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Ultrasound-induced cavitation: applications in drug and gene delivery

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Ultrasound, which has been conventionally used for diagnostics until recently, is now being extensively used for drug and gene delivery. This transformation has come about primarily due to ultrasound-mediated acoustic cavitation – particularly transient cavitation. Acoustic cavitation has been used to facilitate the delivery of small molecules, as well as macromolecules, including proteins and DNA. Controlled generation of cavitation has also been used for targeting drugs to diseased tissues, including skin, brain, eyes and endothelium. Ultrasound has also been employed for the treatment of several diseases, including thromboembolism, arteriosclerosis and cancer. This review provides a detailed account of mechanisms, current status and future prospects of ultrasonic cavitation in drug and gene delivery applications.

Keywords: cavitation, chemotherapy, gene delivery, microbubble, protein delivery, sonodynamic therapy, targeted delivery, thrombolysis, transdermal delivery, ultrasound

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1. Introduction

Ultrasound is a sound wave with frequencies beyond human audible range (> 20 kHz) and has a long history of use in diagnostic applications. Diagnostic ultrasound (2 – 15 MHz) capitalises on transmission and scattering of sound waves while minimising absorbance in other tissues enroute. However, over the last two decades, there has been an unprecedented increase in efforts to facilitate the interaction of ultrasound with cells and tissues and use it for therapeutic applications. For example, therapeutic ultrasound (0.75 – 5.0 MHz) is now an established clinical tool in physical therapy, which employs localised heating of injured tissues. Furthermore, non-thermal ultrasonic effects, which are induced by acoustic cavitation, enhance drug transport in tissues and cells. Cavitation-induced interactions of ultrasound with cells and tissues have been transformed into many interesting applications, including, but not limited to, transdermal drug delivery, gene therapy and microbubble-assisted *in vivo* targeted drug delivery. Consequently, the relevant literature pool has rapidly grown and the high scientific interest in this field is evident by the fact that ~ 50% of the scholarly articles have come about in the past 5 years. This article reviews the underlying mechanisms, current status and future prospects of the role of ultrasonic cavitation in drug and gene delivery applications.

2. Cavitation: the primary mediator of drug and gene delivery

The primary effect of ultrasound waves is to physically perturb the material medium of passage. Ultrasound causes the exposed material to vibrate at an amplitude dependent on the material's compressibility. For densely packed materials, ultrasound is expected to produce low displacement, but for compressible gas bubbles, ultrasound results in high displacements and strains per unit ultrasonic energy applied. Indeed, ultrasound leads to the formation, pulsation and collapse of vapour or gas cavities in materials (predominantly liquids) – a phenomenon

referred to as cavitation. Although acoustic cavitation has long been studied [1], its use in therapeutic applications is a relatively new field of research.

2.1 Transient and stable cavitation

Depending on the oscillatory behaviour of bubbles under acoustic field, cavitation can be broadly classified into two types: transient and stable [2]. These forms of cavitation reflect two extreme cases of bubble dynamics and several intermediate forms, but some forms that fall in both or neither types also exist [3]. In a stricter sense, stable cavitation can be defined as uniform periodic pulsation of bubbles over relatively long intervals of time, as opposed to transient cavitation where short-lived bubbles grow rapidly, often expanding up to two orders of magnitude in size, before undergoing a violent collapse. The extent of cavitation and its nature (stable or transient) depends on several ultrasound parameters, including frequency and pressure amplitude. Lower frequencies or higher pressure amplitudes decrease the threshold size of a bubble that can undergo transient cavitation, and, hence, these ultrasonic parameters are ideal for drug delivery applications.

Transient cavitation events, which last only for a fraction of a second, can have considerable impact on the surrounding tissues or cells. Instantaneous release of highly concentrated energy at the collapse centre causes localised increase in temperature (10^4 – 10^6 K), which leads to the emission of chemically reactive free-radical species and short light pulses (a few hundred picoseconds long) – a phenomena known as sonoluminescence [4]. More importantly, such dissipative collapses produce shock waves, with initial velocities as high as 10^3 m/s and pressure amplitudes exceeding 10^4 atmospheres [5]. If transiently cavitating bubbles collapse near solid boundaries (such as a tissue interface), they can generate microjets travelling at high speeds (~ 100 m/s) towards the rigid surface [6]. Applications of ultrasound in drug delivery, chiefly use these secondary effects of ultrasound (shock waves and microjets) to disrupt biological barriers in a controlled manner. However, it is noteworthy that it is difficult to induce cavitation in dense tissues, hence, ultrasonic delivery applications often rely on external introduction of cavitation nuclei (naturally occurring gaseous cavities in drug solutions or artificial microbubbles). Drug molecules, which are either encapsulated in microbubbles or dissolved in liquid carrying bubbles, can then be efficiently delivered to the surrounding tissue or cells of interest.

The overwhelmingly dominant effects of cavitation, especially those of the transient type, tend to overshadow the other effects of ultrasound exposure on biological systems. Pressure fluctuations imposed by the ultrasound wave have been observed to produce Reynolds stresses in the exposed medium. These pressure gradients in the medium can generate sustained fluid motion, referred to as acoustic streaming [7]. Stably oscillating bubbles, especially those near solid interfaces, can generate small-scale vigorous circulating fluid motions near the bubble surface [8]. This phenomenon, referred to as microstreaming, leads to the formation of convective eddies, which can induce severe shear rates in the order of 10^7/s in proximal tissues [9], and distort neighbouring structures [10]. Although streaming flows, in their own capacity, may assist in dispersion and disruptive infusion of drug molecules in tissues, their strength is several orders of magnitude smaller than shock waves and microjets, which occur during transient cavitation.

2.2 Measurements of cavitation

Traditionally, the parameters for measuring the success of therapeutic efficacy by ultrasound have been related to the basic physical parameters of ultrasound, such as frequency, pressure amplitude, exposure time, duty cycle or the total energy dose [11-13]. However, with increasing evidence for the role of transient cavitation in ultrasound-mediated drug delivery, efforts have been focused on measuring cavitation activity using markers such as acoustic spectrum or sonoluminescence and relating it to biological effects.

A typical acoustic emission spectrum consists of peaks at specific frequencies that are harmonics or subharmonics of the driving ultrasound frequency, and broadband noise that spans the full frequency domain. It is suggested that the broadband noise arises due to the emissions from rapidly changing bubble radii, whereas the subharmonic emissions originate from the prolonged expansion phase and a delayed collapse of oscillating bubbles [3]. Both types of emissions have been reported in the case of transient cavitation, but there is growing support for using broadband noise as an indication of the onset of transient cavitation [14-16]. Amid evidence of subharmonic emissions during transient cavitation, several acoustic studies have also implied a lack of congruence between the two [14,17-19].

Various studies have tried to relate the biological effects of ultrasound to both subharmonic and broadband emissions. For example, Sundaram *et al.* showed that for 3T3 mouse cells, cell viability and uptake of a model dye at several ultrasound frequencies (20 – 100 kHz) correlated best with the magnitude of broadband noise, and no correlation was found with subharmonic emissions [20]. A similar hypothesis has also been put forward in other *in vitro* studies [21,22]. At a tissue level, the effects of ultrasound on transdermal delivery of drugs have been shown to correlate best with broadband noise [23-25]. In another application, it was asserted that ocular delivery of drugs monotonically increased with the broadband noise [26]. On the contrary, there is some evidence in support of subharmonic emissions with ultrasound's biological effects. In two separate studies, controlled cell membrane permeabilisation and consequent haemoglobin release from red blood cells *in vitro* was correlated with the pressure amplitude of the first subharmonic and other ultra-harmonics [27,28]. Strong correlations were also found between cell damage and the emitted total subharmonic energy [29-31]. Similarly, permeabilisation of the blood–brain barrier was shown to correlate well with the second and third harmonics of the driving frequency [32].

Other methods for quantifying transient cavitation have also been reported in the literature. Sonoluminescence was

found to correlate with ultrasonic bioeffects on prostate cancer cells *in vitro* [33]. In another study, free-radical generation due to ultrasonic cavitation was shown to match well with the debilitating effects on tumour growth [34]. Interestingly, cavitation-induced pit-like depressions on soft aluminum foil (held in contact with ultrasonic coupling medium) has been also used a physical marker to quantify the number of cavitation events [35,36].

3. Therapeutic applications of ultrasound

The interactions of cavitation bubbles with biological systems are chiefly responsible for a large number of biomedical applications. In this section these remarkably diverse applications are discussed by dividing them into four broad categories: transdermal drug delivery, gene delivery, delivery into tissues and targeted delivery.

3.1 Transdermal drug delivery

The resistance to drug delivery through skin is due to its uppermost layer, the stratum corneum. This layer possesses a robust structural organisation, similar to bricks (corneocytes) and mortar (intercellular lipid bilayers), and allows delivery of only a handful of low molecular weight (< 500 Da) and highly lipophilic molecules in small doses [37]. Various mechanical [38,39], electrical [40,41] and chemical [42] approaches have been used to breach the skin barrier. Ultrasound has also been shown to enhance the permeability of the skin – a phenomenon termed as sonophoresis or phonophoresis. In a history spanning more than five decades [43], a relatively broad spectrum of ultrasound frequencies (20 kHz – 6 MHz) have been used for transdermal drug delivery. However, in the early- to mid-1990s low-frequency ultrasound (20 – 100 kHz) was shown to be more effective compared with high frequencies, and was shown to deliver high molecular weight drugs, including proteins and nucleotides [37,44-46]. Improved efficacy of low-frequency ultrasound over higher frequencies originates from enhanced transient cavitation in the coupling medium (the liquid present between the ultrasound transducer and the skin). A combined theoretical and experimental approach has shown that the shock waves and microjets generated by transient cavitation can induce strains capable of disrupting the structure of lipid bilayers [35].

Microscopic histology studies *in vitro* [35] and *in vivo* [47,48] showed that stratum corneum retains its microstructure after sonophoresis, with no observable detrimental effects on either corneocytes or the lipid bilayers. However, detailed studies using fluorescence and electron microscopy demonstrated that ultrasound induces nano-scale ultrastructural defects in the lipid regions [49]. The effects of ultrasound on skin structure were highly heterogeneous and manifested themselves as dilated void spaces (referred to as lacunar spaces, ~ 10^2 nm in dimension) between lipid bilayers, which probably led to a connected three-dimensional tortuous drug transport pathway.

Ultrasound, especially under low-frequency conditions, has also been used in applications beyond classical transdermal drug delivery. It has been used for extraction of analytes from the skin for diagnostic purposes [50-52]. Enhanced skin permeability after sonophoresis allows extraction of various solutes, including albumin, calcium, urea, triglycerides, lactate and dextran [50,51]. This opens up the possibility for using ultrasound in the treatment of diabetes by simultaneous continuous monitoring of body glucose level and delivery of insulin [44]. Low-frequency ultrasound has also been proposed for transcutaneous immunisation. Skin is an attractive site for vaccination due to the presence of Langerhans cells and other sentinels of the immune system. Pre-exposure of ultrasound followed by topical incubation of tetanus toxoid resulted in robust immune responses in mice, compared with a simple topical application of the vaccine [47]. Low-frequency ultrasound was also employed to deliver therapeutically significant quantities of antisense oligonucleotides into the skin [53].

Sonophoresis also works synergistically with many other dermal enhancement methods, such as chemical enhancers [36,54,55], iontophoresis [55] and electroporation [56]. The most notable of these synergistic combinations involves the use of sodium-lauryl-sulfate (typically mixed at 1% w/v in the coupling medium), which was shown to reduce the threshold ultrasound energy density needed to permeabilise the skin by 10-fold, compared with ultrasound alone [36].

3.2 Gene delivery

A significant focus of current gene therapy research is on effectively and selectively delivering therapeutic genes to diseased tissues in the body. A number of viral and non-viral approaches have been proposed, but they suffer from limitations, including safety concerns for viral vectors and low expression for non-viral carriers [57,58]. As an alternative, ultrasound has also been used to introduce genes in cells through a phenomenon commonly referred to as sonoporation. Sonoporation is the transient permeabilisation of cell membranes, which enables loading of macromolecules [11,20,59-61]. As the primary cause behind sonoporation is cavitation [62], ultrasound-mediated gene therapy often uses stabilised microbubbles as cavitation nuclei to enhance gene transfer to cells. Microbubbles can be surface-functionalised for highly localised and selective delivery of macromolecules *in vivo*. In this mode, ultrasound offers spatio–temporal control for gene therapy, where microbubbles carrying or mixed with therapeutic genes can be localised and then sonicated for triggered delivery of genes in the tissue of interest.

Ultrasound-mediated gene therapy has been successfully performed, both *in vitro* and *in vivo*. Broadly, *in vitro* experiments have been mainly designed to: i) provide proof of principle for the use of ultrasound in various cell types; ii) optimise ultrasound parameters; and iii) to advance fundamental understanding of intracellular trafficking and kinetics of gene expression. However, *in vivo* studies have largely

focused on demonstrating the applicability of ultrasound in various tissues and animal types.

The literature for sonicated gene delivery finds its origin as early as 1987, when murine fibroblast cells were transfected using a crude tissue-homogenisation sonicator device [63]. Later, in a significant contribution, successful transfection of cultured cells was demonstrated using both continuous and pulsed-wave 1 MHz ultrasound [64]. Since then, a flurry of reports have come about, demonstrating the use of ultrasound at various combinations of frequency (e.g., 24 kHz [12], 500 kHz [12], 1 MHz [64-67] and 2.5 MHz [68,69]), pressure amplitude (mechanical index: 0.1 – 5.0) and duty cycle (pulsed/continuous exposure), to enhance gene expression in several mammalian cell types, including tumour cells [66,67,70-72]. Gene transfection by lithotripter-induced high pressure shock waves has also been shown [66,70,73]. Interestingly, delivery of DNA vectors condensed by cationised polymers [74] and lipids [65,75,76] with ultrasound has also been reported. This is especially advantageous in the wake of direct evidence of DNA degradation due to ultrasound exposure, which following lipid sequestration, is effectively prevented [77].

Having illustrated the beneficial effects of ultrasound in gene delivery, it is incumbent to identify key challenges that this treatment methodology faces. First, successful gene delivery to cells inversely correlates with cell lysis, which, in some cases, has been reported to be as high as 80% [67]. The issue of high toxicity has been addressed to a certain extent by optimising ultrasound parameters, for example, low-intensity continuous wave 1 MHz ultrasound has been reported to increase gene expression with no cytotoxic effects [65]. In another study on prostate cancer cells, the use of high-frequency ultrasound (500 kHz) with contrast agents at high cell concentration (10^7/ml) and 37°C was recommended to achieve best gene transfection *in vitro* [12]. The second major limitation is lack of understanding of the underlying mechanisms in ultrasonic gene therapy. Zarnitsyn and Prausnitz offered some insight by showing that only a fraction of cells with ultrasonically inserted DNA exhibit transfection, which implies the presence of additional transport hurdles [12]. These include cytoplasmic DNA degradation, slow kinetics of perinuclear DNA accumulation, or nuclear entry of the gene. In a recent report, confocal microscopy showed that 1 MHz ultrasound was able to deliver naked-DNA plasmids directly to the nucleus of adherent hamster kidney cells [78]. The nuclear entry of plasmid, which only occurred during extended ultrasound exposure (30 min), was followed by quick protein expression. This is in agreement with a similar report on fast protein expression kinetics after exposure of cells in suspension to 2.25 MHz ultrasound; however, no nuclear entry was reported [69]. Although facilitation of direct nuclear entry of plasmid by ultrasound is an interesting finding, this does not preclude the possibility that it is easier to target the nuclei in flat, adherent cells, compared with that in three-dimensional cells under *in vivo* conditions or in suspension. Overall, there is a great need to advance the mechanistic understanding of DNA trafficking in cells exposed to ultrasound.

Amid considerable recent interest, *in vivo* ultrasonic gene therapy has been demonstrated in several tissue types, including muscles (e.g., myocardium in heart, skeletal muscle, quadriceps), vascular endothelium, fetal tissue, lungs and tumours. This is not surprising, as ultrasound offers a distinct advantage in site-specific gene delivery. Many different protocols have been attempted for ultrasound-mediated *in vivo* gene delivery. In a typical setting, DNA mixed with microbubbles is directly injected to tissues, where subsequent sonication leads to their delivery. Although application of ultrasound without microbubbles also induces some enhancement of gene delivery, the presence of cavitation nuclei leads to a significant enhancement in gene expression. For example, intratumoural injection of DNA plasmids followed by application of focused ultrasound led to a 15-fold increase in protein expression [71]. With a similar method, Manome *et al.* also reported a threefold enhancement in transfection in colon carcinoma tumours implanted in mice [72]. However, when 10% air was co-injected with DNA in tumours and followed by lithotripter exposure, a many-hundred-fold increase in gene expression was observed [79]. Taniyama *et al.* also reported high enhancements in ultrasonic gene delivery by injection of microbubbles and naked-DNA plasmid in pretibial (skeletal) muscle [80]. Along similar lines, gas-filled microbubbles have been extensively used in ultrasonic gene therapy in the vasculature. In this application, genes mixed with contrast agents are introduced into the microvasculature and ultrasound is applied to disrupt microbubbles in order to locally deliver the genes. Examples include two separate reports for the treatment of restenosis, where ultrasound exposure to Optison™ contrast agent mixed with antioncogene p53 [81] or E2F decoy oligodeoxynucleotides [82] was able to partially treat carotid artery injury. In an innovative use of a balloon angioplasty catheter, adenovirus mixed with albumin microbubbles were delivered to the aortic root of rats, which, following sonication, showed improved gene expression [83]. Perhaps the most appealling mode of ultrasonic gene therapy is delivery through gene-encapsulated microbubbles. This topic is discussed in detail in Section 3.4.4. Other appealing modes include endovascular ultrasonic catheters for gene delivery to arteries [84], aerosolised ultrasonic delivery of DNA to lung epithelial cells [85,86] and delivery of lipid-based [74,76] and polymer-based [76,87,88] DNA complexes.

3.3 Transport to tissues

Ultrasound has been used for delivery of drugs to several tissues other than the skin. Ultrasound-induced disruption of the blood–brain barrier has been used for drug delivery to the brain [89-91]. Ultrasound has been shown to induce reversible and non-destructive openings of the blood–brain barrier by disruption of tight junctions present between the capillary endothelial cells [91]. In separate studies, explicit use of cavitation-inducing microbubbles was reported for non-invasive disruption of the blood–brain barrier [32,92]. Ultrasound has also been used to enhance the diffusion of a chemotherapeutic

drug (carmustine) in brain tissue *in vitro* for the treatment of brain tumours (S Paliwal and S Mitragotri, unpublished data). Pulmonary drug delivery approaches have been facilitated by the use of an ultrasonic nebuliser. Ultrasound has been used to aerosolise therapeutics including DNA–lipid complexes [85,86]. This technique has been especially advantageous for delivering surfactant-stabilised proteins [93], which are otherwise difficult to administer by other delivery routes [94,95]. Ultrasound (20 – 880 kHz) has also been proposed for enhancing ocular drug delivery by increasing the transcorneal permeability of drugs. Preliminary experiments, performed using 20 kHz ultrasound, resulted in a fourfold increase in the corneal permeability for β-blocker drugs *in vitro*; however, significant disorganisation of the epithelium was observed [96]. Later studies, which used high-frequency ultrasound at low energy settings, observed up to 10-fold enhancement of transcorneal permeability to a hydrophilic dye [26]. Transient corneal pits due to cell erosion from the surface epithelium were observed, which recovered within 90 min, and no structural changes were observed in the stroma [26,97]. Recently, ultrasonic destruction of intravenously injected microbubbles loaded with luciferase protein was shown to deliver high quantities of the protein to the heart [98]. The treatment showed a sixfold enhancement in protein delivery over controls [98].

3.4 Targeted delivery

The combined use of ultrasound and microbubbles has provided the impetus for targeted drug delivery to different portals in the body. Dissolution of clots in blood vessels, precise nucleotide delivery and localised activation of drugs are among a few areas of intense research. The promise of microbubble-mediated delivery systems arises from several reasons. First, the physical wave-like nature of ultrasound makes it a very adaptable tool that is capable of being extracorporeally operated, penetrable at various depths, being able to be focused at specific regions in the body and adjustable in acoustic intensity and frequency to fit the needs. Second, microbubbles, particularly at low ultrasound frequencies and high intensities, can be imploded at desired locations to selectively permeabilise the biological membranes for drug delivery to the surrounding tissue. Third, microbubbles can be designed so as to carry therapeutic drugs in their shells. Finally, targeting can be achieved by incorporation of tissue/disease-specific antigens on the microbubble surface. Collectively, these features create a favourable scenario, where the adverse effects of drugs such as systemic toxicity, rapid clearance and degradation can be minimised.

Microbubbles have received special attention for intravenous delivery of therapeutics. Ultrasonic destruction of microbubbles has been shown to create extravasation pores in microvasculature (blood capillaries with diameters < 7 μm) [99]. High delivery efficacy of this methodology is evident from the fact that a small extent of capillary rupture is required to deliver large quantities of macromolecules through the microvasculature. Specifically, using polymeric microspheres, it was shown that penetration depths in the order of hundreds of microns into the parenchyma can be achieved [100].

Before discussing various applications of targeted delivery, it is worthwhile to briefly touch on the targeting tools that these applications use. Acoustically responsive microbubbles, which were primarily designed for improving the image quality of diagnostic ultrasound (first application in 1968 [101]), are now the primary tool for ultrasonically targeted drug delivery. The second-generation microbubbles use gases other than air, such as perfluorocarbons, which decrease their solubility and improved their stability *in vivo*. Stabilisation of bubbles by albumin, dextrose or galactose palmitic acid, as well as inclusion of cationic materials such as polymers or phospholipid [102] on the bubble surface, has also improved stability and circulation lifetime of bubbles *in vivo*. Microbubbles are predominantly gaseous entities; therefore, their ability to encapsulate therapeutic molecules is limited. However, the availability of charged shells has enabled bubbles to avidly bind small chemotherapeutic drugs [103], proteins [104], and genetic materials [105]. Furthermore, the third-generation microbubbles feature inclusion of site-specific ligands on their surface. Examples include charge-coupled or direct incorporation of cell-specific adhesion molecules and antibodies (e.g., P-selectin [106] and intercellular adhesion molecule-1 [ICAM-1] [107]) in coated shells and techniques to directly bind ligands to shell components through avidin–biotin-type covalent linkages [108,109].

3.4.1 *Targeting endothelium*

Certain disease markers are upregulated on the surface of endothelial cells and, hence, can be used for targeted drug delivery [110]. Possible pathologies include angiogenesis in solid tumours, atherosclerotic plaques and inflammation [109]. For example, ICAM-1 selectin is upregulated in the areas of the endothelium proximal to early atherogenesis events [111]. Therefore, it has been envisioned that surface-functionalised drug-bearing microbubbles can be used for early diagnosis and simultaneous treatment of diseased endothelium. Villanueva *et al.* prepared lipid shells coated with anti-ICAM-1 antibody, and demonstrated *in vitro* targeting of IL-1-activated coronary artery endothelial cells [112]. Demos *et al.* used acoustically reflective liposomes conjugated to ICAM-1 antibody for targeting atherosclerotic plaques *in vivo* [113]. The case of inflamed vasculature is particularly interesting. Inflammation response is initiated by recruitment of leukocytes at the site, thus resulting in enhanced adhesion of endothelial cells with leukocytes. It was discovered that albumin-coated microbubbles can recruit serum complement proteins and attach to activated leukocytes. Making use of this special characteristic of albumin microbubbles, it was successfully demonstrated *in vivo* that inflammation can be easily diagnosed [114,115] and targeted by delivery of drugs either in the presence [116] or absence of ultrasound [117].

3.4.2 Inducing arteriogenesis

Occlusive vascular disease is marked by inadequate blood flow to organs often leading to malnutrition and ischaemic conditions, particularly involving the myocardium and skeletal muscle tissue. Various approaches for targeted stimulation of neovascularisation have been pursued in the past, including delivery of growth factor proteins or genes [118] and more relevantly, by inducing inflammatory response in the affected vasculature [119]. Ultrasound was hypothesised to initiate angiogenic pathways implicated in inflammation through implosion of microbubbles in ischaemic tissues [120]. Exposure of 1 MHz ultrasound to intravenously injected microbubbles resulted in increased arteriole density, arteriole diameters and nutrient blood flow into the treated rat skeletal muscle [120]. With a similar procedure, microvascular remodelling response was observed in a rat model of arterial occlusion [121]. In a recent mechanistic study, the therapeutic potential of this strategy was shown to originate from the recruitment of growth factor-producing local inflammatory cells [122]. Interestingly, a combined treatment of ultrasound-triggered arteriogenesis and transplantation of bone-marrow-derived mononuclear cells facilitated blood flow restoration by stimulating both angiogenesis and arteriogenesis in an ischaemic rat model [123,124].

3.4.3 Ultrasonic vascular thrombolysis

Arterial thrombosis refers to the formation of insoluble fibrinous clots, due to deposition of atherosclerotic material in the wall of blood vessels, which can cause obstruction in the supply of oxygen and nutrition to an area in the body. Consequently, thrombosis has been implicated in a myriad of circulatory disorders, such as myocardial infarction, non-haemorrhagic stroke and pulmonary embolism. Drug-induced thrombolysis, where clogged vessels are infused with serine proteases for vessel recanalysation, has had limited success and suffers from slow action and low penetration in the clots [125]. Ultrasound (20 kHz – 3 MHz), either by itself or in synergy with clot-dissolving enzymes, has been proposed as a treatment strategy. Although ultrasound-enhanced convective transport (acoustic streaming [126]) of drugs can facilitate clot dissolution, transient cavitation has been demonstrated to be the major mediator of ultrasonic thrombolysis [127]. Cavitation mediates thrombolysis through two effects: first, changes in fibrin ultrastructure makes the clot amenable to further disintegration [128] and, second, cavitation enhances transport of proteolytic agents such as urokinase and tissue plasminogen activator deeper into the clot [129-133]. Wu *et al.* developed a novel targeted approach for thrombolysis using functionalised phospholipid-coated microbubbles carrying a ligand capable of targeting glycoprotein-IIb/IIIa, a receptor expressed in high density on activated platelets in thrombi [134]. Using this strategy, extensive clot lysis was also achieved *in vivo* [134-136]. In an alternative approach, a biotin–avidin–biotin bridge was used to bind a biotinylated lipid-perfluorocarbon microemulsion to avidin-(biotinylated antibody)-fibrin sequence for targeting thrombosis sites [137].

3.4.4 Targeted gene therapy

The beneficial effects of ultrasonic gene therapy in the context of introducing genetic material *in vivo*, either exclusively or as a mixture with contrast agents, was reviewed in Section 3.2. However, such a strategy of injecting genes in unbound form suffers from many drawbacks such as toxicity, gene expression in undesired tissues and low transfection efficiency due to degradation of genes by extracellular enzymes. With the advent of new techniques to incorporate DNA in microbubbles, a targeted approach to ultrasonic gene delivery is feasible. Not only do such ultrasound-responsive gene vehicles avoid the aforementioned limitations, but they enable effective spatial targeting of genes through their cavitation-mediated disruption near the tissue of choice. Binding of therapeutic nucleotides to microbubbles has long been established, with the first conclusive evidence reported approximately a decade ago [105]. It was demonstrated that perfluorocarbon-exposed albumin microbubbles have bioactive albumin on their surface that can bind synthetic antisense oligonucleotides and then release them to tissues in the presence of an acoustic field [116]. Shohet *et al.* prepared adenoviral microbubbles by simple incubation of reporter-gene-containing virus particles with perfluoropropane-filled albumin microbubbles. After intravenous infusion of these novel gene carriers in rats, they showed that ultrasonic destruction of microbubbles near the heart induced specific gene expression in rat myocardium [138]. Later, attaching naked DNA to microbubbles, the authors confirmed *in vitro* [139] as well as in an *in vivo* model [140], that plasmid transgene expression can be obtained with even higher specificity than viral vector. Christiansen *et al.* reported successful transfection of rat hindlimb skeletal muscle by ultrasonic exposure of intra-arterially administered cationic lipid microbubbles with plasmid attached on the bubble surface through charge-mediated interactions [141]. A great extent of myocellular microporations were observed in the treated muscles. Cationic lipid-shelled microbubbles bound to plasmid DNA were also exploited to deliver genes to canine myocardium [142] and recently for myocardial angiogenesis by expression of human VEGF [143]. Using an innovative double-emulsion technique, biodegradable polymer-based echogenic microspheres carrying reporter plasmid were prepared. Polymeric microspheres were locally delivered into the left femoral arterial walls of pigs and high-frequency ultrasound exposure generated enhanced gene expression [144].

3.4.5 Targeted protein delivery

The motivation for new methods of protein delivery derives from the inherent difficulties associated with proteins, such as low diffusion coefficients due to large molecular weights and sensitivity of their structure to the environment. Many attractive strategies involving protection of proteins by novel

materials, followed by triggered-release through ultrasound, have been tried. One such technique employs the use of polymeric microspheres encapsulating proteins, which can be released following ultrasound-induced polymer ablation. In 1934 Szalay first demonstrated that the viscosity of solutions of some natural polymers can be decreased after treatment with ultrasound [145]. However, the effect of ultrasound on protein release from polymeric implants was reported much later [146]. Using a biodegradable microporous copolymer, Agrawal *et al.* confirmed this phenomenon *in vitro* [147]. Kwok *et al.* used an innovative dual-encapsulation approach, where insulin-containing polymeric depot was again coated with an ultrasound-responsive C-18 alkyl chain coating, thus opening the possibility of triggered pulsatile protein release [148]. Another approach for triggered protein delivery is the use of microbubbles. Intracellular delivery of ribosome-inactivating protein toxin was shown *in vitro* with the use of lithotripter-induced shock waves [149]. Mukherjee *et al.* showed in an *in vivo* study that ultrasonic destruction of growth-factor-loaded microbubbles can deliver proteins to the myocardium [104]. In a comparable setup, intravenous delivery of angiogenesis-promoting protein was shown in a canine model of chronic myocardial ischaemia [150].

3.4.6 Targeted chemotherapy

Ultrasound is now an established therapeutic tool for addressing the challenges posed by barriers to delivery of drugs to cell and tissues. Several mechanisms have been proposed, including, generation of radicals, hyperthermia and cavitation-induced transient opening of cell membranes (which represents the dominant pathway) [151]. A large body of *in vitro* and *in vivo* literature exists on enhanced uptake of unbound chemicals into mammalian cells [152,153], and, especially tumour cells [154-158], in the presence of ultrasound. Similarly, ultrasound potentiates the effect of antibiotics on bacteria in culture [159-161] and in animals [162,163]. Ultrasound has also been used for selective delivery of conjugated drugs to their targets, avoiding the harmful side effects. Along the same lines, new delivery vehicles such as drug-loaded micelles, polymer microspheres and liposomes have been used in conjunction with ultrasound. Polymers present at high concentrations can form polymeric micelles enclosing drug within them [164]. In one of the first studies on this topic, Pluronic™ micelles carrying an anticancer drug (doxorubicin), was shown to be highly cytotoxic to cancer cells following ultrasound-triggered release [165]. Using microscopy and flow cytometry studies, it was shown that sonication of micelles enhanced drug loading in cells [166]. In a series of *in vitro* and *in vivo* tumour-model experiments, Nelson *et al.* and Rapoport *et al.* demonstrated the high efficacy of this methodology [167-169]. In a related study, new acoustically responsive micelles that are thermodynamically stable below the critical micellar concentration have been designed [170]. Ultrasound has also been used to trigger the release of drugs from liposomes [103,171]. Bednarski *et al.* prepared MRI-detectable liposomes [172] and Unger *et al.* [103] took advantage of an acoustically active liposphere, to release pharmaceutics from liposomes. Recently, ultrasound has been shown to selectively induce cytotoxicity in skin and prostrate cancer cells *in vitro* in the presence of a common dietary supplement, quercetin [173]. Interestingly, ultrasound in synergy with quercetin was demonstrated to target heat shock proteins with higher specificity in tumour cells, which resulted in cancer-specific cytotoxicity [173].

3.4.7 Sonodynamic therapy

Ultrasound has also been used for targeted activation of chemotherapeutic drugs (sonosensitisers) – a treatment referred to as sonodynamic therapy. The sonochemical effects such as production of free radicals during transient cavitation events have been hypothesised as the mechanisms behind sonodynamic therapy. A dynamic interplay of sonosensitisers with cavitation can activate sonosensitisers either inside or at the gas/solution interface of violently collapsing hot gaseous cavities [174]. A variety of free radicals have been implicated, including reactive hydroxyl radical and hydrogen atom through water pyrolysis, reactive singlet molecular oxygen in the presence of photosensitisers and sonosensitiser-derived alkoxyl and peroxyl radicals [174,175]. The latter have been suggested as the key radical species, primarily due to its longer lifetime and ability to diffuse longer distances, which is much needed for cytotoxic interactions [176]. Free radicals have been shown to induce membrane defects in cells exposed to sonodynamic therapy [151]. However, confusion persists in the literature regarding experimental differentiation of mechanisms by which acoustically active chemotherapeutics exert their cytotoxicity. Although enhanced intake of drugs in cells by means of sonoporation has long been established, it is the sonochemistry of sonosensitisers that makes them special in targeted chemotherapy applications. Sonodynamic therapy has been proposed to enhance the toxicity of many compounds, including several anticancer drugs [177], porphyrin-based photosensitisers [178,179], and others [174]. In an interesting application to target sonodynamic therapy towards cancer cells, a sensitising agent was linked to tumour-targeting antibody [177]. *In vivo* administration of this novel drug conjugate followed by ultrasound application yielded significant inhibition of tumour growth [177]. In another animal study, the sonodynamically activated antitumour effect of porfimer sodium was reported on mammary tumours in rats [180]. Several additional reports documenting similar effects of ultrasound and drugs have been also published [174].

4. Conclusion

Ultrasound is set to have a big impact on modern healthcare, not as a conventional diagnostic tool, but as a means for delivering difficult-to-administer therapeutics to several portals of the human body. This has been possible due to ultrasound-mediated acoustic cavitation, which can be remotely incited in the diseased tissue for targeted and non-invasive

drug delivery. One of the major therapeutic areas where ultrasonic drug delivery has shown immense potential is in the treatment of cancer. Several different approaches for ultrasonic treatment, including direct cell lysis, enhanced drug permeability, localised activation of drug molecules or priming malignant cells towards chemotherapy, have been successfully demonstrated. However, in order to transcend ultrasonic therapies from laboratory-scale studies to clinical practice, more success in the *in vivo* setting, followed by rigorous safety studies, is an imperative.

5. Expert opinion

The safety of ultrasound is critical for wide acceptance of ultrasonic treatment modalities in healthcare. However, ultrasound-induced tissue damage is a potential concern for the use of ultrasound in drug delivery applications. The challenge lies in that the tissue/cellular cytotoxicity stems from the very reason that dictates the success of ultrasound, that is, cavitation. Although controlled cavitation activity can transiently permeabilise cells and tissues for delivery of therapeutics, uncontrolled cavitation may lead to extensive destruction and severe necrosis in the affected tissue/cell. Several studies have reported on using optimal ultrasound parameters to strike a balance between therapeutic effectiveness and cytotoxicity. However, the results from such studies are system-specific, and their extrapolation to other experimental conditions using different transducer designs or tissue types is limited. Further, many studies do not quantitatively capture the toxic effects of cavitation, which makes it difficult to draw generalised conclusions. Some consideration has been given to develop parameters depicting the measure of cavitation activity, such as cavitation dose; however, their usage still remains largely conceptual. There is a need to identify parameters that would accurately describe quantitative estimates of cavitation events, which could then be correlated with the observed end points of ultrasound therapy. Theoretical models of cavitation-induced bioeffects will prove an important step in this direction. However, modelling of multiple cavitation events, even in a cell-free medium (*in vitro*), has proved challenging. Performing such a task under *in vivo* conditions is even more challenging. This is largely due to the complex nature of interactions (e.g., bubble–bubble, bubble–shock wave and bubble–ultrasound) that play an important role. In our opinion, advancement in modelling approaches to describe multiple cavitation events will prove beneficial for precise control of ultrasonic drug delivery, with minimal safety issues.

Many applications of ultrasound-mediated therapies have been tested in humans and some have been approved by the FDA (transdermal drug delivery and thrombolysis to name a few). However, most other applications are in early animal studies, and some at an *in vitro* level. Several challenges need to be overcome to advance these studies to humans, and eentually into routine clinical use. Scaling up from animals to humans is a significant challenge due to prominent differences in human and animal physiology.

There are also several application-specific issues that need further consideration, some of which are presented here. For example, the primary challenge for ultrasound gene therapy is improved transfection efficiency, especially under *in vivo* conditions. This challenge originates largely from the difficulty in generating sufficient cavitation in tissues other than blood. For microbubble-related applications, the challenges are related to their design: i) incorporation of novel ligands through conjugation strategies; ii) effective protection for structure-sensitive therapeutics (including proteins and genes); and iii) long circulation and protection from opsonisation. Further, intracellular uptake of microbubbles has been previously shown; hence, from a safety viewpoint, it becomes critical to avoid unnecessary destruction of bubbles in non-targeted cells.

Finally, the diverse set of ultrasonic drug delivery applications demands an equally diverse range of sonication devices, varying from bulky transducers for direct transcorporeal exposure, to tiny devices for endoscopic applications. With the advent of advance materials, superior fabrication technologies and novel compact designs, we envisage that futuristic transducer designs will come in a wide array of sizes, geometry, acoustic frequency, sound intensity, beam width and other relevant parameters. With the recent advances and the ever-increasing multidisciplinary approach in ultrasound research, we are hopeful that the challenges discussed here will be successfully addressed in future.

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