and/or other biomaterial scaffold. For this exposure scenario, Leskinen and Hynynen[64] thoroughly characterized the acoustic fields generated within plate wells using laser Doppler vibrometry, Schlieren imaging, and pulse-echo ultrasound. In this configuration, the air/media interface produces a reflection that results in the production of a standing wave field in the volume of the plastic well.[53,64] Reflection and transmission from the plate bottom is strongly frequency dependent because of resonance of the plate.[64] Displacements within the well occur both at the carrier frequency and the pulse repetition frequency of the ultrasound exposure.[64] The height of media in the well, and the distance between the transducer and the well bottom, can significantly alter the acoustic field in the well.[64] Mode conversion and shear wave generation can also occur within the well to further complicate the acoustic exposure.[64] Importantly, acoustic coupling between neighboring wells can lead to the production of acoustic fields in wells that were not the target of ultrasound exposure.[64] Placing the ultrasound transducer directly in the well of the cell culture plate also produces standing waves and complex interference patterns in the sample well.[53] To minimize these confounding effects, ultrasound exposure configurations and sample holders should be designed to minimize reflections and indirect heating from sample holders.[53,64]

ULTRASOUND FOR BIOMATERIALS FABRICATION

The sections that follow provide representative examples of innovative approaches for using ultrasound in biomaterial fabrication processes. Ultrasound technologies are finding applications for scaffold fabrication and functionalization, spatial patterning, microvascular engineering, and drug delivery. Specific examples were chosen to illustrate how acoustic mechanisms of heating, cavitation, and/or acoustic radiation forces can be harnessed to direct biomaterials fabrication processes non-invasively and site-specifically. A review of the effects of ultrasound on cells *in vitro* is not an emphasis of this article.

Interactions with Scaffold Materials

New methods are being sought to enable control over the structural characteristics of biomaterials to improve their biological function. Developing new technologies with the capability to produce biomaterials that recreate the physical and biochemical characteristics of native extracellular matrices with spatial fidelity is essential for the fabrication of advanced biomaterials. Ultrasound fields offer a simple, direct, controllable, non-invasive, and non-toxic energy source to enable such control during biomaterials fabrication.

Ultrasound can propagate as a focused beam with good depth of penetration in tissue-like biomaterials. Design of ultrasound exposure parameters, transducers, and beam and scanning configurations can enable non-invasive control of site-specific heating. Thus, ultrasound technologies that utilize localized heating for scaffold and biomaterials engineering applications are being developed. As an example, exposure of collagen type I to ultrasound during polymerization can be used to non-invasively control collagen fiber microstructure within 3-D hydrogels *via* a thermal mechanism.[40] Regional variations in collagen fiber

microstructure, and associated increases in cell migration, were produced by using a focused ultrasound beam to produce site-specific heating within hydrogels.[40] Other studies have used focused, high intensity ultrasound to apply a thermal stimulus site-specifically in fibrin gels to induce local growth factor release from genetically engineered cells embedded in the gels.[112] These studies highlight the potential of using ultrasound-induced heating to establish signaling gradients within biomaterials and locally regulate cell and tissue function.

Ultrasound can also be used to modify the porosity of engineered scaffolds. The physical phenomena associated with acoustic cavitation can alter scaffold porosity during fabrication processes. These applications typically use high-power, low frequency (~20–40 kHz) sonication systems. For example, ultrasonication increased the porosity of 3-D electrospun nanofiber scaffolds resulting in enhanced infiltration of fibroblasts into the scaffold.[62] Other investigations have demonstrated that exposure of polylactic acid polymer foams to high-power, low frequency ultrasound can increase porosity and pore connectivity within the scaffold.[108,109] Ultrasonication of decellularized porcine patella tendon scaffolds opened spaces between collagen bundles to increase porosity and enhance recellularization.[54]

Sonochemistry techniques, based on the generation of inertial cavtiation using high-power ultrasound, have also been investigated for the synthesis of a wide variety of nanostructured materials.[3,41,115] In sonochemistry, the extremely high temperatures and pressures, acoustic shock formation, and high-speed microjet formation associated with inertial cavitation are exploited to drive chemical reaction processes to synthesize unique materials. Sonochemical techniques can be applied for the fabrication of amorphous nanomaterials, the insertion of nanoparticles into porous materials, the deposition or coating of nanoparticles onto surfaces, and the formation of protein micro- or nanospheres.[41,115] Sonochemically synthesized nanoparticles can be produced in various morphologies including nanotubes, nanorods, nanowires, and solid or hollow nanospheres.[3,25,41,115]

Spatial Patterning

The spatial organization of cells and extracellular matrix proteins within 3-D scaffolds provides critical environmental cues that can induce the self-assembly of cells into functional units.[106] Similarly, spatial and temporal variations in growth factor distribution and activity are essential for tissue formation during development.[106] Thus, the ability to spatially position cells, extracellular matrix proteins, and growth factors in defined 3-D geometries or patterns within a biomaterial is an important step in the development of functional engineered tissues and organs. Examples are provided to demonstrate how spatial patterning can be achieved non-invasively with ultrasound.

Acoustic radiation forces associated with an ultrasound standing wave field have been used to trap, levitate, and organize particles or cells to defined locations in suspending media.[14] An ultrasound standing wave field is generated by the interference of an incident and reflected sound field. Ultrasound standing wave fields are characterized by areas of maximum pressure (pressure antinodes) and areas of minimum pressure (pressure nodes). Pressure nodes are spaced at half-wavelength intervals. Therefore, changing ultrasound frequency will predictably alter the spacing between pressure nodes. Primary acoustic radiation forces associated with ultrasound standing wave fields can actively direct particles or cells to pressure node locations.[46–48] The magnitude of the radiation force depends on both the acoustic exposure parameters and the physical properties of the cells or particles and the suspending medium.[46–48] As such, both acoustic and material parameters will affect the patterning of cells and particles.[46–48] The acoustic radiation forces that generate spatial patterning only exist during the application of the ultrasound standing wave field. Thus, a suspending medium that undergoes a phase conversion from a liquid to a solid state during ultrasound exposure can be used to maintain 3-D spatial organization of cells or particles after removal of the sound field.[4,37,39,44,45,73,95]

Ultrasound standing wave fields have been used to spatially pattern cells and microparticles in a variety of applications. As examples, standing wave fields have been used to pattern mammalian cells in collagen hydrogels,[37,39] yeast or red blood cells in agar, alginate, or polyacrylamide gels,[44,45] and acrylic particles in polysiloxane resin.[95] Patterning with ultrasound standing wave fields has been applied to a variety of cell types, including fibroblasts,[39] endothelial cells,[37] neural cells,[5] chondrocytes,[61] and hepatocarcinoma cells.[67] Ultrasound-induced patterning has been shown to enhance cell–cell contact[61,67] and promote cell-mediated collagen reorganization.[39] Ultrasound standing wave fields can control the location and manipulation of cells within microfluidic devices,[111] and can produce localized banding of red blood cells *in vivo*.[28] Although radiation forces exerted on proteins are too small to directly organize proteins, ultrasound standing wave fields have been used to spatially localize cell-bound fibronectin within

3-D collagen hydrogels.[39] Furthermore, different pattern geometries, such as planar bands[39] or concentric cylinders,[61,73] can be produced through design of ultrasound standing wave fields and acoustic sources. In general, ultrasound standing wave fields and exposure parameters are highly controllable, thus allowing design of optimized exposure scenarios for spatial patterning.

Another approach to ultrasound-based spatial patterning of cells combines an acoustic droplet ejection technique with aqueous two-phase exclusion patterning.[32] This technique employs acoustic radiation forces exerted on an air–liquid interface to produce liquid droplets that are ejected from the liquid surface. The droplets are deposited on a plate, and controlled 2-D translation of the plate results in spatial patterning of droplets on the plate. This technique has been used to spatially pattern dextran droplets containing fibroblasts, and can be applied for patterning multiple cell types.[32]

Vascularization

Creating functional microvascular networks within engineered scaffolds and tissues is critical to the fabrication of a wide range of functional tissue constructs. To date, technologies with the capacity to produce functional, small caliber vascular networks within 3-D tissue constructs are limited in number and remain in the early stages of development.[2] Our group has developed the use of ultrasound standing wave fields as a non-invasive method for patterning endothelial cells within 3-D collagen hydrogels. We have shown that acoustic radiation forces associated with ultrasound standing wave fields can rapidly and non-invasively organize endothelial cells into distinct multicellular planar bands within 3-D collagen gels.[37] Ultrasound-induced patterning of endothelial cells accelerates the emergence of capillary-like sprouts, induces collagen fibril alignment, and results in the maturation of sprouts into lumen-containing, branching vessel networks throughout the complete volume of the collagen construct.[37,38] Importantly, the rate of microvessel formation and the morphology of microvascular networks can be controlled by the ultrasound standing wave field pressure amplitude utilized during fabrication.[38] The use of ultrasound standing wave fields for microvascular tissue engineering has several distinct advantages as it is non-invasive, rapid, inexpensive, applicable under standard tissue culture protocols, can be adapted to various dimensions, and does not reduce cell viability.

Drug Delivery

Developing new strategies to enable site-specific drug delivery is a focus area in scaffold and biomaterials engineering. Ultrasound technologies present attractive approaches to enable non-invasive control of drug release both spatially and temporally. Acoustic cavitation, radiation force, and ultrasound-induced heating can be used alone or in combination to engineer new approaches for drug delivery.

Sonoporation describes the transient increase in membrane permeability that can occur in response to ultrasound. Mechanical activity associated with acoustic cavitation is the primary mechanism for sonoporation, therefore the presence or addition of microbubbles can enhance sonoporation efficiency.[118] Sonoporation of the cell membrane can deliver material intracellularly,[24] while permeabilization of the microvasculature can be effective for site-specific drug delivery to tissue.[76,80] A wide body of literature has shown that sonoporation can be an effective method for localized delivery of drugs or genetic material.[11,15,31,34,65,102,104] Design of ultrasound exposure parameters can affect sonoporation efficiency and cell viability.[31,56,89]

Microbubble contrast agents can also be engineered specifically for use as drug delivery vehicles.[11,65,102,104] In these applications, microbubbles are used to carry a drug to a specific location, and spatial and temporal targeting is achieved by using focused ultrasound to rupture the microbubbles and release the drug. Additionally, the use of targeting ligands on the surface of microbubble contrast agents can be employed to deliver drugs to specific cell types.[11,102] Furthermore, primary acoustic radiation forces directed on microbubbles can produce translation of the microbubbles in the direction of sound propagation.[22,23] Acoustic radiation force has been used to push microbubbles to microvessel walls where they can be ruptured to produce local drug delivery.[23,71,94]

Ultrasound can also enable local drug delivery *via* thermal mechanisms. Ultrasound provides an effective means for inducing local hyperthermia. In combination with thermosensitive drug carriers, ultrasound-induced hyperthermia can produce localized heating thereby enabling site-specific drug release. Ultrasound is advantageous because it is non-invasive, focused beams provide excellent spatial resolution, and scanning of the ultrasound beam can be employed to heat larger volumes. Ultrasound-induced hyperthermia has been shown to be effective in activating localized drug release from temperature-sensitive liposomes both *in vitro* and *in vivo*.[26,50,57,72] Others have combined ultrasound-induced hyperthermia with inertial cavitation activity from added microbubbles as an approach

to allow targeted drug delivery with temperature-sensitive carriers.[49,117]

Lastly, ultrasound fields have also been employed to noninvasively direct the controlled release of drugs from engineered biomaterials. Ultrasound-based approaches provide the opportunity to control drug delivery both spatially and temporally. For example, ultrasound exposure significantly increased the release of a test protein from a biodegradable, microporous polymeric implant.[1] Similarly, ultrasound has been utilized to noninvasively control the release of an antibiotic from a polymer hydrogel.[84] Ultrasound-induced heating, cavitation activity, and/or fluid streaming can provide mechanisms for the controlled release of drugs from biomaterials.

ULTRASOUND FOR IMAGING AND BIOMATERIALS CHARACTERIZATION

Although widely used for clinical imaging, ultrasound-based imaging technologies are finding new in-roads for imaging biomaterials for tissue engineering applications. Conventional ultrasound imaging modalities provide rapid visualization of scaffold and tissue structure. High-frequency ultrasound provides enhanced resolution, and the combination of ultrasound with other imaging modalities offers unique visualization capabilities. Quantitative ultrasound and elastography techniques allow for quantitative characterization of material parameters and biological properties of engineered constructs. Representative examples of how these ultrasound imaging and quantitative ultrasound tissue characterization techniques are finding new applications in biomaterials and tissue engineering are provided in the following sections.

Ultrasound Imaging of Engineered Constructs

As described previously, conventional B-scan imaging can provide real-time visualization of the internal structure of biomaterial constructs. For B-scan imaging, frequencies employed typically range from ~1 to 20 MHz, with corresponding wavelengths in water of ~1.5 mm to 75 μm. B-scan imaging (12 MHz) was used to visualize, and the mean gray-scale values of the images were used to monitor, the phase inversion process of an *in situ* forming drug delivery implant comprised of poly(lactic-co-glycolic acid) (PLGA) polymer and 1-methyl-2-pyrrolidinon.[96] Kreitz *et al.*[60] used 13-MHz B-scan ultrasound to image fibrin gels embedded with myofibroblasts over an 18-day period and were able to correlate mean ultrasound gray-scale value with hydroxyproline content in the gels. Pulse-echo ultrasound has been employed to evaluate cell density in β-tricalcium phosphate scaffolds seeded with bone marrow stromal cells.[87]

High-frequency ultrasound (nominally > 20 MHz, resolution ~20–100 μm)[81] offers increased spatial resolution and is advantageous for imaging small-scale structure within biomaterials. High-frequency B-scan (30–40 MHz) can provide qualitative images to visualize cell-embedded hydrogels,[77] as well as collagen fiber structure within acellular collagen gels.[78] High-frequency ultrasound has also been used as a tool to image agarose hydrogels containing varying concentrations of collagen and chondrocytes.[55] Acoustic microscopy at 61 MHz was employed to create B-scan images to monitor the growth and progression of oral mucosal engineered constructs.[113,114]

Multi-modal approaches that combine B-scan imaging with advanced ultrasound imaging techniques or other imaging modalities can provide enhanced capabilities for visualization of engineered constructs. Gessner *et al.*[43] combined three ultrasound modalities to image and characterize decellularized liver scaffolds; high-frequency gray-scale imaging was employed to visualize scaffold structure, dynamic contrast-enhanced perfusion imaging was used to spatially map perfusion, and a novel acoustic angiography technique was developed to visualize microvasculature structure. In another investigation, high-frequency ultrasound (40 MHz) was combined with time-resolved fluorescence spectroscopy and fluorescence lifetime imaging microscopy to characterize changes in extracellular matrix deposition during chondrogenic differentiation of stem cells on PLGA scaffolds.[35] Similarly, time-resolved fluorescence microscopy and high-frequency ultrasound imaging have been combined to evaluate tissue engineered articular cartilage.[98]

Photoacoustic imaging can be combined with ultrasound imaging and/or other imaging modalities to provide structural and functional information, and when combined with nanoparticle targeting agents can provide cellular and molecular imaging capabilities.[29] In photoacoustic imaging, the absorption of pulsed laser light in tissue produces local thermoexpansion leading to the generation of acoustic transients, which are received with an ultrasound transducer at the sample surface and converted into an image.[29] Photoacoustic imaging is finding applications in scaffold and tissue engineering, as illustrated with the following examples. Photoacoustic microscopy has been used to image engineered scaffolds comprised of carbon nanotubes embedded in PLGA polymer *in vitro* and *in situ*,[8,100] and photoacoustic microscopy combined with optical coherence tomography has been able to image microvasculature in polymer scaffolds implanted in mice.[9] Combined photoacoustic imaging

and ultrasound imaging has been used to image and monitor adipose-derived stem cells, loaded with gold nanosphere tracers, cultured in PEGylated fibrin gels over a 16-day period.[13]

Quantitative Ultrasound for Biomaterials Characterization

Advancing the use of engineered scaffolds and tissues for clinical applications requires technologies that can non-destructively quantify the structural, material, and biological properties of the construct both during fabrication and post-implantation. Conventional B-scan imaging provides a rapid tool to visualize scaffold or tissue structure. However, B-scan images are highly dependent upon system settings and therefore only provide qualitative images. B-scan ultrasound does not provide quantitative, system-independent metrics to characterize material properties because gray-scale images are dependent on system parameters, such as the frequency response of the transducer, attenuation, receiver gain, and other system settings.[69] Therefore, images collected with one imaging device and operator cannot be compared quantitatively with other imaging systems.

Quantitative ultrasound tissue characterization describes a variety of measurement and signal processing techniques designed to extract information from radio frequency (RF) ultrasound echo signals in order to characterize the tissue or biomaterial. Quantitative ultrasound parameters that are often employed include speed of sound, absorption and attenuation coefficients, backscatter coefficient, nonlinearity parameter, integrated backscatter coefficient, spectral slope and midband fit, and angular scattering. Many ultrasound tissue characterization techniques are under development for a broad range of normal and diseased native tissues. While ultrasound tissue characterization methods face challenges *in vivo*, due to unknown attenuation, beam distortion, and other factors, the application to engineered tissues *in vitro* can be an ideal measurement situation. Sound speed and attenuation may be accurately determined by transmission or plate reflector methods and phase aberration may be minimal. Unlike B-scan imaging, quantitative ultrasound approaches are independent of the ultrasound system and can provide quantitative metrics for non-invasive, non-destructive materials characterization. Thus, they provide the capability for comparison of measurements between different ultrasound systems, different operators, and for monitoring scaffold development over time.

Quantitative ultrasound techniques are finding new applications in biomaterials characterization for tissue engineering, as demonstrated with the following examples. Measurements of the speed of sound in agarose hydrogels were used to estimate elastic moduli of these gels using elastic and poroelastic models.[107] The speed of sound and frequency-dependent attenuation of high frequency ultrasound have been tested as metrics to characterize the influence of hydrogel scaffold degradation on constructs consisting of chondrocytes encapsulated in polyethylene glycol hydrogels.[92] Another quantitative ultrasound parameter, high-frequency ultrasound backscatter, can distinguish between living, dead, and apoptotic cells.[19,59] A technique based on the integrated backscatter coefficient (IBC) has the capacity to noninvasively estimate cell concentration within cell-embedded hydrogel constructs, and parametric imaging of the IBC can provide quantitative visualization of regional differences in cell concentration within hydrogels.[77] The IBC technique can also detect, quantify and visualize changes in collagen fiber microstructure in 3-D collagen hydrogels that can result from different collagen polymerization conditions.[78] Additional spectral parameters of the backscatter, such as the spectral slope and midband fit, can provide useful metrics to characterize engineered constructs. Spectral slope, midband fit, and signal statistics can detect apoptosis and structural changes in cells.[59,68,70,103] Gudur *et al.*[51] employed the midband fit and spectral slope to evaluate mineral content of collagen hydrogels fabricated with hydroxyapatite. The combination of speed of sound, attenuation, midband fit, and spectral slope has been used to quantify cell number, scatterer size, and monitor osteoblastic differentiation in engineered constructs.[52]

Monitoring the fabrication of engineered scaffolds and tissues requires techniques to measure their mechanical properties, such as modulus, viscoelasticity, elastic nonlinearity, and material anisotropy. Standard mechanical testing methods are destructive procedures and cannot be adapted for longitudinal monitoring of individual samples, or for quantifying mechanical properties *in vivo*. Ultrasound elastography describes a variety of ultrasound-based technologies for quantifying the mechanical properties of tissues non-invasively and non-destructively.[90] There are numerous elastography approaches under development, and the techniques generally differ in the methods used to produce and detect tissue displacement.[90] In quasi-static elastography techniques, a small compression is produced in the sample and the axial component of the displacement is tracked using RF signals from B-scan imaging.[88,105] Dynamic elastography approaches employ harmonic or transient sources to produce shear waves. Sonoelasticity[63] uses an external vibration source to generate low frequency shear waves in tissue. Doppler ultrasound is then employed to detect the resultant strains in the sample

to estimate shear modulus. Magnetic resonance elastography (MRE) employs magnetic resonance imaging to detect tissue displacements. Other approaches (e.g., acoustic radiation force impulse imaging (ARFI),[83] spatially modulated ultrasound radiation force (SMURF),[75] acoustic vibroacoustography,[33] shear-wave dispersion ultrasonic velocity (SDUV),[12] and supersonic shear wave imaging[6]) use focused ultrasound to generate an acoustic radiation force in order to displace a local tissue volume. Diagnostic imaging techniques are then used to track the resultant tissue displacements in order to provide estimates of shear modulus. A large body of literature demonstrates the capability of elastography techniques to estimate the elastic modulus for a wide variety of native tissues, and numerous studies indicate that elastography can be instrumental in detecting diseases that are associated with changes in local tissue stiffness, such as cancer and liver fibrosis.[10,90]

Elastography techniques are also being applied to estimate mechanical properties of biomaterial and engineered tissues non-destructively and non-invasively. Spatially modulated ultrasound radiation force (SMURF) imaging was used to estimate shear moduli of hydrogel constructs, and good agreement was found between SMURF estimates and destructive mechanical testing results.[74] ARFI was used to estimate elastic moduli of thin hydrogels on rigid substrates, as may occur when engineered tissue constructs are fabricated within molds.[66] Ultrasound elastography has been used to monitor the mechanical strength of engineered arterial constructs during fabrication.[27] Others have demonstrated the feasibility of quasi-static elastography to monitor the degradation of a biomaterial scaffold (poly(1,8-octanediol-*co*-citrate)) both *in vitro* and *in vivo*.[58] Quasi-static elastography was also able to monitor non-invasively changes in the mechanical stiffness of three types of porous biodegradable polyurethane scaffolds over a 12-week period post-implantation.[116] A high-resolution elasticity imaging microscope has been developed[16,17] and demonstrated as a useful tool under conditions relevant for tissue engineering applications.[17]

SUMMARY

In summary, ultrasound offers unique capabilities for scaffold fabrication and tissue engineering. Technologies that take advantage of the ability of ultrasound to interact with biomaterials through thermal and/or mechanical mechanisms provide innovative approaches to control biomaterial fabrication processes non-invasively and site-specifically. Ultrasound-based imaging modalities provide capabilities for real-time, volumetric visualization and quantitative monitoring of engineered tissue constructs during fabrication and post-implantation. Innovative ultrasound technologies promise to provide exciting opportunities for advancing the fields of biomaterials and tissue engineering.

ACKNOWLEDGMENTS

The authors thank Dr. Stephen McAleavey, Eric Comeau, and Karla Mercado (University of Rochester) for providing images.

CONFLICT OF INTEREST

No conflicts of interest exist.

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