Ultrasound-enhanced drug delivery for cancer

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Introduction: Ultrasound, which has traditionally been used as a diagnostic tool, is increasingly being used in non-invasive therapy and drug delivery.
Areas covered: Of particular interest to this review is the rapidly accumulating evidence that ultrasound may have a key role to play both in improving the targeting and the efficacy of drug delivery for cancer. Currently available ultrasound-triggerable vehicles are first described, with particular reference to the ultrasonic mechanism that can activate release and the suitability of the size range of the vehicle used for drug delivery. Further mechanical and thermal effects of ultrasound that can enhance extravasation and drug distribution following release are then critically reviewed.
Expert opinion: Acoustic cavitation is found to play a potentially key role both in achieving targeted drug release and enhanced extravasation at modest pressure amplitudes and acoustic energies, whilst simultaneously enabling real-time monitoring of the drug delivery process. The next challenge in ultrasound-enhanced drug delivery will thus be to develop a new generation of drug-carrying nanoparticles which are of the right size range for delivery to tumours, yet still capable of achieving initiation of cavitation activity and drug release at modest acoustic pressures and energies that have no safety implications for the patient.

Keywords: adenovirus, cancer, cavitation, ultrasound therapy, virotherapy

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1. Introduction of therapeutic ultrasound

The intense research which has advanced the utility of ultrasound for diagnostic imaging has also more recently helped redefine and expand its role, so that it may now be considered one of the most promising therapeutic modalities under development.

Therapeutic ultrasound may be considered to be any application of ultrasound which instigates a direct improvement in disease outcome or enhances the activity of a co-applied conventional therapy. A huge range of exposure conditions and applications are therefore covered by this definition. These include the use of high-intensity focussed ultrasound (HIFU) for thermal ablation of diseased tissue [1,2], cataract treatment by phacoemulsification [3], and the break-down of calculi such as kidney stones and gall stones so that they can be passed from the body, a process known as lithotripsy [4]. In addition, low-intensity ultrasound is widely used for stimulating tissue and bone repair [5] and to reversibly disrupt the blood–brain barrier [6]. Therapeutic ultrasound is also known to enable targeted drug delivery [7,8] and localised enhancement of drug activity for applications ranging from sonothrombolysis for stroke therapy [9] to cancer therapy [8]. It is this final application and the underlying therapeutic ultrasound mechanisms responsible that will be the focus of this review.

Ultrasound is the alternation between compressional and rarefactional pressure fluctuations with a frequency greater than 20 kHz. Therapeutic ultrasound tends to use frequencies in the range 0.5 – 5 MHz [1]. A key advantage of using

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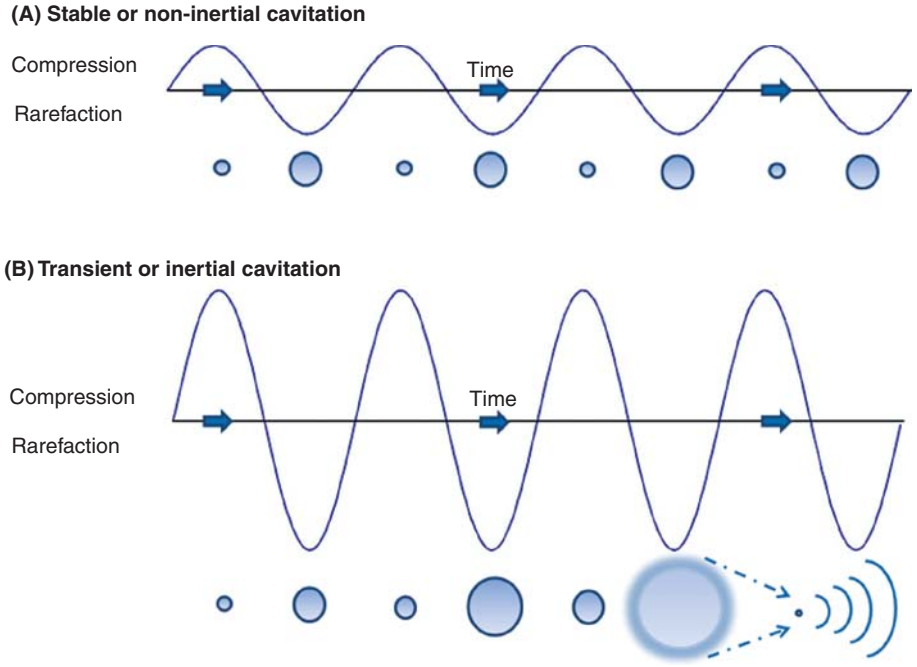

Figure 1. Schematic diagram illustrating the effects of acoustic fields of identical frequency but differing pressure on microbubble behaviour. A. Under stable cavitation, microbubbles oscillate periodically between small and relatively larger size under compression and rarefaction, respectively. **B.** In the case of inertial cavitation, the higher peak-to-peak pressure causes microbubbles to collapse very rapidly to a small fraction of their initial volume.

therapeutic ultrasound is its ability to exert a specifically localised effect from the surface of the skin up to 15 – 20 cm into the body. Such non-invasive and targeted application contrasts markedly with surgical intervention and chemo- and radiotherapy which have a high potential to damage neighbouring healthy cells. Lower-frequency ultrasound waves give a greater penetration depth in soft tissue but are less focused; for instance, 1 MHz ultrasound waves will propagate to more than 12 cm within the body, whilst 4 MHz waves will become heavily attenuated within 3 cm [1].

Depending on the exposure conditions used (e.g., frequency, pressure, duration) ultrasound can instigate three different categories of effects that can be used to enhance drug delivery: thermal, cavitational and acoustic radiation force effects [10-13].

Thermal effects arise from the absorption of the sound waves by tissue and can be used to achieve temperature increases that are moderate enough to cause no direct tissue damage but are sufficient to give release of drug from thermoresponsive carrier systems [7,8].

Cavitation effects are instigated by acoustic excitation of microbubbles present within the target tissue [10,12]. These microbubbles may be spontaneously nucleated by ultrasound in the tissue or may be introduced by injection in the form of ultrasound contrast agents (UCAs) [14]. Originally developed to enhance contrast in diagnostic ultrasound imaging, UCAs are now being applied to provide 'cavitation nuclei'. These inert gas-filled bubbles are of a diameter ranging from 0.5 to 10 μm, and are coated to prevent bubble dissolution and thereby improve stability *in vivo* [15]. However, the half-lives of these microbubbles within the bloodstream are around 1 min [16,17], indicating multiple injections may be required for sustainable inertial cavitation in *in vivo* experiments. There are many commercially available and biodegradable microbubbles, including Albunex (air in albumin shell), Definity (perfluoropropane in lipid shell), Imagent (perfluoropropane in lipid shell), Levovist (air inside galactose shell), Optison (perfluoropropane inside albumin shell), Sonazoid (perfluorobutane inside lipid shell) and SonoVue (sulphur hexafluoride inside lipid shell). Importantly, because UCAs are very strong scatterers of ultrasound, they can be detected and mapped non-invasively using conventional B-mode ultrasound, even before they are excited using therapeutic levels of ultrasound for drug delivery [18,19].

The response of microbubbles to acoustic excitation is termed acoustic cavitation, and two qualitatively different behaviours can be discerned: inertial (formerly referred to as transient) and non-inertial (formerly known as stable) cavitation [8,20]. Non-inertial cavitation (**Figure 1A**) occurs when bubbles oscillate linearly or non-linearly about their equilibrium radius over several acoustic cycles without collapsing. The rarefaction phase of an ultrasound wave causes the microbubbles to expand, whilst the compression phase makes the microbubbles contract. Stable cavitation has been found to

result in microstreaming, i.e., enhanced momentum transfer to the surrounding liquid that can enable the micropumping of drugs [8,21]. Inertial cavitation (**Figure 1B**) occurs when the pressure amplitude becomes sufficiently large to initiate unstable bubble growth during the rarefactional half-cycle of the ultrasound wave, resulting in microbubble expansion typically greater than a factor of 2; this catastrophic growth results in a very rapid and violent collapse during the compressional half-cycle, under the effect of the inertia of the surrounding liquid [8]. Inertial cavitation tends to generate heat, free radicals, shock waves, shear forces, and microstreaming [22]. Microstreaming is believed to be the main driving force used to deliver therapeutic agents deep into solid tumours [23].

The third potential mechanism for drug delivery, acoustic radiation force, results directly from the energy lost by the ultrasound wave as it propagates through tissue and is absorbed and scattered by particular structures [24]. This results in a net force which tends to push particles away from the ultrasound transducer enhancing extravasation, convection, and diffusion through tumour matrix [12,13]. It should be noted that acoustic radiation force can only act on particles which are not small compared to the ultrasonic wavelength.

The majority of ultrasound-enhanced drug delivery research during the twentieth century was performed *in vitro* using cell and tissue cultures. Since then a growing number of studies have reported the beneficial effects of ultrasound-enhanced drug delivery *in vivo*, demonstrating targeted release, improved delivery, and enhanced extravasation of therapeutic agents into solid tumours. The following section reviews various ultrasound-enhanced drug delivery vectors and the different types of drugs encapsulated within such vectors.

2. Encapsulation of drugs

Ultrasound has been used to trigger the release of anti-cancer drugs from delivery vectors including liposomes, polymeric microspheres and self-assembled polymeric constructs [8,23]. This release has been achieved using the mechanical or thermal effects of ultrasound. Mechanical effects have been exploited to release micelle-encapsulated doxorubicin [25], liposome-encapsulated thrombin [26], and nanoemulsions [27], while thermal effects have been used to trigger doxorubicin release from temperature-sensitive liposomes [7,8].

2.1 Ultrasound-enhanced delivery vehicles

Many studies have attempted to co-administer cavitation nuclei and non-encapsulated therapeutics (such as drugs or genes reviewed in Section 2.1) and then apply therapeutic ultrasound [8,26,28-35]. Such a strategy can result in the temporary formation of pores in target cell membranes through which therapeutics can achieve efficient passage into the cell. Despite showing promise *in vitro* [36,37], this 'sonoporation' method (discussed in Section 3.1) is limited *in vivo* by the non-specific interaction of therapeutics with non-target tissues and the requirement for the therapeutic to co-localise with the agent used as a cavitation nuclei. The applicability of sonoporation has also been limited by poor transfection efficiencies, which have in general not exceeded 3% of the target tissue volume, resulting in limited therapeutic effect [38]. To reduce non-specific interactions and improve the co-localisation of the therapeutic and the cavitation nuclei, therapeutics have been incorporated into vectors designed to have intrinsic sonosensitivity, i.e., possess the ability to undergo inertial cavitation in response to ultrasound. A range of 'all-in-one' acoustically active delivery vectors including microbubbles, liposomes, and nanobubbles are now under development [8,12,32].

Traditionally used solely as ultrasound contrast agents (UCA), microbubbles have more recently been formulated to serve as both delivery vectors and cavitation nuclei [26,28-31]. Traditional formulations of UCAs include either lipid or polymer shells, resulting in different toxicity, stability and acoustic response [14]. Therapeutic agents can be loaded within the microbubble shell or can be conjugated directly to the surface of the shell. Under ultrasound-induced cavitation, these microbubbles are designed to collapse and release entrapped therapeutic agents within target tumours [8,30,32]. However, microbubbles do not readily extravasate into tumours due to their relatively large diameter which is usually in the range of 0.5 – 10 μm, with a mean diameter of about 2.5 μm [16]. As the gaps between neighbouring tumour-associated endothelial cells have been calculated to be between 400 and 600 nm [39] or 100 and 1000 nm [40], the vast majority of micron-sized bubbles will be prevented from extravasating through these gaps and gaining access to the tumour. Once through the endothelial gaps, therapeutics still need to penetrate through the tumour interstitium, a high pressure environment with dense extracellular matrix and intercellular spaces which have a mean diameter of 1.7 μm and range from 0.3 to 4.7 μm [41]. It is also apparent that many of these formulations are cleared rapidly from the bloodstream due to poor stability and capture by the reticulo-endothelial system (RES) and entrapment in the capillary beds of the lung and spleen [42,43]. Their micron size and poor pharmacokinetics may therefore ultimately limit the ability of microbubble formulations to benefit from the enhanced permeability retention effect (EPR) [44,45] and achieve enhanced passive accumulation in tumours. In response to these limitations, smaller and more robust sonosensitive delivery vehicles (e.g., liposomes and nanobubbles) are being developed.

Liposomes are vesicles comprised of a bilayer of either natural or synthetic phospholipids. Usually therapeutic agents are entrapped inside the liposome for stability and the external layer of the liposome is frequently coated with tumour-cell targeting ligands and/or protective polymer to minimise recognition by the RES [7,26,46]. Different types of liposomes have different sizes, but those used clinically usually measure between 100 and 400 nm [47]. Initially, ultrasound-enhanced

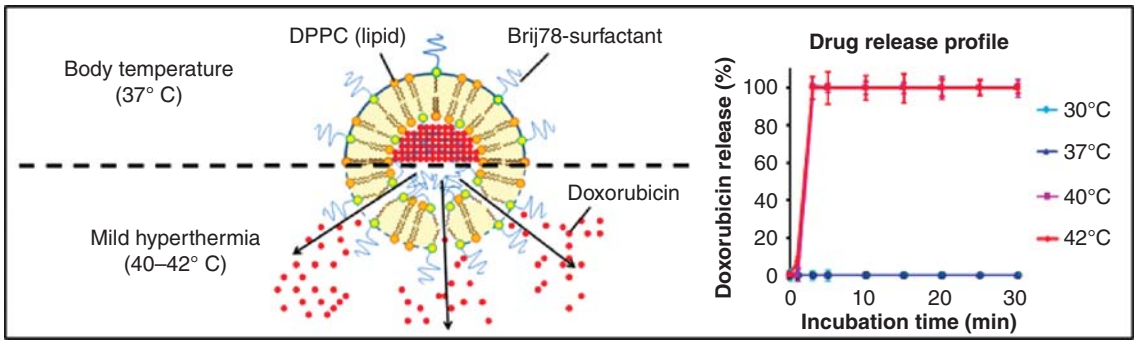

Figure 2. Thermosensitive liposomes stably entrap doxorubicin at physiological temperature, and upon exposure to mild hyperthermia (4 – 5°C temperature elevation) by external and localised sources (e.g., ultrasound, light, microwave), phospholipid bilayers of liposomes disintegrate and release drug content.
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liposome-delivery experiments were performed in the presence of ultrasound-contrast agents such as microbubbles [48]. More recently, there have been reports of liposomal formulations that can achieve triggered release in response to the thermal [6,15] or mechanical effects of ultrasound [47,49-53] even in the absence of microbubbles.

Thermosensitive liposomes (TSL) were first synthesised by Weinstein *et al.* in 1980 to treat solid L1210 xenograft tumours in mice [54], but it was not until 2007 that Dromi *et al.* demonstrated therapeutic ultrasound can serve as a source of hyperthermia and trigger doxorubicin release from these formulations [7]. Control non-thermosensitive liposomes (NTSL) do not release entrapped drugs at physiological or raised temperatures, whereas most TSLs are designed to release entrapped drugs only upon exposure to a temperature of 42°C or higher [55]. The sensitivity of TSLs to a slight and non-lethal temperature elevation of around 4 – 5°C does not therefore require a continuous ultrasound exposure to achieve a traditional 'ablation' effect [2], even though it requires the use of HIFU. Non-continuous 1 MHz ultrasound with low duty cycle (10%), pulse repetition frequency (1 Hz), and 120 pulses can be used [7,56], but with pressure exceeding 4 MPa in order to deposit sufficient energy in tissue to raise the temperature by a few degrees. A schematic representation of how doxorubicin release occurs from thermosensitive liposomes is provided in **Figure 2**. It should be noted that this promising approach will nonetheless always require a specialised HIFU transducer generating super-diagnostic pressure levels to produce the required temperature rise for release.

Where mechanical response is concerned, the recent work of Evjen *et al.* [49,50] is of particular interest and has improved the understanding of how phospholipid composition can be modulated to enhance sensitivity to mechanical disruption. However, the ultrasound exposure parameters (40 kHz, 100% duty cycle, up to 6 min) used in these reports are unlikely to be clinically applicable and ultimately mechanically triggered release will require the inclusion of gas to provide cavitation nuclei. Indeed, Huang *et al.* have suggested that most liposome preparations can actually entrap gas to a certain degree if freeze-dried phospholipid cakes are formulated in the presence of mannitol under vacuum pressure of 100 torr. One hypothesis for this process is that defects in the liposomal bilayer created during the lyophilisation process expose hydrophobic surfaces which, upon re-suspension in buffer, allow contact with and entrapment of air [52]. Huang *et al.* later used this procedure to formulate 780 ± 70 nm calcein-containing liposomes comprised of egg PC, DPPE, DPPG, and CH. These liposomes were reported to contain a payload of one-third gas and two-thirds drug in aqueous solution and could be acoustically triggered *in vitro* by applying 1 MHz ultrasound at 2 W/cm^2 for 10 s [53]. Studies have revealed considerable heterogeneity in the samples prepared by this method, with substantial numbers of liposomes having diameters exceeding 1 μm [52]. Evidence that these larger liposomes are not solely responsible for the sonosensitivity reported would be useful in emphasising how much of a departure from standard 'microbubbles' this strategy represents.

Several studies have reported the use of empty liposomal bubbles as ultrasound contrast agents without entrapping any therapeutic agents [57-59]. Suzuki *et al.* reported the preparation of liposomal bubbles with a mean diameter of 1 μm ± 300 nm as measured by dynamic light scattering. These were injected intraperitoneally in combination with a non-encapsulated luciferase reporter gene plasmid into mice implanted with S-180 cells while 1 MHz, 1.0 W/cm^2 ultrasound was applied transdermally for 1 min and 50% duty cycle. The luciferase signal from the transfected cells was two orders of magnitude higher when plasmid was combined with liposomal bubbles and ultrasound than when combined with liposomal bubbles only. It is unclear from these studies how such an ultrasound exposure would have benefitted transduction with this plasmid in the absence of the bubbles [60].

Kheirolomoom *et al.* combined the advantages of both liposomes and microbubbles by successfully conjugating liposomes to the microbubble shell. This 1.5-μm liposome-microbubble hybrid oscillated in a similar manner to control microbubbles

in response to a single 200 kPa, 2.25 MHz acoustic pulse and could successfully deliver fluorescent cholesterol to a monolayer of PC3 cells. After microbubble oscillation or disruption, the liposomes were detached from the microbubbles, but the study did not characterise the liposome release profile. While such a strategy should enable the micron-sized bubble-liposome formulation to release drug within tumour vasculature, thereby allowing penetration of free drug into the tumour, at a size of 1.5-µm this formulation will not be able to benefit from the augmented passive tumour accumulation provided by the EPR effect [61].

A recent innovation is the creation of nanobubble cavitation nuclei, which are echogenic and small enough to passively extravasate through the gaps in the tumour-associated endothelium [62-64]. There are currently two types of nanobubbles under development: free nanobubbles [64,65] and nanobubbles trapped on the surface of solid nanoparticles [62,63]. Wang *et al.* prepared coumarin-6 and sulphur hexafluoride-loaded lipid nanobubbles comprised of tween-80, cholesterol, and soybean lipid with a reported diameter of 300 nm. To date, there are no reports of the combined use of these nanobubbles with ultrasound to instigate drug delivery [65]. In similar studies, Gao *et al.* synthesised perfluoropentane 300 to 800-nm nanobubbles with doxorubicin as a payload, a formulation which demonstrated a few interesting and potentially useful properties. In *in vitro* tests, although these nanoemulsions were very stable at room temperature, upon heating to physiological temperature they fused to form micron-size bubbles. Provided this can be achieved over the desired timescale, and such fusion does not occur immediately post injection, this may allow nano-sized bubbles to accumulate within the tumour by EPR and then fuse and expand to become micron-scale and ultrasound-responsive [64]. Indeed in studies using these agents, Rapoport *et al.* showed that such a nano- to micro-scale conversion was detected within 1 min following direct intratumoural injection and even following intravenous injection into mice with tumours of 2000 mm^3. Although such conversion could not be detected in smaller tumours (60 mm^3), impressive retardation of tumour growth was instigated by the use of nanobubbles in combination with ultrasound in mice with 100 – 150 mm^3 tumours [27].

Nanodroplet vaporisation is an emerging technology that has yet to be tested for its echogenic properties [66,67]. While Lattin *et al.* have recently demonstrated a new method of entrapping 50 – 100-nm droplets inside liposomes, the study has yet to demonstrate the utility of applying ultrasound to induce inertial cavitation and release drugs from within such liposome formulations [66]. Sheeran *et al.* showed that 200 – 300-nm decafluorobutane droplets can yield microbubbles (1 – 5 µm) once vaporised under clinical diagnostic ultrasound (5 MHz); however, to apply this same experimental setup in the clinical setting might be challenging given that the *in vivo* stability and circulation half-life of these nanodroplets are hard to be controlled and that the exact nanodroplet location cannot be identified using ultrasound [67].

The second type of nanobubble, in which gas is entrapped on the hydrophobic surface of solid nanoparticles, is also a notable development. Wagstaffe *et al.* recently used this approach to demonstrate that LUDOX®-coated polystyrene nanoparticles with rough surface morphology can significantly lower the threshold for cavitation, i.e., the minimum pressure required for ultrasound to instigate inertial cavitation. LUDOX® is a commercially available silicon oxide of 15 – 25 nm in diameter, and the LUDOX®-coated nanoparticles produced were in the size range of 300 – 600 nm. In *in vitro* tests these rough-surfaced nanoparticles lowered the cavitation threshold of water from 5 MPa to less than 0.5 MPa [63], and should this formulation prove to be compatible with *in vivo* delivery it may represent a significant step forward in providing cavitation nuclei in the nanoscale range.

2.2 Therapeutic agents encapsulated in ultrasound delivery vehicles

Therapeutic ultrasound has been demonstrated to significantly improve the uptake of anti-cancer molecules of various sizes and molecular masses (e.g., plasmid DNA [33], 6-nm titanium oxide nanoparticles [34], 72 kDa clotting protein thrombin [26], 150 MDa 120-nm adenovirus [35]) in cells and tissues. According to the official U.S. Food and Drug Administration (FDA), there are presently 141 FDA-approved anti-cancer drugs. However, the majority of the ultrasound-enhanced drug delivery studies have focused on a few selected therapeutic agents. The following section reviews some of the drugs and therapeutic nucleotides (genes and siRNA) that have been more frequently used in combination with ultrasound.

2.2.1 Conventional chemotherapies and small molecules

Doxorubicin is one of the drugs most commonly tested for ultrasound-enhanced delivery. This anthracycline has been entrapped within liposomes, nanobubbles and microbubbles [11,25,27,46,68]. Another chemotherapeutic that has been tested in combination with ultrasound is the mitotic inhibitor paclitaxel [69-71]. In addition, ultrasound has been applied to enhance the diffusion of radionuclides to pancreatic tumour cells *in vitro* [31] and to inhibit the growth of leukaemia cells alongside the delivery of chemotherapeutic drug cytosine arabinoside *in vitro* [72]. Application of focused ultrasound in conjunction with microbubbles has been shown to enable non-invasive delivery of epirubicin across the blood–brain barrier in mouse models [73].

In addition to conventional chemotherapeutics, biological therapeutics such as peptides and antibodies have also recently been used in combination with ultrasound. Thrombin has been used as a 'natural clotting drug' in several delivery studies [74]. Klibanov *et al.* showed that the release of thrombin from liposomes conjugated to the surface of microbubbles was triggered by a 1 MHz ultrasound treatment at 5 pulses using a 100,000 cycle pulse length, resulting in significant acceleration

of *in vitro* blood clotting [26]. Lee *et al.* also demonstrated similar thrombin-release effect using 3.5 MHz ultrasound (4.7 MPa peak rarefactional pressure) applied to thrombin-loaded microbubbles *in vitro* [75].

2.2.2 Nucleotide-based therapeutics

Nucleotides offer powerful therapeutic benefit due to their intrinsic 'amplification' potential, with each successfully delivered copy providing many therapeutic events. Unfortunately, nucleotide-based therapeutics are often restricted *in vivo* by their relatively large extended structure and size and high net negative charge. These characteristics can inhibit extravasation from the circulation into the tumour, limit movement through the tumour interstitium and restrict passage across the plasma membrane of cells. Technologies, such as ultrasound, that can help remove these limitations therefore hold considerable potential therapeutic benefit. Ultrasound has been used in conjunction with nucleotide sequences which will instigate a therapeutic effect, such as the suppression of tumour growth, or act as a marker molecule (e.g., GFP and luciferase), to allow quantification of the efficiency of delivery. Current techniques in genetic engineering have even enabled the synthesis of hybrid therapeutic and reporter genes [76]. Therapeutic genes can be delivered using viral or non-viral vectors; while viral gene delivery will be discussed in Section 2.2.3 of this review, this section focuses mainly on non-viral ultrasound-enhanced gene delivery.

Taniyama *et al.* reported an *in vivo* study performed in a rabbit model testing the delivery of a non-encapsulated luciferase reporter plasmid to skeletal muscle cells in the presence and absence of Optison microbubbles and ultrasound (1 MHz and 2.5 W/cm^2) for 30 s. Compared to administration of the plasmid alone, the application of plasmid and ultrasound gave a 10-fold enhancement of reporter gene expression, perhaps suggesting the instigation of a sonoporation event. Notably this enhancement was increased to 70-fold when the cavitation nuclei Optison was also included. Even though this model did not utilise tumour cells, there is a strong implication that the same ultrasound-enhanced delivery mechanisms can be applied to the treatment of cancer [33]. However such a strategy may ultimately be limited to situations where direct administration into target tissue is possible, as non-encapsulated plasmid would be rapidly eliminated following intravenous administration. Suzuki *et al.* transfected S-180 cancer cells with liposomal bubbles containing plasmid DNA encoding the luciferase gene. When bubbles with plasmid were used in combination with ultrasound the magnitude of luciferase expression was two orders of magnitude higher than in the three controls: plasmid-only transfection, ultrasound-guided plasmid transfection in the absence of liposomal bubbles, and plasmid transfection with liposomal bubbles with no ultrasound exposure [60]. The liposomes used in these studies measured 1 μm and so have an average size that is still likely to exclude them from the tumour interstitium if administered intravenously. Many other studies performed using luciferase [35,57,77] and GFP [35,78] reporter genes or calcein [26,79] have demonstrated that ultrasound alone may have a direct effect on gene expression even without the introduction of microbubbles. However, a greater level of delivery was achieved when ultrasound was applied with cavitation nuclei in all these studies, compared to the absence of ultrasound and/or cavitation nuclei.

The use of ultrasound to enhance delivery of therapeutic nucleotide such as siRNA [79-83] and suicide genes [84-86] has been the focus of much recent research. Vandenbroucke *et al.* [81] and Otani *et al.* [80] were able to conjugate siRNA to the surface of microbubbles and instigated the cavitation nuclei at 1 MHz (10% duty cycle, 2 W/cm^2, 2 s) and 1 MHz (20% duty cycle, 2 W/cm^2, 10 s), respectively. In the presence of ultrasound, the siRNA-loaded microbubbles were able to silence more luciferase expression (90%) than the free siRNA control (10%) in HUH7 cells [81]. Otani *et al.* further demonstrated that the siRNA-loaded microbubbles were able to knockdown around twofold more PTEN (a tumour suppressor gene) than control siRNA alone in both adipose stromal cells and mesenchymal stem cells [80]. Negishi *et al.* [83] and Endo-Takahashi *et al.* [82] successfully formulated siRNA within liposomal bubbles for ultrasound-enhanced delivery; in contrast to previously discussed studies, in both these studies the siRNA was entrapped inside the liposomal bubbles, potentially improving *in vivo* applicability. Furthermore, no additional ultrasound-contrast agents were present, and so siRNA-entrapped liposomes served as the sole cavitation nuclei at 2 MHz (50% duty cycle, 2.0 W/cm^2, 10 s) for both experiments; again statistically significant knockdown of desired genes in tumour cells were observed [82,83]. It is notable that all these formulations measure in the micron size range and that all these studies have been performed *in vitro* and so the challenge will now be to test these technologies in *in vivo* studies.

Of the therapeutic genes tested, herpes simplex thymidine kinase (HSVtk) has been a popular choice in studies of ultrasound-enhanced delivery of genes to cancer cells [84,85]. As a suicide gene, HSVtk imparts sensitivity to ganciclovir (GCV) upon cancer cells by encoding a protein that metabolises non-toxic GCV into a phosphorylated product. This produces a toxic nucleoside analogue that induces apoptosis by inhibiting the function of DNA polymerases [87]. Aoi *et al.* demonstrated that HSVtk plasmid transfection efficiency in both *in vitro* and *in vivo* experiments was greatly increased in the presence of Optison microbubbles and ultrasound (1 MHz, 1.3 W/cm^2, 50% duty cycle, 10 s). *In vitro* experiments showed that the relative number of A549 cells that underwent apoptosis in the presence of Optison, ultrasound, and HSVtk gene was around 20 times higher than following exposure to just Optison and ultrasound. However, the experiment did not address how this enhanced gene delivery compared to the apoptotic effect of transfection of the HSVtk gene only or of exposure to ultrasound alone. *In vivo* results

indicated that tumour size was reduced by a factor of 4 after 30 days in the ultrasound-enhanced gene therapy group compared to gene therapy alone. Aoi *et al.* suggested that sonoporation (discussed later in this review) is the main driving force behind improvements to suicide gene delivery, and demonstrated that the exposure of same ultrasound parameters alone did not induce any cell damage [84].

Carson *et al.* recently reported the synthesis of lipid microbubbles (1.9 – 2.3 μm) entrapping perfluorobutane with HSVtk plasmid attached to the surface and demonstrated that such attachment provided efficient protection from DNAse digestion. Following intravenous injection of this formulation into mice bearing C3H/NeJ carcinoma cells, a 1.3 MHz ultrasound at 1.8MPa was applied to the tumour region. Notably, the interval between each ultrasound pulse was adjusted to allow enough time for the microbubble to reperfuse in the tumour site. Ultrasound-targeted microbubble delivery was shown to increase expression of the payload gene (either GFP or HSVtk) and retard tumour growth [85]. Interestingly expression was localised mainly in endothelial structures, again emphasising the important potential that these micron-sized systems may have in application to anti-angiogenic strategies.

Further studies with suicide genes have been performed by Ogawa *et al.* who introduced the recombinant fcy::fur gene, and exposed LNCap cells to this plasmid *in vitro* in combination with 1 MHz ultrasound at 0.5 W/cm^2 and 10% duty cycle for 60 s in the absence of any UCAs. The fcy: fur gene expression was enhanced 15-fold in the sonicated sample compared to control with no ultrasound [86]. As no UCAs were introduced in this study, it is unclear whether this effect is the result of the presence of gas bubbles in the media which may have served as cavitation nuclei or whether the ultrasound mediated a direct effect on the cell membranes.

Most of the ultrasound-enhanced gene and drug delivery studies reviewed here rely on sonoporation and do not distinguish the effects of sonoporation on cancer cells of therapeutic agents. These experiments are mainly performed *in vitro* [26,75,81-84], and direct intratumoural administration was used in the case of most *in vivo* studies with little consideration or testing of the circulation half-life of these agents [58,64]. The few studies that have used intravenous administration have relied on the use of microbubbles, which possess size limitations that may make them most effective when utilised in anti-angiogenic strategies rather than when used for direct tumour cell kill [33,65,84].

2.2.3 Ultrasound-enhanced tumour-targeting virotherapy

Clinical trials using oncolytic virotherapy for the treatment of tumours were performed as long ago as 1956 [88]. Since then, this field of research has generated many useful vectors and some impressive clinical results. However, the use of viruses in combination with ultrasound was not reported until 2006 [89]. Adenoviral vectors encoding the GFP reporter gene (Ad-GFP) were reconstituted in the absence or presence of microbubbles, and a 30-min at 37°C incubation with 60 mg/mL human complement was used to inactivate free non-incorporated Ad-GFP as shown by reduced infection of a cancer cell line monolayer. The Ad-GFP–microbubble complexes were administered to DU-145 and H23 cells plated on glass coverslips, which were then exposed to 2.5 MHz ultrasound at 535 kPa continuously for 1 min. Strong fluorescence signal was observed when the Ad-GFP–microbubble formulation was used in combination with the ultrasound exposure. However, it remains unclear how the levels achieved differed from those resulting from the simple addition of the same amount of non-modified Ad-GFP [89].

In further studies, Greco *et al.* showed that intravenous administration of oncolytic Ad-GFP–microbubble complexes gave greatly enhanced GFP expression in an ultrasound exposed (2.25 MHz ultrasound 1.8 MPa continuously for 10 min) tumour compared to a non-ultrasound exposed tumour in the same mouse. This important study represents the first demonstration of such an effect and to develop this work the authors then tested two therapeutic adenoviruses for efficacy in two xenograft models. Intriguingly, regardless of the virus or model used no significant decrease in the growth of the right ultrasound exposed tumour with respect to the left non-exposed tumour was observed. The authors postulate that such an effect was the result of the immune 'bystander' effects from the successfully infected ultrasound exposed tumour providing systemic inhibition of the growth of the non-ultrasound treated tumour. Unfortunately, the absence of a control group of Ad-microbubble-treated mice in which neither tumour received ultrasound prevented this hypothesis being fully validated. Hence, whilst this study represents substantial progress in testing the application of ultrasound for viral delivery, it is unclear whether ultrasound only provided enhancement of the release of the virus from the microbubbles or whether the amount of virus delivered and/or the intratumoural distribution of these viruses was also improved. A better understanding and quantification of the mechanisms responsible (inertial cavitation, stable cavitation) will also be an important step in developing this work [90].

Instead of using conventional phospholipid microbubbles as UCAs, Mannel *et al.* introduced magnetic microbubbles (MMB) into their ultrasound-enhanced virotherapy strategy. The advantage of using MMBs is that an external magnetic field can be applied to concentrate these magnetic UCAs within the tumour [91]. Mannel *et al.* prepared rrl-CMV-eGFP lentiviral particles and iron oxide MMB by mixing at a ratio of 300 femtogram of iron per viral particle. Human dermal microvascular endothelial cells (HMEC) were cultured and placed under a neodymium iron boron magnet for the induction of a magnetic field. All experiments were

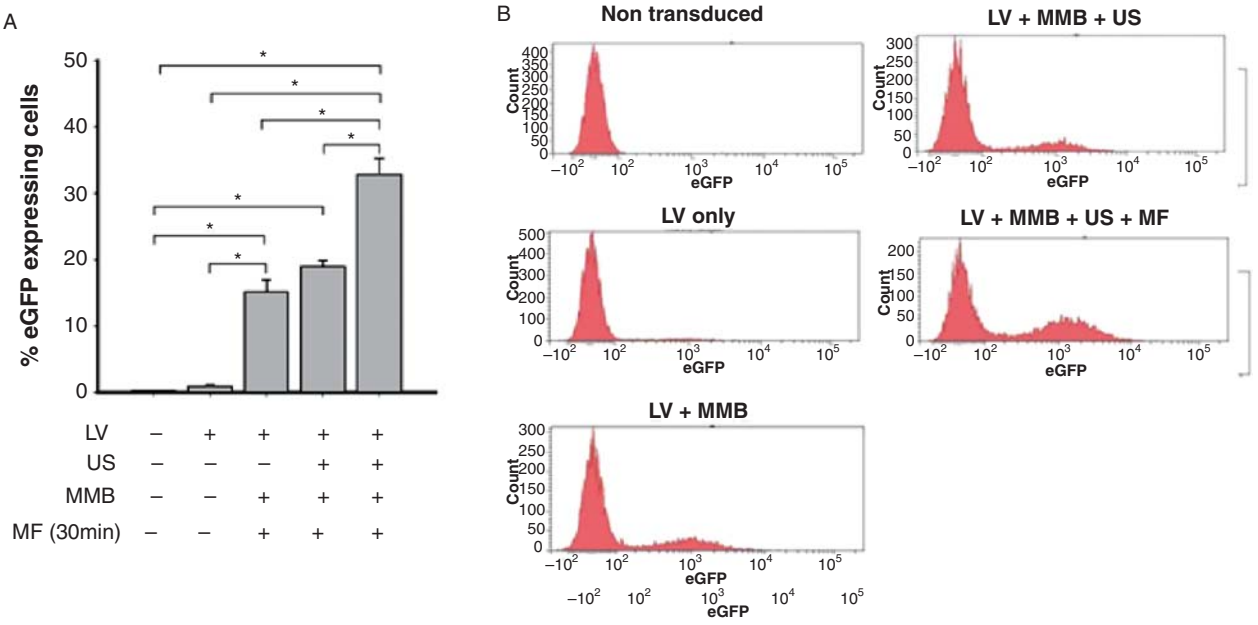

Figure 3. Lentiviral particle (LV) infection of HMEC in the absence and presence of ultrasound (US), magnetic microbubbles (MMB) and/or magnetic field (MF). The percentage of GFP expressing cells and distribution of GFP intensity of the same cells were shown in parts A and B, respectively. Statistical significance with $p < 0.05$ was shown with *.
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carried out at five lentiviral particles per HMEC and 1 MHz ultrasound (2 W/cm^2, 50% duty cycle, and 30 s) was applied to the media. Measurement of viral transduction was carried out 72 h later using flow cytometry as shown in **Figure 3**, and in contrast to many other studies reviewed here, care was taken to characterise the influence of each treatment component in isolation as well as in combination [91]. The combined use of virus, MMB and ultrasound provided substantial and significant improvements in infection efficacy compared to all other combinations tested. This method has therefore provided promising results, and if the use of MMB and magnetic fields can be translated for *in vivo* use it may have important clinical applications [91].

Virotherapy is an important emerging therapeutic modality, especially when direct intratumoural injection of these self-amplifying tumour cell killing agents can be achieved [92]. However, the use of virotherapy for the systemic treatment of metastatic disease may ultimately be limited by poor extravasation into tumours and inefficient intratumoural spread. It now seems that therapeutic ultrasound may offer a solution to this limitation and the combination of these two relatively new and powerful technologies offers much promise. A glut of recent *in vitro* studies have clearly demonstrated ultrasound can provide a powerful stimulus in this context and the test will now be whether these findings transfer to *in vivo* studies. It is also clear however that understanding, quantifying and optimising the underlying mechanisms by which ultrasound instigates enhancement of virotherapy will be essential in achieving this goal.

3. Ultrasound mechanisms for enhanced delivery and extravasation

Biological effects of ultrasound in the tumour environment include sonoporation, convection and extravasation of drugs. Cavitation effects and radiation force are the main driving forces behind improved extravasation and convection of drugs.

3.1 Sonoporation

A well-established mechanism for ultrasound-enhanced increased gene uptake to tumour cells is sonoporation, a process in which ultrasound is used to alter the permeability of the cell plasma membrane [36,37]; collapsing bubbles are believed to create transient holes in the cell membrane, through which large molecules can enter cells [8,36]. Sonoporation can provide a very specific and high concentration of delivered drugs at the site of interest while minimising the overall exposure of the rest of the body to the drug [37]. It may be of particular advantage for the delivery of free nucleotide which would otherwise have limited passage across the plasma membrane due to its large size and net negative charge.

Several recent studies have tried to measure the cell membrane pore sizes opened under ultrasonic sonoporation by SEM imaging. These reports have all used commercially available microbubbles as cavitation nuclei and produced measurements of ~ 75 nm in rat mammary carcinoma cells exposed to 1.15 MHz ultrasound (400 kPa and 10 s) [93], ~ 56 nm in DU

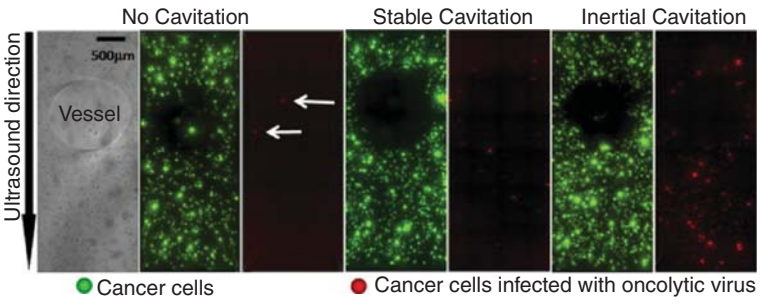

Figure 4. BT-474 cells embedded in tumour-mimicking model containing a vessel; co-administration of SonoVue microbubbles and adenovirus through the flow vessel while 0.5 MHz ultrasound applied at 0 MPa (no cavitation), 360 kPa (stable cavitation), and 1.25 MPa (inertial cavitation). Extravasation of the oncolytic virus is greatly enhanced in the presence of stable and in particular inertial cavitation, with much of the enhancement being observed in the direction of sound propagation.

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145 prostate-cancer cells exposed to 24 kHz ultrasound (700 kPa, 10% duty cycle, and 20 acoustic pulses) [94], and 110 ± 40 nm in *Xenopus laevis* oocytes exposed to 1 MHz ultrasound (300 kPa and 0.2 s) [95]. Even in the absence of microbubbles or liposomes, ultrasound alone still increased the drug or gene delivery to target tumour cells at 1 MHz [86,96] in *in vitro* experiments, and one possible explanation is that there are still adequate levels of dissolved gas in the experimental cell media solution to allow cavitation activity. This raises concerns regarding the clinical applicability of such effects as the efficient removal of bubbles from the circulatory system means that there may not be a sufficient supply of cavitation nuclei [23].

The micron-sized cavitation agents required for sonoporation will have restricted direct contact with tumour cells and so may only impact upon the tumour-associated vasculature. Hence in the context of drug delivery to solid tumours, sonoporation may ultimately be most effective in enhancing strategies that seek to destroy tumours by killing their supporting endothelial cells.

3.2 Enhanced extravasation and convection

For the purpose of this review, convection is defined as the collective movement of fluid (e.g., blood plasma and interstitial fluid) within tumour vasculature and tissue, whereas extravasation refers to the ability of desired therapeutic agents to penetrate deeply and spread as widely as possible within tumour tissue.

There are two mechanisms by which ultrasound may increase the convection of liquid. First, acoustic streaming produced by an external ultrasound source may instigate flow of fluid in the direction of the sound propagation [8,77]. The second mechanism of increased convection is known as microstreaming in which stable or inertial cavitation instigates oscillating bubbles (such as UCAs) to expand and shrink repeatedly to create shear flow in the surrounding liquid [21,77].

Stieger *et al.* demonstrate that convection (resulting from 1.00 and 2.25 MHz ultrasound exposure with microbubbles) is the dominant transport mechanism enhancing vascular permeability and delivery of therapeutic agents in a chorioallanotoic membrane model for *in vivo* visualisation of these ultrasound effects on blood vessels. Lipid-shelled perfluoropropane-filled microbubbles and FITC-labelled dextrans were co-administrated into the vascular system of the embryo. The study suggested the threshold pressure for extravasation was 0.5 MPa for 1 MHz and 1.6 MPa for 2.25 MHz ultrasound; the remaining ultrasound parameters for both frequencies are 10-cycle pulses at PRF of 500 Hz over 5 s. The control group (with no ultrasound exposure) had no sign of extravasation of the labelled dextran and showed close to zero flow velocity, whereas the flow rates of dextran inside the vasculature was increased up to 188 ± 75 and 362 ± 150 μm/s by 1 and 2.25 MHz exposure, respectively. The precise role and importance of the microbubbles in mediating these effects is hard to gauge as the influence of these exposures on this model were not tested in their absence [97]. Arvanitis *et al.* utilised quantitative passive cavitation detection techniques to demonstrate conclusively for the first time that inertial cavitation is the key acoustic mechanism by which ultrasound can enhance the extravasation of drugs [77]. Several recent studies have also demonstrated that drugs exposed to ultrasound in the presence of microbubbles achieve greatly enhanced extravasation into solid tumours [33,64,65,84,98].

Attempts to better understand and control extravasation mechanisms were recently made by Bazan-Peregrino *et al.* [35]. A phantom vessel running through agar gel containing breast cancer cells was developed as an *in vitro* 3D tumour model for these studies. Oncolytic adenovirus AdEHE2F-Luc was introduced through the phantom vessel, and a focused 0.5 MHz HIFU transducer was used to instigate acoustic cavitation in the presence and absence of UCAs (SonoVue). While delivering the same amount of

acoustic energy, the ultrasound exposure parameters were optimised to maximise either the ultraharmonic content (360 kPa, 90% duty cycle and PRF of 10 Hz for stable cavitation) or broadband spectral content (1.25 MPa, 6.5% duty cycle and PRF 10 Hz for inertial cavitation) of the acoustic emissions produced in the presence of UCAs [35]. In the absence of ultrasound, very few cancer cells were successfully infected by the oncolytic virus. However, the instigation of cavitation correlated with increased adenovirus extravasation resulting in increased infection efficacy. Interestingly small increases in the total amount of adenovirus transferred into the agar upon the use of SonoVue and ultrasound compared to the use of ultrasound alone lead to much more marked increases in the transgene expression achieved. Stable cavitation caused a twofold increase in the number of viral particles extravasated, which translated into a 10-fold increase in expression at 24 h. Yet more impressively inertial cavitation caused a fourfold increase in viral concentration which translated into a 200-fold increase in luciferase expression, with much of the increase being observed in the direction of sound propagation (**Figure 4**). This indicates the key role that therapeutic ultrasound in general and inertial cavitation in particular can play in improving both the level of therapeutic agent transferred and the distribution of the agent throughout the target tumour [35]. Whether such findings are applicable to the *in vivo* setting has yet to be reported.

4. Therapy safety

Ultrasound therapy offers a safe, convenient, non-invasive, and tissue-specific system to deliver drugs and genes to solid tumours [60,65,84]. A crucial prerequisite for any cancer therapy is that the benefits of killing cancer cells outweigh deleterious side effects and damage to healthy cells. All studies discussed in this review used 0.5 – 2.5 MHz ultrasound transducers with up to 2.0 MPa pressure and showed no significant difference in cell viability in the absence or presence of therapeutic ultrasound alone [35]. Although inertial cavitation and sonoporation might create short-life pores in cell membranes for delivery of therapeutic agents, these pores quickly close upon the removal of ultrasound exposure [8,36]. The majority of sonoporation studies showed no decrease in cell viability with ultrasound alone, demonstrating that increases in cancer cell death were instigated by the anti-cancer therapeutics [86,96].

Furthermore, in theory, because ultrasound is an external wave with acoustic energy concentrated at the focus (e.g., within the solid tumour), all tissues and organs in the path of the ultrasound wave but outside the focal region should not suffer ultrasound-induced damage. Whilst the concern that ultrasound-induced inertial cavitation may create shear forces that induce the breakup of primary cancer cells thereby enhancing metastasis was recently assuaged. Oosterhof *et al.* showed that there is no difference in the observed metastases between control and ultrasound-treated xenograft mice [99]. In addition, Wu *et al.* studied the effect of therapeutic ultrasound in human patients and concluded that it does not increase the chance of metastasis [100].

5. Conclusion

Ultrasound is completely non-invasive, targetable, controllable, and poses little risk to the operator or patient, making it a potentially ideal technology for targeted drug delivery. Both thermal and mechanical effects of ultrasound can be used to achieve release of a wide array of agents, ranging from conventional chemotherapeutics to oligonucleotides, from a variety of delivery vehicles such as liposomes or nanoemulsions. Mechanical effects of ultrasound, and acoustic cavitation in particular, have also been found to enhance the extravasation and distribution of drugs into and within tumours, which may ultimately increase the efficacy of current and future therapeutic agents.

6. Expert opinion

The mainstream non-invasive therapies for treating solid tumours are chemotherapy and radiotherapy. These methods are limited by sub-optimal specificity for cancer rather than normal cells and the possibility that they induce suppression of the host anti-cancer immunity.

Therapeutic ultrasound is a non-invasive modality which does not impair immune function and is not associated with any common physiological insult. As such it represents an ideal technology for use in enhancing the specificity and efficacy of mainstream therapies. On the other hand, ultrasound therapy is not a cure-all method either and does have its limitations. Sound wave propagates poorly in air and through bone, so therapeutic ultrasound is not appropriate to target tumours in or close to lung or bowel tissue and is difficult to apply through the skull. If a sufficient acoustic window is not present or if the targeted tissue or organ is situated too close to other healthy tissues, it is not possible to treat these tumours with therapeutic ultrasound.

The next challenge in ultrasound-enhanced drug delivery is to develop a new generation of delivery vehicles which are tailor-made for cavitation-enhanced drug delivery for cancer. Increasing bloodstream stability and pharmacokinetics and achieving co-location of the delivery vehicles with cavitation nuclei are important goals. However, just improving the delivery vehicles alone is not sufficient. Ideally, researchers can produce 'real' acoustically active nanobubbles that show a monodisperse size distribution below the 500 nm range, allowing optimal extravasation into the tumour interstitial space. To date, there is an absence of compelling evidence that ultrasound-responsive bubbles that are truly nanoscale have been formulated. The invention of sonosensitive solid nanoparticles is a promising approach, and more studies are needed to validate and understand the mechanisms of these 'indestructible' UCAs.

The exploitation of inertial cavitation for ultrasound-enhanced drug delivery presents another major advantage

which no other modality can offer: cavitation can be detected remotely, and can therefore act not only as a promoter but also as a marker of successful drug delivery. The recent development of a novel technique known as passive acoustic mapping [18,19] provides a unique opportunity for spatio-temporal mapping of cavitation activity using conventional ultrasound imagers, and could thus also provide a method for informing clinicians of when and where a therapeutic agent has been successfully delivered.

To translate this technology into clinical trials, more robust ultrasound exposure parameters must be defined, ideally with low pressure required for cavitation initiation. Given the current knowledge acquired from *in vitro* and *in vivo* studies, short ultrasound exposure time (less than a few minutes), frequency between 1 and 2.5 MHz and pressure up to 2.0 MPa are recommended for safe and effective ultrasound therapy.

The integration of virotherapy with therapeutic ultrasound is another encouraging field worthy of further investigation, not least because the self-amplification of the virus can overcome issues associated with delivering sufficient therapeutic dose. Cancer cell-specific viruses can be genetically engineered to provide and enhance specificity for tumours while avoiding viral infection to other healthy tissues. Given the many therapeutic systems already in clinical [8,101] use and the approval by the FDA for several adenoviruses to be used in Phase III clinical trials, ultrasound-enhanced virotherapy may well be rapidly approaching clinical applicability.

Declaration of interest

The authors state no conflict of interest and have received no payment in preparation of this manuscript.

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