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Ultrasonic drug delivery – a general review

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Ultrasound has an ever-increasing role in the delivery of therapeutic agents, including genetic material, protein and chemotherapeutic agents. Cavitating gas bodies, such as microbubbles, are the mediators through which the energy of relatively non-interactive pressure waves is concentrated to produce forces that permeabilise cell membranes and disrupt the vesicles that carry drugs. Thus, the presence of microbubbles enormously enhances ultrasonic delivery of genetic material, proteins and smaller chemical agents. Numerous reports show that the most efficient delivery of genetic material occurs in the presence of cavitating microbubbles. Attaching the DNA directly to the microbubbles, or to gas-containing liposomes, enhances gene uptake even further. Ultrasonic-enhanced gene delivery has been studied in various tissues, including cardiac, vascular, skeletal muscle, tumour and even fetal tissue. Ultrasonic-assisted delivery of proteins has found most application in transdermal transport of insulin. Cavitation events reversibly disrupt the structure of the stratus corneum to allow transport of these large molecules. Other hormones and small proteins could also be delivered transdermally. Small chemotherapeutic molecules are delivered in research settings from micelles and liposomes exposed to ultrasound. Cavitation appears to play two roles: it disrupts the structure of the carrier vesicle and releases the drug; and makes cell membranes and capillaries more permeable to drugs. There remains a need to better understand the physics of cavitation of microbubbles and the impact that such cavitation has on cells and drug-carrying vesicles.

Keywords: cavitation, chemopotentiation, chemotherapy, DNA, hyperthermia, liposomes, micelles, microbubbles, protein, targeted drug delivery, ultrasound

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1. Mechanisms of ultrasonic-enhanced drug delivery

Ultrasound has been employed to enhance the delivery and activity of drugs for the past two decades. In the past 6 years, however, research in ultrasound-activated drug delivery has blossomed with the introduction of gas bubbles. In order to understand the mechanisms of ultrasound-enhanced drug delivery, one must first understand the physics of ultrasound and of cavitation, the keystone of this novel approach to drug delivery.

1.1 Physics of ultrasound

Just as audio sound is the transmission of pressure waves through a medium, such as air or water, ultrasound is the same type of transmission of pressure waves, but at frequencies above human hearing, or ≥ 20,000 Hz. As with light waves, these ultrasonic waves can be reflected, refracted (bent), focused and absorbed. Unlike light waves, ultrasonic waves are very physical in nature; they are actual movement of molecules as the medium is compressed (at high pressure) and expanded (at low pressure), and thus ultrasound can act physically on biomolecules and cells.

Most importantly, unlike visible light waves, ultrasonic waves are absorbed relatively little by water, flesh and other tissues. Therefore, ultrasound can 'see' into the

body (e.g., diagnostic ultrasound) and can be used to transmit energy into the body at precise locations. This safe, non-invasive and painless transmission of energy into the body is the key to ultrasonic-activated drug delivery.

1.2 Hyperthermia

The traditional use of ultrasound in medicine is for diagnostic imaging (which occurs at low average intensities and high frequencies) and for tissue heating (which occurs at higher intensities and higher frequencies). The intensity (also called power density) of an ultrasonic beam is measured in terms of power carried/cross-section area of the beam, typically having units of W/cm^2. If a beam is focused down to a small size on a targeted tissue, the power/area becomes very large and significant thermal energy can be absorbed from the beam by the tissue, resulting in heating. Such hyperthermia has been traditionally employed in physical therapy to warm tissues [1], in drug delivery to 'melt' drug-containing liposomes [2], and in medical therapy to kill or ablate tissue [3-5]. Thus, hyperthermia in targeted drug delivery accomplishes the role of heating the drugs, drug carriers and/or the tissues receiving the drugs.

1.3 Cavitation

1.3.1 Nature of cavitation

A relatively recent and novel application in drug delivery takes advantage of the remarkable ability of ultrasound to produce cavitation activity. Cavitation is the formation and/or activity of gas-filled bubbles in a medium exposed to ultrasound [6]. As the pressure wave passes through the media, gas bubbles of any size will expand at low pressure and contract at high pressure. If the resulting oscillation in bubble size is fairly stable (repeatable over many cycles), the cavitation is called 'stable' or 'non-inertial' cavitation. Such oscillation creates a circulating fluid flow (called microstreaming) around the bubble [7-9], with velocities and shear rates proportional to the amplitude of the oscillation. At high amplitudes, the associated shear forces are capable of shearing open red cells [10] and synthetic vesicles such as liposomes [7].

As the ultrasonic intensity increases, the amplitude of oscillation also increases to a point in which the inward moving wall of fluid has sufficient inertia that it cannot reverse direction when the acoustic pressure reverses, but continues to compress the gas in the bubble to a very small volume, creating extremely high pressures and temperatures [11,12]. This type of cavitation (called transient, inertial or collapse cavitation) can be detrimental to cells or vesicles because of the very high shear stresses in the region of the collapse, the shock wave produced by the collapse, and the free radicals produced by the high temperatures. The collapsed bubble often fragments into smaller bubbles that serve as cavitation nuclei, grow in size, and eventually collapse again [11,12]. Figure 1 shows a streak optical photograph before, during and after a cavitation collapse [12]. On the left is the original microbubble. The middle frame is a high-speed streak photograph showing the boundaries of the bubble when subjected to a five-cycle driving pulse at 2.5 MHz and 1.6 MPa; the pressure measurement is recorded at the top of the photograph. The right frame shows the resulting fragments of the bubble. Cavitation is a violent phenomenon that concentrates the energy from ultrasound into a small volume.

If the collapse is near a solid surface, an asymmetrical collapse occurs that ejects a liquid jet at sonic speed toward the surface [11,13]. Figure 2 illustrates this type of collapse. If the rigid surface is a blood vessel wall, skin, a large cell or a semi-rigid vesicle, then the jet can pierce the surface.

Collapse cavitation can be damaging to biological tissues and, therefore, there has been much research into the conditions under which it is produced. In general, the likelihood and intensity of collapse cavitation increases at higher intensities and lower frequencies, as has been demonstrated by experiments [11,14,15] and theory [16]. The size of the bubble and its physical properties (i.e., gas species, interfacial tension, surface rigidity) also affect the cavitation process [17-21].

1.3.2 Ultrasound-induced mechanisms of drug delivery

1.3.2.1 Enhanced transport

Ultrasonication and cavitation can be involved in drug delivery by several mechanisms. The simplest mechanism derives from the oscillatory motion of the insonated fluid and can occur in the absence of cavitation. The oscillating fluid increases the effective diffusivity of molecules; thus, the transport of any drug, whether free or bound to a carrier, will be augmented by the oscillatory motion of the fluid. Such ultrasonic-enhanced transport may occur within blood, cells or extracellular fluids.

When a strong ultrasonic beam is directed through a partially absorbing liquid, some momentum from the beam is transferred to the fluid, imparting a large-scale convective motion to the fluid that can also increase the overall rate of drug transport [22]. Although this so-called 'acoustic streaming' could enhance drug transport *in vitro*, we opine that it has little, if any, application for *in vivo* systems, because fluid convection in the vascular system is already very fast, and in tissues beyond the vascular system there are few reservoirs of fluid that can be accelerated by an acoustic beam.

In the presence of oscillating bubbles, drug transport is enhanced by orders of magnitude compared with transport by diffusion alone. At least two mechanisms are reported for creating convection in the presence of a stable oscillating bubble. The first phenomenon, called microstreaming, is the creation of circulating eddies around the oscillating bubble [7-9]. Such eddies can transport drugs at high velocities. For example, the velocity of water near the surface of a 10 µm (diameter) bubble, with a 2 µm oscillation amplitude, are in the order of 10 m/s [9]. Even more surprising are the extremely high viscous shear rates near the surface of the bubble, which, in this example, are on the order of 10^7/s [9]. This shear rate is equivalent to shearing water in a 1 mm gap between parallel plates in which one plate is stationary and the other moving at

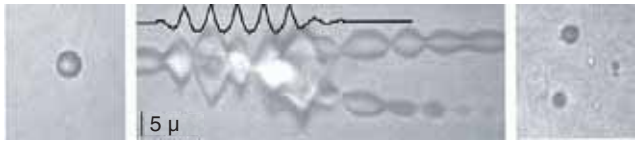

Figure 1. Optical images of a 2.5 µm radius microbubble exposed to five cycles of 2.5 MHz ultrasound at 1.6 MPa pressure amplitude. The left panel shows the bubble before exposure. The central panel shows a streak photograph (an optical M-mode image of a line through the centre of the bubble as a function of time) with the measured pressure superimposed at the top of the panel. The right panel shows the bubble fragments produced by the collapse of the cavitating bubble. Used with permission from MAY DJ, ALLEN JS, FERRARA KW: Dynamics and fragmentation of thick-shelled microbubbles. *Ieee Transactions on Ultrasonics Ferroelectrics and Frequency Control* (2002) **49**(10):1400-1410 [12], © 2002 IEEE.

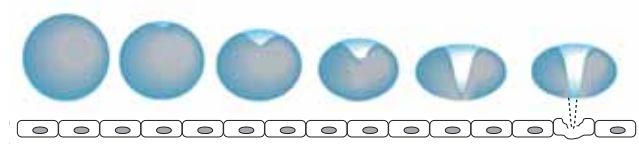

Figure 2. Illustration of an asymmetric collapse of a bubble near a surface, producing a jet of liquid toward the surface.

10^{10} m/s. This extremely high shear rate stresses cells and vesicles, as will be discussed later.

The second phenomenon related to oscillating bubbles is called acoustic pressure, which is a net force acting on other suspended bodies in the vicinity of an oscillating bubble. If the body is more dense than the suspending liquid, the body is pushed toward the bubble; if it is less dense, the body is repelled from the bubble [23]. Most drug-carrying liposomes and micelles are more dense than water, and will be convected toward the bubble, thus increasing the dispersive transport of the drug carrier, particularly if the vesicle is drawn into the microstreaming field around the bubble, and is sheared open by the high shear rate (releasing the drug). Marmottant and Hilgenfeldt show a fascinating example of this phenomenon [7]. If the vesicle is another microbubble, it will be dispersed away from the primary oscillating bubble because it is less dense. Therefore, a field of microbubbles, such as an injected bolus of contrast agent, will tend to spread itself in the ultrasonic field, and at the same time attract and shear more dense vesicles, such as suspended cells or introduced liposomes.

1.3.2.2 Perturbation of the drug carrier

This leads to the second major contribution of ultrasound toward drug delivery: the disruption of drug carriers by ultrasound. As mentioned above, vesicles more dense than the surrounding liquid will be sucked into the shear field surrounding an oscillating bubble. If the shear stress exceeds the strength of the vesicle, it will rupture and spill its contents. In the case of a liposome (and perhaps a micelle), the vesicle will probably reform, often at a smaller size than before the encounter with the shear field. The case of a liposome resizing into a number of smaller vesicles, with the same amount of surface area (assuming no phospholipids are lost), presents an interesting method of drug release. If a large spherical vesicle is fractured into a number of smaller vesicles and the surface area is conserved, the total volume of the smaller vesicles is less than the original volume. Thus, some interior liquid must be released during the fracture event. If a drug is soluble in the aqueous core of a liposome, some will be released following fracture, even if the liposomes reform into smaller vesicles. Collapse cavitation is not required for this method of drug release; a stable oscillating bubble is sufficient.

Other mechanisms of vesicle perturbation arise from collapse cavitation. The collapse event produces a shock wave that can be envisioned as an expanding thin shell of dense water. As this spike of dense fluid passes over a vesicle, a shear stress is produced at the vesicle surface that can rupture the vesicle if the critical stress is exceeded [24,25]. As the shock wave expands spherically, its energy density decreases, such that, beyond a certain distance from the bubble, there is no longer sufficient shear to disrupt vesicles [25].

As mentioned previously, the sonic jet of fluid produced by collapse cavitation near a solid surface also generates extreme shear stresses that can shear open or perhaps pierce nearby vesicles. Some of these methods of drug delivery are illustrated in Figure 3.

It is well known that low-frequency, high-intensity ultrasound (conditions promoting collapse cavitation) can reduce the molecular weight of soluble polymers [26-28]. Polymer chain scission is attributed to viscous shear stresses that pull the backbone apart and to the generation of free radicals that may react with and break the chain. In cases employing drugs bound to a polymeric carrier, such reduction in polymer molecular weight will increase the diffusivity of the polymer–drug fragments. In some cases, the drug might be sheared from the polymer backbone. An interesting example is given for the ultrasonically activated delivery of cobalamin (vitamin B_{12}) from a carrier. The release is attributed to free radicals, generated by collapse cavitation, that react with, rearrange, and break the bond connecting the cobalamin to the drug carrier [29].

1.3.2.3 Cell permeabilisation and capillary rupture.

The third major contribution of ultrasound to drug delivery relates to the stresses inflicted on cells and tissues as a result of cavitation events. Ultrasound by itself, in the absence of cavitation, is thought to have little, if any, effect on cells and tissues apart from some heating that may occur at higher frequencies and intensities [6,23,30,31]. As with vesicles, cells in an environment of cavitation events are subject to shear from

microstreaming, shock waves and sonic jets. It is possible that a large semi-rigid cell, adjacent to a small cavitating bubble, could induce an asymmetric bubble collapse, by which a small jet of liquid would shoot directly into the cell at sonic speeds, probably rupturing the cell membrane. Similarly, a collapse of a microbubble near a capillary or blood vessel wall will cause the liquid jet to shoot right into the wall. Such a collapse may be the source of the large amount of extravasation that is caused in tissue exposed to ultrasound in the presence of microbubble contrast agents [32-35]. There is some concern that a blood vessel injury by sonic jets, shear stresses, or radicals could generate a nidus, initiating subsequent thrombus formation.

1.3.3 Biological and physical consequences of cavitation

Much has been written about the biological consequences of ultrasonic cavitation in living systems, and little, if any, is positive with respect to the health of the organism or tissue [6,23,30,31]. Obviously, a plethora of bubbles undergoing collapse cavitation is not a healthy environment for a cell. Shock waves and shear forces are shearing the cell membrane. Liquid microjets at sonic velocities may be shooting right through cells (and lysing them). Free radicals may be interfering with essential biochemical processes. At the other extreme, a field of quivering bubbles experiencing mild stable cavitation probably has no negative biological consequences to most cells, and may even be beneficial in terms of increasing convection of oxygen or nutrients to cells [36].

Targeted drug release is a very active process, and these same energetic cavitational phenomena, which may be detrimental to tissues, are requisite in promoting the physical or chemical release of drugs from a carrier, or in promoting the transport of drugs into cells. Low-intensity stable cavitation may not be adequate to effectively execute drug delivery. Therefore, some cavitational activity must be tolerated to accomplish the medicinal goal. Somewhere between these harsh and mild extremes lies the desired realm of ultrasonic drug delivery. This would be a cavitational level that produces bubble activity sufficient to permeabilise cell membranes without killing the cells, a level that creates a sufficient number of microjets to allow extravasation from capillary walls without killing the endothelial cells or causing thrombosis, or a level that generates sufficient microstreaming to break open liposomes, or other vesicles, without lysing red or other host cells.

1.4 Types of drug carriers

The delivery of free drug, not associated with a carrier system, can be enhanced by ultrasound, as will be discussed in Section 2.3.1. However, the disadvantage of free drug is that it often interacts with non-targeted tissues if it is delivered systemically.

To preclude the non-targeted interaction of free drug with tissues, the drug is sometimes attached to a polymeric carrier from which it is released at the target site by degradation of a linker, via enzymes or pH at the target site. Ultrasound has been used to release covalently-bound cobalamin from a carrier [29], but this was a very specific application and may be difficult to extend to drugs in general.

Liposomes and micelles can sequester hydrophobic drugs within their lipophilic membrane (liposome) or core (micelle), and liposomes can sequester hydrophilic drugs in their aqueous interior. These vesicles appear to prevent general and/or premature release of the drug [37-39]. In theory, liposomes and micelles should not be acoustically active if they do not contain any gas. However, they can be drawn towards and then sheared open by the action of cavitating bubbles. In addition, cell membranes can be sheared by cavitation events, rendering them more permeable toward liposome or free drug uptake. Some studies suggest that even carefully prepared liposomes contain some gas, and thus they can become acoustically active during insonation and release their payload following cavitation-induced fragmentation [40,41]. Unger has prepared a liposome–microbubble hybrid, in which gas and stabilising oil are introduced into the standard liposome [42]. This 'liposphere' can be considered a hybrid between a pure liposome and microbubble, and can also be used as an ultrasonic contrast agent.

Microbubbles are distinguished from liposomes in that they contain gas and do not necessarily have a bilayer structure; they are simply gas bubbles stabilised by a surfactant at their surface. Proteins such as albumin can be used in place of conventional phospholipid surfactants. As mentioned, when microbubbles of gas-carrying lipospheres also carry drugs, they are dually effective drug delivery vehicles, being both the carrier and the activator for the ultrasonic drug delivery.

Some drugs can spontaneously associate with the surface of liposomes and microbubbles. An example is the association of negatively-charged DNA and RNA with liposomes and microbubbles composed of cationic surfactants. When the liposome or microbubble is fragmented by cavitation events, the drug can be released, although it may yet retain some association with the cationic surfactant. The free drug can be taken up by normal mechanisms, whereas the drug associated with fragmented surface could be taken up by pinocytosis, or related mechanisms.

Having completed this basic introduction to ultrasound, cavitation and drug carriers, this review is organised according to the general type of drug delivered. First the delivery of the largest and most sensitive macromolecules, DNA, to targeted tissue will be discussed. The focus will then turn to smaller and/or globular macromolecules (i.e., proteins). Finally, strategies for the ultrasonic delivery of conventional small chemotherapeutic drugs will be presented.

2. Ultrasonic-assisted delivery of therapeutic molecules

2.1 DNA and gene delivery

Gene delivery is a topic of intense interest in targeted drug delivery [43-49]. The use of ultrasound in gene delivery has

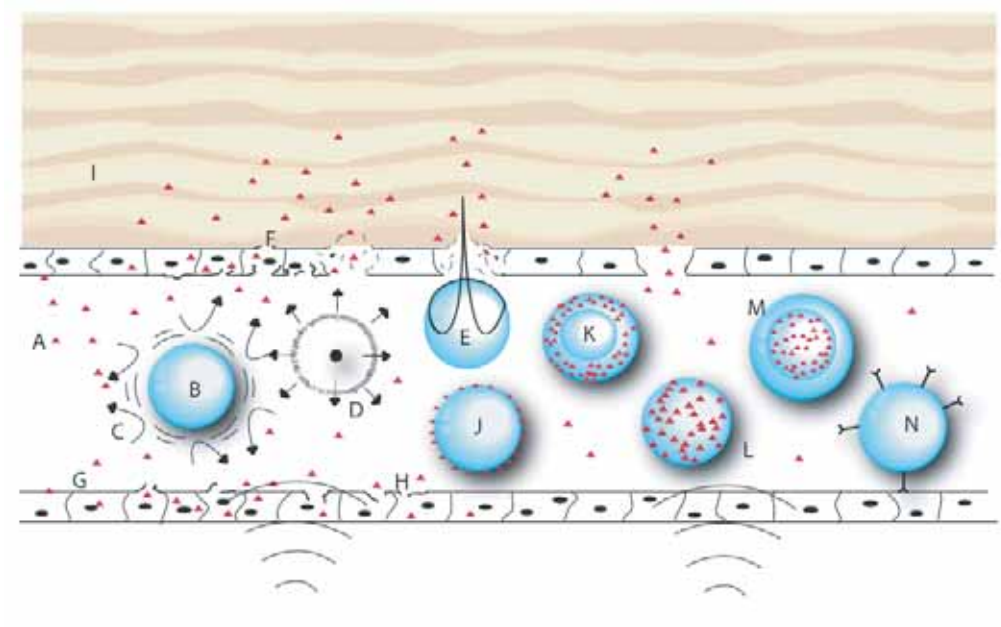

Figure 3. Schematic representation of various modes by which drug delivery can be enhanced by ultrasound. (A) Therapeutic agent (triangles); **(B)** gas bubble undergoing stable cavitation; **(C)** microstreaming around cavitating bubble; **(D)** collapse cavitation emitting a shock wave; **(E)** asymmetrical bubble collapse producing a liquid jet that pierces the endothelial lining; **(F)** completely pierced and ruptured cell; **(G)** non-ruptured cells with increased membrane permeability due to insonation; **(H)** cell with damaged membrane from microstreaming or shock wave; **(I)** extravascular tissue; **(J)** thin-walled microbubble decorated with agent on surface; **(K)** thick-walled microbubble with agent in lipophilic phase; **(L)** micelle with agent in lipophilic phase; **(M)** liposome with agent in aqueous interior; **(N)** vesicle decorated with targeting moieties attached to a specific target.

exceptional potential because the beam can be focused on a particular tissue. The trick is to release the genetic material only at the targeted site. A further complication is that the DNA (or RNA) must enter the targeted cells before it is degraded by DNase, or carried to other tissues by the blood. This section will first discuss gene carriers, and then review examples of targeting specific tissues.

2.1.1 Ultrasonically activated gene carriers

2.1.1.1 Microbubbles

Gene delivery using insonated microbubbles was first reported in 2000 [50,51] and has since become a topic of intense study. The popularity of this technique arises from the availability of commercial ultrasound contrast agents, combined with the versatility and ease of their use. The microbubbles can be injected upstream of the target region, which is easily imaged at low intensity by the presence of the bubbles. When such imaging demonstrates that the ultrasound is precisely focused on the target tissue, the ultrasonic intensity can be increased to create collapse cavitation. The collapse events appear to permeabilise the vessel walls and provide pathways for extravasation of DNA that is freely floating with the bubbles, or that is associated with the bubble surface [47,52]. Lawrie *et al.* [53] showed that transfection was not related to free radical production from collapse cavitation, but attributed transfection to transient holes in the cell membrane produced by other cavitation phenomena.

A convenient manner to prepare gene-carrying microbubbles is to insonate a mixture of surfactant, such as albumin, in the presence of gas (air or perfluorocarbon) and the plasmid or DNA fragment to be delivered [54]. If commercial contrast agents are available, the genetic material can be mixed directly with the contrast agent and injected [52,55,56]. This is often naked DNA, but one can also mix the genetic material with a cationic stabilising agent, such as poly(ethylene imine) or poly(L-lysine) [57].

2.1.1.2 Liposomes

Liposomes have been used for decades as drug carriers, and recently as gene carriers, particularly cationic liposomes. During the past decade, ultrasound applied in combination with gene-carrying liposomes has enhanced the transfection rate both *in vitro* [58-61] and *in vivo* [62].

Cationic liposomes are particularly effective in delivering DNA because the DNA stays associated with the liposome until it is acoustically activated. Most cationic liposomes contain dioleoylphosphatidylethanolamine, although some contain quaternary ammonium compounds or cationic derivatives of cholesterol [46,59,63].

2.1.1.3 Free genetic material

It is noteworthy that, in the absence of microbubbles, liposomes, or other acoustically active agents, ultrasound still enhances transfection, although not as much as with them present [64]. As an *in vitro* example, ultrasound without any extraneous cavitation agents increased transfection of human endothelial cells and vascular smooth muscle cells slightly (compared with a non-ultrasound control), whereas when microbubbles were present, the transfection increased by ≥ 1000-fold [64]. Such transfection is usually attributed to sonoporation of cell membranes by acoustic activity, but the mechanisms leading to sonoporation are not proven in the absence of microbubbles. It is plausible that *in vitro* there is sufficiently dissolved gas and enough organic molecules with a surfactant nature that the insonation itself generates cavitation bubbles that subsequently grow, collapse, and shear cell membranes. On the other hand, postulating an *in vivo* source of cavitation nuclei is more problematic because the lungs are very efficient at clearing small bubbles from the circulatory system [6,23].

Another disadvantage of using free DNA is that ultrasound causes DNA fragmentation [28,65], which reduces the transfection efficiency [57]. Complexing the DNA plasmids with cationic polymers such as poly(ethylene imine) and poly(L-lysine) preserves the integrity of the DNA on exposure to 20 kHz ultrasound [57]. Another strategy to protect the DNA is to expose the tissues to ultrasound and subsequently perfuse the genetic material into the targeted region [66].

2.1.2 Examples of ultrasound-assisted gene delivery

Ultrasonically enhanced gene delivery to cultured cells *in vitro* has been abundantly reported. However, this review will focus on published examples of gene delivery to tissues *in vivo*.

2.1.2.1 Cardiac tissue

A major thrust in ultrasound-enhanced gene delivery is the combination of visualising coronary arteries or other heart structures, and then delivering genes or drugs to the diseased tissues [67]. In most published studies, a marker gene has been delivered to show efficacy of the concept. One of the long-term goals of cardiac gene delivery is to deliver genes that will inhibit, or reverse, stenosis of coronary arteries or to regrow missing or diseased tissue.

Bekeredjian *et al.* imaged and delivered a luciferase transgene marker to the left ventricle of rat hearts using both commercial and custom gas microbubbles mixed with the genetic material [68,69]. The sonography (1.3 MHz) was triggered by electrocardiographic signals to expose the microbubbles every four ventricular contractions. Genetic expression was significantly greater using triggered rather than continuous insonation [70]. The resulting gene expression was limited to the heart, with slight expression in the liver and pancreas, and no expression in the brain, muscles or lung, thus validating their goal of targeted delivery. The microbubble destruction caused very little change in the regulation of host genes in the heart [71].

Shohet *et al*. mixed an adenovirus delivery system encoding a β-galactosidase gene to albumin microbubbles and injected them into the jugular vein of rats, with or without insonation at 1.3 MHz [50]. Gene expression was enhanced 10-fold when the genetic material was mixed with the microbubbles and insonated. If the virgin microbubbles were insonated, and the genetic material subsequently infused, gene expression was enhanced only twofold compared with gene infusion without any insonation. This experiment indicates that gene delivery is most efficient when applied concomitantly with insonation; apparently the cell permeability to genetic material decreases with time after insonation.

2.1.2.2 Vascular tissue

There are many reports of ultrasound-enhanced gene delivery to arteries, with the goal of developing a delivery system that can be used to treat stenosis and other arterial diseases. Both endovascular ultrasound and extracorporeal ultrasound have been studied. An endovascular ultrasonic catheter was employed in a rabbit femoral artery model of overdilation [72]. An adenovirus delivery system expressing a blue fluorescent protein (*BFP*) gene or a non-viral plasmid with the *BFP* gene, was infused with or without application of 2 MHz endovascular ultrasound. Insonation increased plasmid-mediated gene expression 12-fold and viral-mediated gene expression 19-fold over their non-insonated controls.

Beeri *et al.* [55] mixed an adenovirus encoding a reporter gene with albumin microbubbles and delivered it to the aortic root of rats. Extracorporeal ultrasound was employed both to image and to disrupt the microbubbles. Genetic expression in the aortic tissue was increased if the blood flow was transiently stopped during insonation. Some incubation time appears to be helpful in allowing transport of the gene into the cells.

Also employing a rat overdilation model, Taniyama *et al.* [64] delivered a naked plasmid encoding a luciferase gene to the carotid artery. Neither plasmid DNA alone, nor plasmid DNA with ultrasound induced significant gene expression. However, when albumin microbubbles were mixed with the plasmid and insonated, and the data showed ≥ 1000-fold increase in luciferase activity compared with plasmid DNA alone.

Huber *et al.* [73] employed a rabbit carotid artery model and used high-intensity focused ultrasound at 0.85 MHz to deliver a plasmid reporter gene. Consistent with the above results, they found that gene expression after exposure to plasmid alone or plasmid plus microbubbles was minimal. Although insonation of the vessel with plasmid increased the gene expression, insonation with plasmid and albumin microbubbles increased gene expression even more.

In an *ex vivo* model, Teupe *et al.* [54] exposed excised porcine arteries to albumin microbubbles mixed with plasmid DNA with a reporter gene. Ultrasound alone, or microbubbles alone, produced a minimal gene expression, whereas the combination of 2.2 MHz diagnostic ultrasound with microbubbles significantly increased gene expression. The perfusion flow rate through the artery affected the expression,

with a maximum expression at 2 ml/min (compared with no flow or higher flows).

In a slight twist of the conventional reports above, Du *et al.* [74] synthesised echogenic gas-filled solid polymer microspheres containing a reporter plasmid. These were injected into the femoral artery of pigs and subjected to diagnostic Doppler imaging. Gene expression was subsequently found (6 days later) in all experiments. Unfortunately, there were no control experiments in which genes were delivered without ultrasound. Solid echogenic microspheres appear to deliver genes, but at this point the transfection efficiency cannot be compared with microbubble delivery systems.

2.1.2.3 Tumour tissue

There is considerable interest in genetic targeting of tumours as an anticancer therapy, and several studies have examined the feasibility of such a therapy. For example, Manome *et al.* [75] grew MC38 murine colon carcinoma in mice and injected a naked plasmid with a reporter gene, directly into the tumour. Application of 1 MHz transcutaneous insonation increased the reporter activity threefold over the non-insonated control. Higher power densities increased reporter activity, as did increasing insonation time up to 30 s.

Using a cationic lipid–cholesterol transfection complex, Anwer *et al.* [66] delivered an *IL-12* gene to a mouse tumour model. Insonation significantly increased the gene expression, and the transfected tissue was limited to the tumour vasculature. The expression of *IL-12* was sufficient to inhibit tumour growth compared with the control conditions. Although the authors did not expressly introduce microbubbles, or mention their possible existence, it is possible that the synthesis of the DNA–lipid–cholesterol complex created some liposomes, some of which could contain gas and become acoustically active during insonation. McCreery describes a similar procedure for plasmid gene delivery to human adenocarcinoma grown in nude mice, with similar results [62].

Huber *et al.* [76,77] delivered a naked plasmid DNA reporter gene into subcutaneous Dunning prostate tumours in rats. Insonation at 0.85 MHz produced a 10- to 15-fold increase in reporter activity compared with non-insonated controls.

In a related procedure, Bao reports the use of a lithotripter to deliver a luciferase reporter to B16 melanoma grown in mice [78]. A lithotripter generates a shock wave containing a spectrum of acoustic frequencies and can produce cavitation phenomena. The plasmid was injected directly into the tumour, and in some cases, 10% air (relative to the tumour volume) was also injected. Various regimens of shock waves were subsequently applied, and the luciferase expression evaluated. The results showed that shock waves with direct injection enhanced expression ~ 15-fold relative to direct injection alone, and that application of air produced a further 7-fold increase in expression. More information on *in vitro* gene delivery by lithotripter shock waves is reviewed elsewhere [49].

2.1.2.4 Skeletal muscle and bone tissue

Skeletal muscle is one of the largest tissue systems in the body, and as such can express a large quantity of therapeutic protein into the circulatory system to eventually find another target tissue, or to produce a 'whole body' therapy. Other applications aim at promoting neovascularisation for tissue repair. Several articles have been written on ultrasound-enhanced gene delivery to skeletal muscles.

In some *in vivo* experiments in rats, the triceps brachii or gastrocnemius was exposed to diagnostic ultrasound. A mixture of plasmid encoding for luciferase and commercial microbubble contrast agents was injected into the muscle, followed immediately by insonation. The results showed that insonation in the presence of microbubbles and plasmid resulted in higher luciferase activity than injection of plasmid alone, plasmid plus microbubbles (without insonation), or plasmid lipofection. Virtually no luciferase activity was observed in other muscles or organs.

In similar experiments, Christiansen *et al.* injected a luciferase reporter coupled to cationic lipid microbubbles by intra-arterial or intravenous routes [79]. Only the rat hind limb skeletal muscle was insonated. Transfection was 200-fold greater with the intra-arterial than intravenous route, and was similar to intramuscular injection of plasmid. No transfection was observed if the plasmid was administered without microbubbles. These results indicated that insonation of plasmid–microbubbles at a remote site (away from the injection site) produced sufficient extravascular deposition and DNA incorporation leading to genetic expression. Other groups have also used luciferase and microbubbles to transfect rodent skeletal muscle [80,81].

Schratzberger *et al.* [82] investigated the effect of duty cycle and power density on naked DNA transfection in rabbit quadricep muscle. A plasmid reporter gene was injected via the intramuscular route, and insonation was immediately applied. Consistent with the above findings, the plasmid reporter gene showed very little expression when DNA, but not ultrasound, was applied. The group concluded that gene expression increased as the duty cycle (the fraction of time that ultrasound is activated in a pulse sequence) or the power density increases, consistent with the hypothesis that cavitation is involved in the gene delivery. No microbubbles were used in these experiments.

2.1.2.5 Fetal tissue

Gene transfection on a mouse fetus was carried out *in utero* using a plasmid encoding a fluorescent marker [56]. In this procedure, an incision was made on a pregnant mouse and the uterus was externalised. Then, plasmid mixed with microbubbles was delivered to specific locations by micropipette, the ultrasound applied, the uterus replaced, and the fetus developed for another 24 – 48 h. Gene expression with naked DNA alone, DNA with microbubbles, or DNA with ultrasound was low and showed no significant difference between these conditions. However, application of 1 MHz ultrasound to plasmid and

microbubbles produced ~ 1000-fold enhancement in gene expression. Micrographs showed some disruption to the fetal skin under conditions of ultrasound with microbubbles.

2.1.2.6 Brain

It would be advantageous to express genes directly in the brain that may ameliorate debilitating brain diseases, such as Alzheimer's. So far, there are no examples of *in vivo* enhancement of gene delivery using ultrasound. However, there are some publications of *in vitro* delivery to neural tissue in culture [63,83], and reports of employing ultrasound to breach the blood–brain barrier [35,43,84-87].

2.1.2.7 Lung

Lung tissue contains gas and, therefore, reflects and scatters ultrasound; thus transcutaneous ultrasound cannot be used to deliver therapeutics to the lungs. An alternative use of ultrasound in gene delivery is to create an aerosol using an ultrasonic nebuliser. Cationic DNA complexes have been delivered to mice, rats and guinea-pigs, and their lung epithelial cells transfected [88,89].

2.1.3 Needs in gene delivery

As most of the recent progress in gene delivery involves microbubble cavitation, the most pressing need is a better understanding of cavitation physics and ultrasound–microbubble interactions. Of course, this will aid not just in gene delivery, but in all aspects of drug delivery.

Another need is to develop better protection for the genetic material so that it is not degraded by fluid shear forces or enzymes before it can be delivered to the cells. In addition, the loading of genetic material needs to be optimised such that the genes are delivered only to the target tissues and not to tissues downstream from the insonated site.

2.2 Protein delivery

Here, proteins will be differentiated from other macromolecules by their size and characteristic polypeptide backbone. This review will consider proteins as having a molecular weight of ≥ 2000 Da. Smaller polypeptides can be treated as low-molecular-weight drugs. Compared with low-molecular-weight drugs, proteins have very different transport and solubility characteristics in tissues, and, therefore, their delivery is usually much more complex. Specifically, proteins do not diffuse easily through solids and most gels. A simple bilayer lipid membrane is sufficient to preclude the transport of a protein. Two systems with large surface area, the skin and the gastrointestinal (GI) tract, are problematic. The skin is designed to be impermeable to protein transport, and the GI tract hydrolyses proteins into smaller peptides and amino acids for absorption. The high surface area of the lungs makes the pulmonary system attractive for protein delivery. However, lung tissue blocks ultrasound, as previously mentioned. These limitations relegate most protein delivery to injection with some applications employing inhalation.

Regulatory hormones are the centre of focus for controlled protein delivery; specifically, insulin delivery for diabetes therapy comprises the vast majority of protein delivery research, with some research effort in growth-related hormones and birth-control hormones. With respect to ultrasound-assisted protein delivery, nearly all research is focused on insulin delivery, and the great majority of that research is on transdermal delivery. Ultrasonic delivery is ideal, because a small transducer can be placed on the skin surface for a painless, non-invasive delivery route.

2.2.1 Transdermal protein delivery

There is a tremendous amount of literature on the use of ultrasound to enhance the permeability of skin for transdermal drug delivery, including several excellent reviews [90-97]. Therapeutic levels of ultrasound (1 – 3 MHz, 1 – 3 W/cm^2) have been used for years to drive small hydrophobic molecules, such as steroids, into or through the skin [91,92,96-101]. Chemical enhancers have been used to further increase the permeability [102-104]. However, no significant transport of protein could be achieved until 10 years ago when Mitragotri *et al.* [102-114] showed that low-frequency ultrasound was much more effective than higher frequencies and provided evidence as to the mechanism involved. Skin permeability increased with decreasing frequency, and with increasing time of exposure and intensity (beyond a threshold), thus identifying collapse cavitation as a causative mechanism [105-107,110,111,115].

The current theory is that cavitation events open reversible channels in the lipid layers of the stratum corneum, and provide less tortuous paths of transport for proteins, such as insulin [90,105-107,110]. Electron microscopy on skin exposed to low frequency ultrasound revealed the removal of surface cells and the formation of large pores and pockets (~ 20 µm), large enough to accommodate transport of proteins and other large molecules [116-119]. Tezel and Mitragotri [24,120] have formulated a model of the shock wave and of microject cavitation events and their impact on skin permeability. Although this model can be fit to the data of this group, it is based on many assumptions and parameters, and more direct evidence is needed to conclusively reveal the mechanisms of ultrasound-enhanced transdermal protein delivery.

The future of ultrasound-enhanced transdermal protein delivery is brimming with potential, but it has not yet appeared in the clinic. Because large pores and channels are opened through the natural skin barriers, many hormones and proteins could be candidates for transdermal delivery [105,106,121-127]. The effect of the ultrasound on the protein conformation and/or activity needs to be addressed in more detail.

2.2.2 Other protein delivery

2.2.2.1 Activated protein depot

Kwok *et al.* [128] studied insulin-loaded drug depots of poly(2-hydroxyethyl methacrylate) and poly(ethylene glycol) with a surface layer of C18 alkyl chains. In the absence of

insonation, the alkyl chains appeared to form an organised and less permeable barrier to proteins; on insonation the surface organisation was apparently disrupted, and protein within the depot matrix escaped. In this research very little insulin was released until 1.1 MHz ultrasound was applied. On termination of insonation, the low permeability of the lipid-like surface layer was restored. Such a device is envisioned as a subcutaneous depot with an external transducer positioned over the depot that can be activated either automatically or on demand as insulin is required.

2.2.2.2 *Thrombolytic enzymes*

The transport of tissue plasminogen activator (tPA) and other lytic proteins, such as urokinase, into clots is beneficial in increasing the rate of fibrinolysis of clots [129,130]. In most studies, the protein is delivered to the clot via intravenous catheter, and the ultrasound is applied transdermally.

Francis *et al.* [131] showed that 1 MHz ultrasound increased the rate of uptake and the depth of penetration of tPA into clots. They speculated that enhanced transport could be due to fluid motion from shock waves, microstreaming, or penetration of the clot by microjets. Later studies showed that pulsed low frequency ultrasound (27 kHz) is more effective than higher frequencies in enhancing fibrinolysis by tPA, again supporting a non-thermal cavitation-related transport into the clots [132].

The same group also showed that water permeability through a fibrin gel was increased by ultrasound (in the absence of any lytic enzymes) [133]. This increase was reduced when the fibrin gels were degassed, and was thus attributed to cavitational activity. Other authors have shown that high-intensity, focused ultrasound produced echo-dense material in the clot, most probably by creating cavitation bubbles [134].

Although ultrasound by itself is beneficial in enhancing thrombolysis, the addition of microbubbles appears to enhance thrombolysis even more [135-139]. In 1995, it was shown that application of ultrasound with albumin-based contrast agent significantly increased fibrinolysis by urokinase of thrombus *in vitro* [135-137]. *In vivo* studies using tPA and other enzymes led to the same conclusions [138,139].

Another advance in this technology is to attach receptors for the thrombus material to the microbubbles, thus attaching the bubble to the thrombus surface during the ultrasonic exposure. These ligands often bind to the glycoprotein IIb/IIIa receptor of platelets, which are expressed when platelets coalesce into a clot, and are used with or without a thrombolytic protein present [137,140]. The authors envision that bubbles cavitating on the thrombus surface will produce microjets that can mechanically disrupt the clot.

2.2.2.3 *Pulmonary delivery by ultrasonic nebulisation*

There are several reports of protein delivery to the lungs via inhalation of ultrasonically aerosolised protein solutions. Nebulisation requires rather severe cavitation and, therefore, it has been found beneficial to protect the proteins by the addition of certain surfactant stabilisers [141,142]. These additives appear to complex with the proteins, and protect them from degradative shear stresses and perhaps from free radical attack. It is possible that the surfactants may form micelles or liposomes that sequester the proteins and protect them from cavitation stresses. A short list of ultrasonically nebulised protein delivery includes IFN [143], platelet-activating factor [144], lactate dehydrogenase [141], superoxide dismutase [145], α_1-protease inhibitor [146,147], urokinase plasminogen activator [148] and aviscumine [142].

2.2.3 *Needs in protein delivery*

As mentioned at the beginning of this section, protein delivery is fairly limited at present by very slow diffusion of proteins through skin and polymeric depots, and by the proteinolysis that occurs in the upper GI tract. The authors foresee a need for technology in which a polymeric depot can be implanted, perhaps in subcutaneous, intraperitoneal or intramuscular locations. Timed drug release could then be activated by transdermal ultrasound. Polymers in a depot can be degraded by ultrasound [27,149], but this is a fairly slow process, and the same physicochemical mechanisms that degrade the polymers may degrade the proteins in the depot. What is needed is technology that will open and close the depot to protein transport. The technology of Kwok *et al.* [128] is a good start in this direction, but the depot needs to completely shut off protein delivery when not insonated.

It would also be advantageous if the depot were activated by stable cavitation or some other low intensity phenomenon related to ultrasound. Collapse cavitation may be capable of opening depots, but repeated ultrasonic exposure over long periods may start to have adverse effects on the healthy tissue in the region of the depot. Finally, it would be beneficial if the depot were eventually degradable, so that it did not need to be surgically removed when it was emptied. A refillable depot would also be useful.

2.3 Ultrasound-enhanced small chemical delivery

In the last two decades, ultrasound has been investigated as a delivery mechanism for a variety of therapeutic agents to diseased cells throughout the body. Section 2.3.1 will discuss the use of ultrasound in the delivery of conventional chemotherapy agents for vertebrate tissues, whereas Section 2.3.2 will focus on the enhanced activity of antibacterials in the presence of ultrasound.

2.3.1 *Traditional chemotherapy*

The use of ultrasound as a drug release and potentiation mechanism in traditional chemotherapy has been studied extensively. This section will discuss ultrasonic chemotherapy delivery in free, micellar and liposomal forms.

2.3.1.1 *Free drug*

A synergistic effect between the pharmacological activity of chemotherapeutic drugs and ultrasound has been reported for a variety of agents. Loverock *et al.* [150] have shown that 1 h of

exposure to ultrasound (2.3 W/cm^2 at 2.6 MHz) rendered doxorubicin significantly more toxic to Chinese hamster lung fibroblasts. Exposure to ultrasound alone did not affect cell viability. Flow cytometry revealed an increase in doxorubicin concentration inside the cells, but the authors did not attribute the increased uptake to any particular mechanism.

Tachibana *et al.* [151] studied the effect of 0.3 W/cm^2 and 48 kHz ultrasound on the cytotoxicity of cytosine arabinoside (Ara-C) towards human leukaemia (HL60) cells. Insonation for 120 s in the presence of Ara-C, reduced the number of observed colonies 100-fold when compared with cells incubated with the same concentration of the drug. This increase in cell death was not caused by hyperthermia, as the temperature increase was ≤ 0.2°C throughout the experiments. Scanning electron microscopy of insonated cells revealed a decrease in the total number of microvilli and 'a slight disrupted cell surface with flap-like wrinkles' under the action of ultrasound. They concluded that low intensity ultrasound altered the cell membrane, which resulted in the increase of Ara-C cell uptake.

In another study, Tachibana *et al.* [152] have shown that the exposure of HL60 cells to 255 kHz ultrasound and MC 540 for 30 s formed pores in the cell membrane. They claimed that sonoporation caused the cytoplasm of HL60 cells to extrude through pores formed in their cell membrane. The same effect was not observed when cells were exposed to ultrasound alone, thus implicating a synergism between the drug and ultrasound.

Saito *et al.* [152] have shown that exposure to ultrasound increased the permeability of corneal endothelium cells. The increase in permeability appeared to be reversible and the cells regained their membrane integrity after several minutes.

Rapoport *et al.* [154] investigated the increase in intracellular drug uptake by HL60 cells as a result of ultrasonic irradiation (67 kHz and 2.5 W/cm^2) using fluorescence techniques. It was found that the amount of doxorubicin that intercalated the DNA increased after 1 h of insonation. In another related study, Munshi *et al.* reported that the IC_{50} (inhibitory concentration 50%) for doxorubicin was reduced substantially when HL60 cells were sonicated for 1 h at 80 kHz in the presence of doxorubicin [155]. In both studies, the temperature of the cell suspensions was kept at 37°C using a thermostatic bath and, therefore, hyperthermia was not part of the mechanism of this ultrasonic enhanced killing.

Yu *et al.* [156,157] investigated drug-ultrasound synergism applied to doxorubicin-resistant and cisplatin-resistant human ovarian carcinoma cell. They concluded that the synergism reported was not due to the decrease of the multidrug resistance (*mdr1*) gene level by insonation. Thus, multidrug resistance gene expression is not inhibited by ultrasound. The group did not report whether any changes in drug accumulation occurred.

Some investigators attribute this synergism between drug activity and ultrasound to an increase in the local temperature of the sonicated area (hyperthermia) [158-160]. Saad and Hahn [159] exposed Chinese hamster cells to ultrasound (average intensities in the range 0.5 – 2 W/cm^2) and to several drugs at temperatures in the range 37 – 43°C. The study showed that at lower intensities (0.5 W/cm^2) the cytotoxicity of doxorubicin was significantly enhanced when the temperature was raised from 37 to 41°C. At higher ultrasonic power densities (1 W/cm^2), the cytotoxic effect of doxorubicin increased threefold when the temperature reached 41°C. The study concluded that the 'temperature threshold' decreases as the power intensity of ultrasound increases.

In summary, ultrasound has been used in combination with chemotherapeutic agents for increased efficacy. Insonation appears to enhance the transport of drugs into cells and tissues. Considering the physics of cavitation processes, it is the authors' opinion that ultrasound produces transient micropores in the cell membrane, which would increase the passive accumulation of the drugs in the cells and tissues. Although the cytotoxic effect of chemotherapeutic agents has been shown to increase with insonation, this effect has not been shown to be independent of any increase in drug uptake.

Most studies attribute ultrasonic enhanced killing of cancer cells in the presence of drugs to a phenomena called chemopotentiation. The use of the term chemopotentiation is rather misleading, as it suggests that ultrasound alters the structure of the drug and renders the chemical more potent. Most data reported in the literature support the hypothesis that ultrasound permeabilises the membrane so that more chemotherapeutic drug molecules are able to diffuse into the cells. In some cases, the permeabilisation appears truly synergistic in that both drug and ultrasound are required simultaneously to render the cell membrane more permeable. In general, at sufficiently high intensities the ultrasound permeabilises the membrane, most probably through shear stresses in the membrane from microstreaming or shock waves.

A major problem associated with whole body chemotherapy is not totally alleviated by ultrasound; the drug is still delivered systemically, thus causing systemic side effects. For this reason, research in recent years has focused on developing molecular vehicles that can sequester the drug inside a package, and release it using ultrasound stimulus at the tumour site. Two types of drug delivery molecules have been developed for this purpose: polymeric micelles and liposomes.

2.3.1.2 Micelles

Polymeric micelles have been used to improve site-specific drug delivery in cancer therapy. The technique relies on the small size of these carriers to extravasate at the tumour site where the drug can diffuse into the tumour and carry out its therapeutic effect [161-164]. Although several groups have investigated the use of polymeric carriers to deliver chemotherapeutic as well as other drugs, the only group that has reported the use of polymeric micelles in conjunction with ultrasound is Rapoport, Pitt and colleagues at the University of Utah and Brigham Young University. Their micelles are made from the Pluronic® (BASF Corporation) family of block copolymers.

Pluronic polymers are triblock copolymers of poly(ethylene oxide) (PEO)–poly(propylene oxide)–PEO, which form micelles at sufficiently high aqueous concentrations [165-170]. These micelles have several advantages as drug delivery vehicles. They are stable in blood and other biological fluids, and are large enough to escape renal excretion, while being small enough to extravasate at the tumour site. Antineoplastic agents can be easily sequestered inside the core of these polymeric micelles by the simple act of mixing [154,171], thus avoiding the complexities involved with covalently bonding the drug to the polymeric carrier [172]. Several studies have reported the effect of Pluronic surfactants in overcoming multi-drug resistance [173-175]. Furthermore, the PEO chains on the micelle exterior prevent its recognition by cells of the reticuloendothelial system (RES).

The feasibility of using ultrasound with Pluronic micelles to deliver anticancer agents *in vitro* was first reported by Munshi *et al.* [155] and Husseini *et al.* [39], who reported that a combination of 70 kHz ultrasound and Pluronic–P105-encapsulated doxorubicin substantially increased the cytotoxicity of the drug. The enhanced toxicity on insonation was attributed to the release of the agent from micelles under the action of ultrasound [176,177].

Using fluorescent microscopy and flow cytometry, they also reported that insonation enhanced the intracellular uptake of Pluronic micelles and their internalisation into the nucleus of HL60 cells [178-183].

The main challenge facing the use of micelles to deliver chemotherapy drugs is in maintaining the concentration of the polymer above the critical micellar concentration, to guarantee that the micellar structures remain intact and do not dissolve and prematurely release the drug before reaching the target site. Pruitt *et al.* [184] have stabilised Pluronic micelles with an interpenetrating network using *N*,*N*-diethylacrylamide to form Plurogels™. The network expands at room temperature, allowing the drug to accumulate inside its hydrophobic core, and contracts ≥ 31°C, thus trapping the drug at body temperature. The network-micellar structure is eventually degraded after a few days (the half-life is ~ 17 h [185]). Plurogel micelles have also been shown to release doxorubicin after exposure to ultrasound [186].

Recently, *in vivo* studies examined the feasibility of acoustically-activated drug delivery from Plurogel. Nelson *et al.* [187] showed that exposure to 70 kHz ultrasound of doxorubicin encapsulated in Plurogel significantly decreased the size of colorectal cancer tumours in rats. When unencapsulated doxorubicin was administered, the same dose was lethal to the rats within 2 weeks of injection. Gao *et al.* [188] studied the intracellular distribution of fluorescently-labelled non-stabilised Pluronic P105 and Pluronic P105 stabilised using PEG-diacylphospholipid. The study showed that insonation at 1 MHz was able to enhance the accumulation of these labelled micelles at the tumour site in ovarian cancer-bearing *nu-nu* mice. Using the same *in vivo* mouse model, Rapoport *et al.* [189] showed that micelle accumulation was significantly higher in the ultrasonicated tumour than in the non-insonated tumour in the same mouse.

The mechanisms of this acoustically-activated micellar drug delivery system are still being investigated, and there is a strong correlation with insonation frequency and power density that suggests a strong role of stable and transient cavitation.

2.3.1.3 Liposomes

Unlike micelles, liposomes can sequester both hydrophilic and hydrophobic drugs in their aqueous interior and lipid bilayer membrane, respectively. Liposomes are also larger than the polymeric micelles; the average diameters of liposomes are in the range of 150 – 200 nm, compared with 5 – 30 nm for micelles. Herman *et al.* [190] showed that liposomes are able to encapsulate doxorubicin, and reported a decrease in the cardiotoxicity of the drug. Zvi *et al.* [191] reported that liposome-encapsulated doxorubicin accumulates preferentially in cancerous muscle tissues when compared with tumour-free muscles. The studies mentioned above rely on the increased accumulation of liposomes at the tumour site due to increased extravasation (passive targeting). Recently, studies have been reported in the literature in which ultrasound is used as an active targeting mechanism to release drugs from liposomes [32,42,101,192].

Ning *et al.* [37] demonstrated that ultrasound-induced hyperthermia, in addition to enhancing drug antitumour activity, accelerated the release of doxorubicin from long-circulating liposomes. The group reported that by increasing the temperature from 37 to 41°C, the rate of release of doxorubicin was increased sixfold after 1 h of sonication at 2 W/cm^2. The accumulation of doxorubicin in RIF-1 tumour cells was 10-times higher when introduced in liposomes at 42°C compared with when introduced as free drug at 37°C. Several other reports have shown that other drugs can be released from liposomes using ultrasonic hyperthermia [2,193-195].

2.3.2 Antibacterial chemotherapy

Rediske *et al.* [198-200] showed that ultrasound increased the killing of bacteria both in planktonic suspension [196,197] and biofilm forms in the presence of antibiotics. This synergistic killing effect was most pronounced at lower frequencies and decreased as the frequency of insonation increased [201,202]. The effect was more pronounced in *Escherichia coli* and *Pseudomonas aeruginosa* (Gram-negative bacteria) than in *Staphylococcus epidermidis* (Gram-positive bacteria). They found that this enhanced killing synergistic effect is prevalent with certain antibiotics (the aminoglycosides), but does not exist when others are used. They hypothesised that stable cavitation or sonoporation might be involved in increasing the transport of antibiotics into the bacteria either by reducing the mass transfer boundary layer around the cells, or by altering the cell membrane, thus allowing the antibiotics to diffuse through newly formed membrane pores.

In vivo experiments in a rabbit model of an implant infection confirmed the increased toxicity of gentamicin

against *E. coli* biofilms in the presence of low frequency ultrasound (28.48 kHz and 0.3 W/cm^2) [203]. Pulsed ultrasound applied for 48 h with gentamicin was effective in eliminating *E. coli* infections [204], but was less effective against *P. aeruginosa* [205]. Vancomycin combined with ultrasound was somewhat effective against *S. epidermidis* infections in the same rabbit model [206].

2.3.3 Needs in ultrasonic-enhanced small-chemical delivery

The role of ultrasound in small-chemical delivery involves permeabilisation of the cell membrane, such that these molecules can enter more easily. At present, very little is known about how ultrasound permeabilises the cell membrane. Are small transient holes formed for µs timescales, allowing diffusive entry? Or are larger, more permanent holes or disorganised regions formed? Is a different treatment needed for molecules of different sizes?

Neither is much known about how ultrasound may regulate gene expression and protein production. Does ultrasound cause a stress response (similar to heat shock) that may enhance or interfere with the action of drugs? Is this response similar for all cells, and does it differ for various ultrasonic frequencies and intensities? These and other questions about cell physiology need to be addressed.

Finally, liposomes used in drug delivery are thermodynamically stable, but are cleared by the RES. Alternatively, micelles may not be cleared by the RES, but they are thermodynamically unstable when diluted in blood. Neither system is ideal and, thus, there is a need to make the liposomes more stealthy and the micelles more stable.

3. Expert opinion

3.1 The future of ultrasonic-activated drug delivery

It is clear that the field of ultrasonic-enhanced drug delivery has expanded tremendously during the past decade, and we expect that this trend will continue as our understanding and technology increases. Furthermore, the use of microbubbles to assist drug delivery has exploded in the past 5 years, and we expect that much of the technological growth in the next decade will be in the clever use of microbubbles and ultrasonic pulse sequences.

The published literature regarding ultrasound and microbubble in gene delivery is solid, makes important contributions, and is definitely worth studying. This review has cited many innovations, and more are sure to come. We propose that ultrasound-enhanced gene delivery will experience the greatest growth and provide the greatest medical contribution because ultrasound and microbubbles allow genes to be delivered through non-viral technologies to specific locations. Such genetic therapy can make monumental contributions to the treatment of heart disease, vascular disease, cancer, autoimmune diseases, and much more. As microbubble collapse is controlled and fine-tuned, we speculate that it can be applied to deliver genetic therapy to the brain to treat neurological diseases.

The area of protein delivery has been limited in the past to insulin delivery technology, and will probably remain so, with some small expansion into delivery of a few other small regulatory hormones. Despite 10 years of solid research on transdermal delivery of proteins, there are still limitations in the rate at which a protein can pass through the skin without inflicting permanent damage. Transport can be increased by increasing the surface area, but a larger treatment area and heavy transducers may lead to difficulty with patient compliance. We foresee some growth in this area, but not wide clinical application until the size of the transducer is reduced, such that it can be easily attached and carried at work or at home.

Technological developments for small chemical delivery will follow those of gene delivery. For cancer chemotherapy or antibiotic therapy, delivery to specific tissues will be targeted by decorating the carriers or microbubbles with antibodies or other site-specific adhesive molecules.

3.2 What are the critical needs?

The basis of future technological advancement requires a better understanding of the behaviour of microbubbles in ultrasonic fields. This will require more modelling and experimental understanding of the physics of microbubble oscillation and collapse as a function of microbubble size, internal gas composition, membrane or wall thickness, mechanical properties (i.e., modulus, shear strength, viscosity), acoustic frequency and pressure amplitude. At present, most ultrasound/microbubble drug delivery is done with imaging transducers at high frequencies that are not necessarily optimised for cavitation or drug delivery. What are the optimal bubble sizes and ultrasonic frequencies for drug delivery? The goal of such research should be to develop the acoustic parameters and perhaps the pulse sequences that can excite a bubble to cavitate without imposing mechanical or thermal damage to tissues. Some modelling effort has commenced [17,207], but much more is needed.

If bubble physics dictate that the optimal frequencies are different than those frequencies available in imaging transducers, then a new generation of transducers must be developed for use in ultrasound/microbubble drug delivery. Even better would be a multi-frequency transducer that can perform three functions, perhaps all at different frequencies: target imaging, bubble destruction, and cell membrane permeabilisation.

As mentioned, in conjunction with transdermal drug delivery, small-sized low-frequency transducers need to be developed so that patients can wear them for continuous insulin delivery. Although some intra-luminal transducers are available in a catheter format, at present, these are designed for imaging; catheter-based transducers also need to be developed for drug delivery.

Finally, we must not forget the issue of biological response to these new applications of ultrasound in drug delivery. Is

the frequency for optimum drug release from a carrier also the optimum frequency for permeabilising a cell membrane, without destroying the cell? It is very probable that frequencies that optimise membrane permeability are different than frequencies that optimise drug release. Thus, the response of cells and their membranes to ultrasound must be studied, so that in our efforts to optimise drug release, we do not produce collateral damage to the target or adjacent tissues.

3.3 What are the best technologies?

It is our opinion that the best technologies at present are those that employ gas bubbles, with the therapeutic agent in or on the bubble, such as DNA decorating the exterior of a surfactant-stabilised microbubble, or a thick-shelled microbubble [12,42] that can carry the drug inside, either in an oil or aqueous phase. In these systems the drug and cavitation agent are intimately mixed, and the drug is, therefore, released at the location where tissues are stressed by cavitation. Micelles and liposomes without gas are less useful because the cavitation is not necessarily produced at the same time or place as the drug. Application of free drug or free DNA is least efficient because it is not sequestered, and can, therefore, interact with non-targeted tissue, or be cleared (or degraded) before it can reach therapeutic concentrations. Gas liposomes or microbubbles constructed from native proteins (such as albumin), or with PEO chains on the surface, may have an advantage in that they will not be recognised and cleared as fast from the circulatory system [208,209].

Another current and innovative technology in which we anticipate more growth is the attachment of targeting molecules to microbubbles or drug carriers. The targeting molecules may be different for each application (such as unique or individualised antibodies), and, therefore, it may be advantageous to attach a generic binder to the microbubbles. The specific antibodies could then be attached to a complementary binder. The target-specific antibodies could then be mixed with the generic microbubbles to create custom drug delivery systems with the drug attached to the bubbles. Although biotin and streptavidin have been suggested for such a system [210], these proteins may elicit an antigenic response. Thus, other systems should be investigated, such as a self-assembled biological system or a chemical system, such as maleimide–thiol bond formation.

Without a crystal ball, one cannot predict what specific technology will be in place 10 years from now. However, we can predict that ultrasonic-activated drug delivery will play an ever-increasing role in targeted drug therapies.

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- Targeting moieties were attached to microbubbles.

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