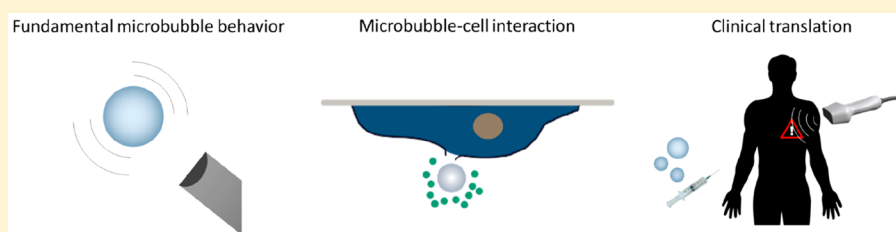


The Role of Ultrasound-Driven Microbubble Dynamics in Drug Delivery: From Microbubble Fundamentals to Clinical Translation

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ABSTRACT: In the last couple of decades, ultrasound-driven microbubbles have proven excellent candidates for local drug delivery applications. Besides being useful drug carriers, microbubbles have demonstrated the ability to enhance cell and tissue permeability and, as a consequence, drug uptake herein. Notwithstanding the large amount of evidence for their therapeutic efficacy, open issues remain. Because of the vast number of ultrasound- and microbubble-related parameters that can be altered and the variability in different models, the translation from basic research to (pre)clinical studies has been hindered. This review aims at connecting the knowledge gained from fundamental microbubble studies to the therapeutic efficacy seen in in vitro and in vivo studies, with an emphasis on a better understanding of the response of a microbubble upon exposure to ultrasound and its interaction with cells and tissues. More specifically, we address the acoustic settings and microbubble-related parameters (i.e., bubble size and physicochemistry of the bubble shell) that play a key role in microbubble–cell interactions and in the associated therapeutic outcome. Additionally, new techniques that may provide additional control over the treatment, such as monodisperse microbubble formulations, tunable ultrasound scanners, and cavitation detection techniques, are discussed. An in-depth understanding of the aspects presented in this work could eventually lead the way to more efficient and tailored microbubble-assisted ultrasound therapy in the future.

INTRODUCTION

Over the last few decades, ultrasound has become an essential tool in diagnostic imaging due to its cost-effective, noninvasive, and safe nature.^{1,2} In ultrasound imaging, an ultrasound beam is transmitted through the body, where it is scattered and reflected at the different interfaces (e.g., fibers, bones, etc.) present.³ The tissue's distinctive properties result in acoustic impedance mismatches between the tissues leading to different echo intensities, which are displayed in the resulting image as contrast differences. In contrast to other commonly used diagnostic techniques, such as MRI and PET, ultrasound does not need expensive and often stationary equipment or the injection of possibly harmful radioactive agents, which makes it an ideal tool for imaging in precarious cases such as pregnancy.¹ However, standard (B-mode) ultrasound imaging suffers from poor contrast, which renders the images hard to interpret to an untrained eye.

By serendipity, it was found that injecting saline containing a small dose of tiny gas bubbles tremendously improved the contrast in an ultrasound image.^{4,5} Later, the first clinically approved gas-filled microbubbles for diagnostic imaging were

introduced. These micrometer-sized (1–10 μm in diameter) gas bubbles can be intravenously administered and consist of a gaseous core surrounded by a protective shell to extend their lifetime. To further prolong the microbubble lifetime, typically the microbubbles are filled with high-molecular-weight gases with low aqueous solubility such as perfluorocarbons.^{4,6} The microbubble shell can consist of a variety of materials such as proteins, lipids, or polymers. The stabilizing shell has been shown to dramatically affect the response of a microbubble to a driving ultrasound pulse, and different physical properties result in a different acoustic response.^{7–9} Bioinspired phospholipid-coated shells are of particular interest because they provide high flexibility while maintaining sufficient resistance to gas diffusion. Consequently, they are the main

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bubble type encountered both in research and in the clinic, and will therefore be the focus of this review. Apart from the commercial phospholipid-coated microbubble formulations, such as Definity (Lantheus), Sonazoid (GE Healthcare), and Sonovue (Bracco), custom-designed lipid-shelled microbubbles are frequently used in *in vivo* and *in vitro* studies because they allow the coupling of targeting agents,^{10–13} drug carriers,^{10–12,14,15} and multimodal imaging agents.^{16–18}

The compressibility of the gas core gives microbubbles the ability to contract and expand in response to an ultrasound field. These volumetric oscillations generate harmonic echoes much stronger than the (linear) tissue echoes.¹⁹ This unique feature is at the origin of the “contrast mode” of clinical ultrasound machinery and boosts the microbubble signal to tissue ratio. Since microbubbles are blood pool agents, this feature also enables the visualization and quantification of organ and/or tumor perfusion. Contrast-enhanced ultrasound imaging has become indispensable in the field of echocardiography and is proving its use in other imaging applications as well. For a more extensive overview of the use of microbubbles in ultrasound imaging, the reader is referred to other reviews.^{20–23}

Volumetric microbubble oscillations are not only key for contrast-enhanced ultrasonic imaging but are also crucial for drug delivery applications.^{5,19,24,25} Microbubbles oscillating near a cell membrane generate streaming in the surrounding liquid, which puts mechanical stress on the nearby cell. This stress can result in cellular deformation and the creation of small, temporary openings in the cell membrane and in between cells.^{26,27} These openings can lead to an enhanced uptake of drug molecules in the tissue when drugs are coadministered with microbubbles.²⁸ Drugs and other therapeutic agents can also be directly loaded onto the microbubble shell, and ultrasound triggering ensures that they are released only at the desired time and location, which gives the added benefits of preventing their leakage into unwanted tissue or premature degradation of the drug molecule.^{2,11,29,30} This can be especially useful for drugs that have difficulties in reaching the target site in sufficiently high concentrations without inducing side effects, such as chemotherapeutic and anti-inflammatory drugs or in the case of easily degradable drugs, such as gene and protein therapeutics. In this way, contrast agent microbubbles become theranostic tools that enable disease monitoring, drug carrier visualization, and local drug delivery simultaneously while strongly enhancing drug uptake into the target tissue.

With this review, we aim to connect the knowledge gained from fundamental microbubble studies with a focus on ultrasound-driven microbubble behavior and cellular interactions, to the therapeutic efficacy seen in *in vitro* and *in vivo* studies. In other words, we aim to connect the dots between ultrasound parameters and microbubble characteristics used in both the fundamental and clinical fields and provide a comprehensive account of the current knowledge. An in-depth biophysical understanding of microbubble–tissue interactions should ultimately lead to a more predictable, more controlled, and more efficient translation to the (pre)clinical field.

■ PART 1: ACOUSTIC MICROBUBBLE RESPONSE AND ITS IMPORTANCE TO ULTRASOUND-TRIGGERED DRUG DELIVERY

Understanding the response of a microbubble upon exposure to ultrasound is an essential prerequisite to an effective microbubble-assisted ultrasound therapy. Therefore, a vast amount of work has focused on elucidating the mechanical response of a microbubble to ultrasound in terms of the acoustic parameters on one hand and microbubble characteristics (i.e., bubble size and physicochemistry of the bubble shell) on the other hand. In this part, we describe what determines the dynamics of a microbubble in response to an ultrasound driving pulse and summarize which aspects are relevant to drug delivery.

Characteristics of Ultrasound-Driven Microbubble Oscillations. As already mentioned, microbubbles will respond to an incoming ultrasound wave through volumetric oscillations. The amplitude of these oscillations highly depends on the ultrasound frequency, pressure and pulse duration, and it is strongly influenced by the microbubble shell and microbubble size. The dynamics of a free microbubble in response to an ultrasonic field are theoretically described by the Rayleigh–Plesset equation.^{5,6} The equation is widely used because it can accurately represent the different microbubble oscillation regimes mentioned below. It can be modified to account for the viscoelastic coating,^{31–34} the presence of a nearby substrate³⁵ and nonspherical microbubble oscillations.³⁶ The Rayleigh–Plesset equation is a nonlinear differential equation and, for any driving pressure, it can be solved to give the radius of a microbubble as a function of time.^{6,37} To gain insight into microbubble resonance behavior, it can be linearized. Assuming a sinusoidal acoustic driving pulse, small amplitudes of oscillation with respect to the equilibrium radius (i.e., $R = R_0(1 + x)$ where $x(t) \ll 1$), and the neglect of higher-order terms, linearization of the Rayleigh–Plesset equation leads to the classic equation for a driven mass–spring system in which the gas can be represented by the spring while the surrounding liquid can be regarded as the mass

$$\ddot{x} + 2z\omega_0\dot{x} + \omega_0^2x = \frac{p_A}{\rho R_0} \sin \omega t \quad (1)$$

with x being a small variation in the microbubble radius R , \dot{x} and \ddot{x} being the first and second time derivatives of x respectively, z being the damping of the system, ω_0 being the bubble oscillation eigenfrequency, p_A being the driving pressure amplitude, ρ being the liquid density, R_0 being the equilibrium bubble radius, and ω being the angular ultrasound frequency.

A key characteristic of such a mass–spring system is that it contains a resonance frequency $f_0 = \omega_0/2\pi\sqrt{1 - z^2}$, where the oscillation amplitude will be maximal. Simply put, a gas bubble will oscillate in response to an ultrasound pulse due to two phenomena that push the liquid–gas interface in opposite directions: the resistance against compression of the gas inside the microbubble on one hand and the inertia of the liquid on the other hand.^{25,38} This competition corresponds to a specific time scale that, at a certain frequency, synchronizes with the ultrasound, resulting in a maximum radial response. This frequency is called the eigenfrequency of the microbubble. However, the microbubble system experiences damping resulting from a loss of energy due to viscous dissipation in

the surrounding fluid, radiation of sound, and thermal losses.⁶ Owing to this damping (z), the resonance frequency is slightly reduced (usually around 1 to 2%) compared to the eigenfrequency of the system. The damping also defines the range of frequencies over which the bubble will have a measurable response, which is typically 1 MHz around the resonance frequency. As a result, matching the incoming ultrasound frequency to the resonance frequency of the microbubbles as closely as possible will be crucial to maximizing the acoustic response,^{5,19,24} as evident in Figure 1A.

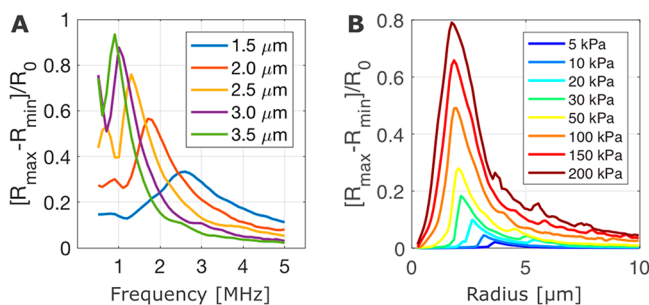


Figure 1. Relative oscillation amplitude of a microbubble with a shell stiffness of 2.5 N/m, a shell viscosity of 6×10^{-9} kg/s, and an initial surface tension of 0.02 N/m, as derived from the nonlinearized Rayleigh–Plesset equation. (A) Varying frequency and microbubble size at a constant pressure of 100 kPa. Microbubbles of different sizes display their maximum amplitude of oscillation at different frequencies of insonation, corresponding to their resonance frequency. The maximum oscillation amplitude decreases for smaller microbubbles due to an increase in damping. (B) Varying microbubble size and acoustic pressure at a constant ultrasound frequency of 2 MHz. The maximum amplitude of oscillation is obtained when the frequency of the incoming ultrasound wave matches the resonance frequency of the microbubble, which is dependent on its size and shell characteristics. When the pressure becomes larger than 50 kPa, the radius of maximal response remains the same because the resonance frequency is no longer influenced by shell characteristics.

ω_0 can be defined as

$$\omega_0 = \sqrt{\frac{3\kappa P_0}{\rho R_0^2}} \quad (2)$$

with κ being the polytropic exponent of microbubble filling gas and P_0 being the ambient pressure. This shows that the resonance frequency is directly related to the microbubble size; therefore, the amplitude of microbubble oscillation is governed by the coupling between the incoming ultrasound frequency and the size of the microbubble. Equation 2 can be simplified by filling in the constants ($\kappa = 1.1$ for heavy gases, $\rho = 1000$ kg/m³, $P_0 = 100$ kPa) to yield eq 3, derived by Minnaert³⁹ for uncoated bubbles, namely,

$$f_0 R_0 = 3.3 \mu\text{m MHz} \quad (3)$$

where f_0 is the resonance frequency and R_0 is the radius of the microbubble at rest.^{5,38} Even though eq 3 describes uncoated gas bubbles and thus disregards coating effects and surface tension, it has been proven to be a good estimate of the resonance frequency contrast microbubbles.³⁷

However, typical microbubble ultrasound contrast agents have a broad size distribution, with bubble diameters ranging from 0.5 to 10 μm . Therefore, only a small fraction of the

microbubbles will resonate at the acoustic driving frequency, and only a small fraction of the total bubble population is driven into (maximum) oscillation. To obtain a maximal response, the incident wave should match the resonance frequency of the majority of microbubbles in the population (i.e., the peak in the size distribution). While imaging pulses remain rather broadband ($\sim 70\%$) to allow for a good resolution, this matching becomes even more critical in drug delivery application where longer bursts (and thus a narrower bandwidth) are used to allow the bubble to exert a measurable effect on the target tissue. Another approach to maximizing the response of a polydisperse suspension is to use frequency sweeps, also called chirps, where a range of frequencies are used within the same incident wave in order to drive all microbubbles within the population into oscillation.⁵ Alternatively, efforts have been made to either sort polydisperse microbubbles according to size^{40–44} or to produce a monodisperse bubble population using microfluidic flow-focusing techniques.^{45–47}

Microbubbles subjected to an ultrasound field can change in equilibrium size and therefore in resonance behavior as well. Gas leakage out of the bubble through diffusion resulting in bubble shrinkage can occur as a result of the changing permeability of the bubble shell during oscillations.⁴⁸ In contrast, rectified diffusion can promote bubble growth due to a net influx of gas into the microbubble as a result of both a larger surface area allowing for gas exchange during bubble expansion than during compression and a lower pressure in the expanded bubble, which temporarily decreases the saturation concentration at the bubble surface, thereby accelerating gas intake.⁴⁹ As a result, the resonance frequency can change over time. Furthermore, bubbles can cluster due to acoustic radiation forces (which are discussed in more detail later in this work). The resonance frequency of the bubble cluster is different from that of the single bubbles and will attain an effective resonance frequency of a large bubble with the total gas volume of the bubble cluster.^{5,50} Even so, resonance is not based on bubble size alone. Other factors such as physicochemical properties of the lipid coating and the presence of nearby structures can alter microbubble resonance as well, although the former is mostly important at low driving pressure amplitudes (< 50 kPa, Figure 1B), which are typically less relevant for drug delivery applications.

The lipid coating surrounding the microbubble, while necessary to lower the microbubble's surface tension and prevent gas dissolution in order to extend its lifetime under ultrasound exposure, also hinders the bubble's ability to oscillate to a certain extent. Even though lipid coatings are more flexible than other types of coatings such as polymer coatings, they still add a substantial stiffness and viscosity to the bubble.⁵¹ Practically, for acoustic driving pressures of < 50 kPa, the presence of a stiff elastic shell increases the microbubble's resonance frequency by a factor of 2 to 4.⁸ This effect becomes negligible for the pressure ranges typically employed in imaging and drug delivery (> 50 kPa) because the shell can rupture at large amplitude oscillations. Consequently, the bubble can recover its free gas bubble surface tension and is no longer governed by the microbubble shell. Thus, for driving pressures exceeding 50 kPa, the resonance frequency of a coated microbubble can be accurately estimated using the Minnaert equation (eq 2). The viscosity of the shell does not typically affect the resonance frequency but dampens the microbubble oscillations significantly because it dissipates

energy through intermolecular friction between the lipid molecules. Since damping increases with increasing shell viscosity, changing the composition of the shell can have a significant effect on the microbubble oscillation amplitude.^{19,52} Hence, even bubbles that are the same size and consist of the same type and ratio of shell constituents can have significant variations in their acoustic response.^{53–55}

Microbubble Oscillation Regimes at Varying Driving Pressures. Various oscillation regimes can be distinguished on the basis of the microbubble oscillation amplitude: (i) linear spherical oscillations such as those described by eq 1, (ii) nonlinear and/or nonspherical oscillations, and (iii) inertial cavitation characterized by violent inertial collapses, bubble fragmentation, and the emission of broadband noise.^{5,19} Regimes i and ii are also termed stable cavitation. It is important to keep in mind that the pressures mentioned below apply only to a microbubble driven at its resonance frequency.

At very low excitation pressures (<15 kPa), microbubbles will oscillate along with the frequency of the incident wave and show stable, symmetrical, and relatively small radial oscillations governed by the microbubble coating.^{5,19} Under these low excitation pressures, the echo that is reradiated by the oscillating microbubble mainly contains the fundamental driving frequency (i.e., the frequency of the incoming ultrasound wave (Figure 2, black curve)).

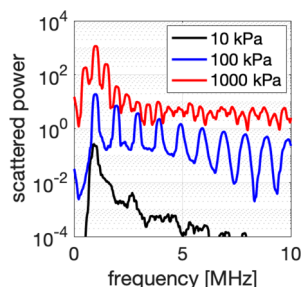


Figure 2. Frequency spectrum of echoes arising from a 3 μm microbubble (shell stiffness 2.5 N/m, shell viscosity 6×10^{-9} kg/s, and initial surface tension 0.02 N/m) cavitating at 10, 100, and 1000 kPa under 1 MHz ultrasound, as simulated using the Rayleigh–Plesset equation. The spectrum at 10 kPa contains mostly the fundamental driving frequency, while the spectrum at 100 kPa shows signs of nonlinear behavior with defined peaks at the harmonic frequencies. Both are examples of stable cavitation. Finally at 1000 kPa, the signal increases at the frequencies between the fundamentals, indicative of broadband inertial behavior.

Increasing the excitation pressure above 15 kPa will increase the amplitude of the radial oscillations, at which point a threshold for nonlinear and/or nonspherical microbubble oscillations is achieved. Nonlinearity is caused by the fact that the expansion phase and compression phase of the microbubble oscillations are no longer equal,^{8,56,57} while both coating effects (i.e., buckling, stretching, and breaking,^{48,58,59} although relevant only for pressures <50 kPa) and intrinsic gas core behavior (i.e., surface modes^{60,61}) can lead to a nonspherical response. In addition, the proximity of a rigid wall in studies where bubble dynamics are investigated against a substrate, can be a secondary source of nonlinear and nonspherical effects.^{36,62–66} This nonlinear and/or nonspherical response is evident from the microbubble echo that now contains not only the fundamental driving frequency but also harmonics and subharmonics thereof (Figure 2, blue

curve). Nonlinear and nonspherical behavior can lead to microstreaming patterns around the microbubbles because the fluid around the microbubbles will be affected in an inhomogeneous, asymmetric way.^{31,38} Microstreaming can transport molecules to and away from the microbubbles, such as drugs, for instance, and can generate shear stresses on structures around the microbubbles.^{57,67} The ability to induce mechanical stress is particularly interesting for therapeutic applications because it can open cellular tight junctions⁶⁸ and create pores in cell membranes.⁶⁹ Since the echoes from the microbubbles contain harmonics and subharmonics of the driving ultrasound frequency under this regime, it is especially useful for clinical ultrasound imaging as it allows a discrimination between the signal from the microbubbles and the signal scattered from surrounding tissues. The harmonics and subharmonics are interesting for discerning stable from inertial cavitation during drug delivery experiments as well.

If the excitation pressure increases even further (approximately >300 kPa), then the microbubble behavior becomes chaotic and the oscillation amplitude grows so large during rarefaction that the inertia of the surrounding fluid will eventually overcome the pressure inside the gas bubble, followed by bubble collapse and/or fragmentation into smaller bubbles.^{5,19} This regime is called inertial cavitation. In terms of therapy, the inertial cavitation regime can cause shock waves and jet formation that can have significant effects on surrounding cells ranging from pores in the cell membrane to cell death.^{66,70} The regime no longer elicits well-defined harmonic echo signals but rather broadband noise over a broad range of frequencies¹⁹ (Figure 2, red curve). Since the microbubbles are destroyed, resulting in a loss of contrast, and because the violent collapses may cause hemorrhage and tissue necrosis in the surroundings, this regime is less suitable for imaging purposes. In practice, the threshold between inertial and stable cavitation is not well defined, and bubbles start to cavitate inertially over a range of acoustic pressures ranging from roughly 200 to 500 kPa.

Since the ultrasound pressure is an important parameter to determine the oscillation regime, it stands to reason that this ultrasound parameter is most often reported in the literature. However, as mentioned above, the relation between the acoustic pressure and microbubble dynamics applies only when microbubbles are excited near their resonance frequency. In reality, microbubbles are often driven out of resonance. Therefore, it proves difficult to make clear conclusions and comparisons between the results from different research groups based on solely the acoustic pressures reported. To find a more suitable parameter for comparing microbubble behavior, other options could be explored, such as the relative amplitude of oscillation, $R_{\text{max}} - R_{\text{min}}/R_0$, as used by Kooiman et al.,⁷¹ Helfield et al.,²⁷ and Luan et al.⁷² This takes into account the size and coating-related factors as well as the impact of the ultrasound regime. Unfortunately, relative oscillation amplitudes can be determined only with ultrahigh-speed imaging systems operating at an imaging frequency that must exceed the ultrasound frequency multiple times⁷³ and is therefore not in reach of the majority of research groups working on microbubble-assisted drug delivery. Alternatively, the use of monodisperse microbubbles results in more controlled bubble dynamics due to a homogeneous acoustic response;⁷⁴ therefore, comparing the results based on such formulations will potentially be more straightforward.

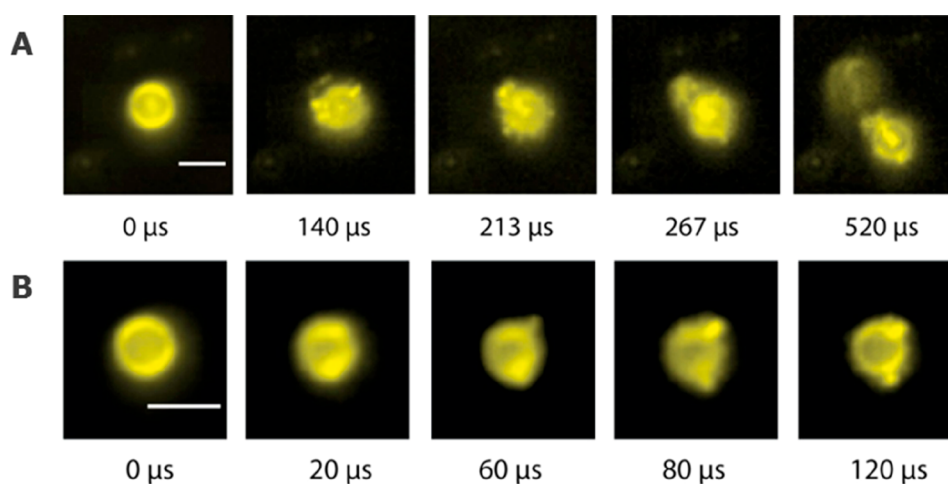


Figure 3. DPPC-based microbubbles with fluorescent DiI in the shell to show the shedding events under ultrasound radiation at a 1 MHz center frequency. (A) Shell material is released from the microbubble core under the influence of an ultrasound pulse of 170 kPa and 500 cycles, whereas (B) no shell shedding could be observed at 255 kPa, 100 cycles. Instead, shell budding was observed. In both cases, the scale bar represents 5 μm . Reproduced from Luan et al.⁷² with permission from Elsevier.

Microbubble Shell Shedding to Release the Therapeutic Payload of Drug-Loaded Microbubbles. Since microbubbles can be designed to hold various therapeutic payloads on their surfaces, it is important to consider if, when, and how this payload is being released. It was already suggested by Borden et al.⁴⁸ that because of the surface area reduction in the compression phase, excessive shell material could be expelled. This could result in the shedding of the lipid material; therefore, the therapeutic payload will be shed as well. Subsequent studies have focused on the requirements for microbubble shell shedding in response to ultrasonic radiation and have found that the shedding of the microbubble shell is strongly correlated with a reduction in the surface area leading to an oversaturation of the lipid shell material.^{75–77} Later, a relative bubble oscillation amplitude of at least 30% was observed as the threshold for lipid shedding of DPPC-coated microbubbles.^{67,72} This could already be achieved with pressures of around 200 kPa (Figure 3).⁷² Below this shedding threshold, the shell material was not released, but rather a rearrangement of the shell constituents and buckling was observed.^{58,72}

Shell shedding is again highly dependent on the microbubble shell composition. For example, Borden et al. showed that longer acyl chains resulted in coatings that were more resistant to acoustic dissolution, which in turn lowered their propensity to shedding.⁴⁸ This can be explained by the higher cohesiveness (intermolecular forces) and elasticity of these kind of shells. Van Rooij et al. confirmed this by showing that DPPC-based microbubbles are less acoustically stable than their DSPC (C18) variants.⁷⁸ Additionally, Kooiman et al.⁵⁸ demonstrated that a relative vibrational amplitude of 30% was not enough to initiate shell shedding of DSPC bubbles but rather resulted in enhanced lipid bucking and folding in the surface structure of the bubbles instead. The shedding of the shell components can also be affected by loading therapeutic agents onto the surface of the microbubbles, as had been found by Luan et al.⁵⁷ They saw that a higher pressure was needed for vibration and shedding when nanoparticles were covalently coupled onto the microbubble shell as a result of the higher viscosity of the shell, in particular for larger microbubbles.⁵¹ In conclusion, whereas the microbubble shell has little influence

on the resonance frequency at the acoustic pressures used for therapy, it may have a dramatic effect on the shedding of therapeutic compounds loaded into or onto the microbubble shell through shell damping. Hence, it is important to keep in mind that whenever alterations to the microbubble composition are made, for example, to allow the coupling of therapeutic agents or targeting agents, careful consideration of the effects on the acoustic response is required as well.

Radiation Forces Leading to Microbubble Translation. Apart from radial oscillations, microbubbles can also respond to an incoming ultrasound wave by translating under the influence of the so-called acoustic radiation forces, or Bjerkness forces^{19,24,25,38,79} (Figure 4). These forces arise at any acoustic pressure, but their effects become increasingly important with longer acoustic pulses and higher intensities and result in a net time-averaged force directed away from the transducer.^{24,25,38} Furthermore, because the microbubbles themselves act as a secondary source of ultrasound waves when cavitating, they will also exert secondary forces onto neighboring bubbles.¹⁹ As a result, microbubbles oscillating in phase in close proximity to each other will experience a net attractive force (Figure 4B). This can result in microbubble aggregation and can eventually lead to microbubble coalescence.^{5,19,79,80} Primary radiation forces can be useful for molecular imaging purposes because they can be applied to push targeted microbubbles toward ligands lining the vessel wall in order to stimulate microbubble binding.^{81–83} Additionally, these forces can be involved in therapeutic applications as they can be used to push drug-loaded microbubbles toward their target in order to maximize drug delivery,^{84,85} as shown in Figure 4A.⁸⁶

Presence of Nearby Structures. The presence of nearby substrates such as rigid membranes present in *in vitro* studies and even more compliant viscoelastic boundaries found *in vivo* can influence the microbubble oscillation mode and as such also acoustic radiation forces and microbubble shell shedding. In terms of microbubble oscillation dynamics, it is known that structures in the vicinity of an oscillating microbubble can affect the resonance frequency and maximum oscillation amplitude.⁴ For example, microbubble vibrations were shown to be smaller in the case of vascular confinement.⁸⁸ Depending

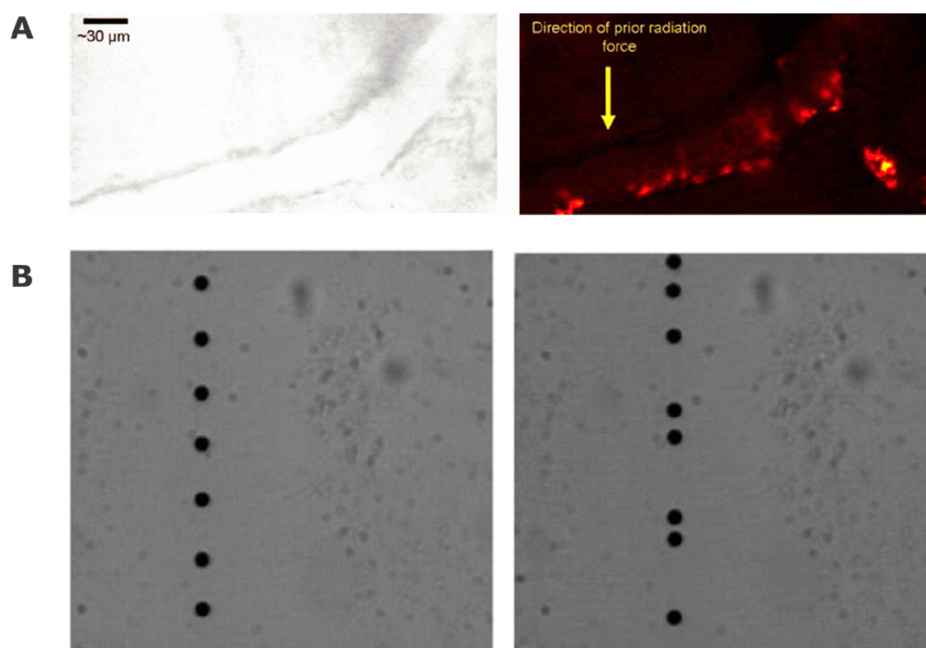


Figure 4. (A) Deposition of fluorescent model drugs attached to microbubble-like acoustically active lipospheres on an ex vivo capillary wall after a specific acoustic radiation force fragmentation ultrasound pulse sequence. The primary acoustic radiation force is used to push the microbubble-like vehicles in the direction of the distal wall, where the payload can be deposited using the fragmentation pulse. (B) Secondary radiation forces cause individual air bubbles ($53 \mu\text{m}$) to move toward each other under a single pulse of 110 kPa when their oscillations are in phase. The same mechanism applies for microbubbles. Copyright 2018 IEEE. Reprinted, with permission, from Shortencarier et al.⁸⁶ and Palanchon et al.⁸⁷

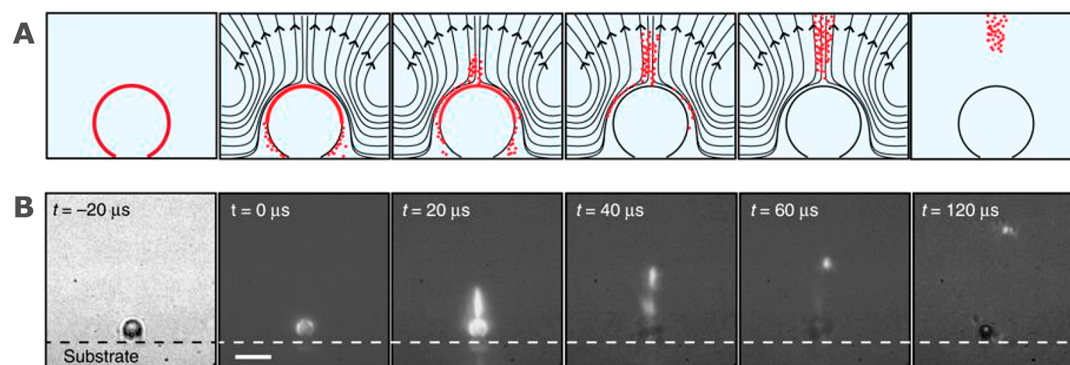


Figure 5. (A) Schematic representation of shell shedding when a microbubble is in close proximity to an acoustically reflective wall. The red dots represent microbubble shell lipids, and the black lines show the calculated streamlines. (B) Asymmetrical shell shedding as observed in a side view at a pressure of 331 kPa . The scale bar represents $10 \mu\text{m}$. Reproduced from Lajoinie et al.³⁶ under the Creative Commons Attribution 4.0 International License: <http://creativecommons.org/licenses/by/4.0/>.

on the mechanical properties of the substrate, theoretical studies have shown that the microbubble resonance frequency can either increase or decrease, although this needs further investigation.^{89,90}

Second, when a microbubble is oscillating next to a membrane or a wall with a higher acoustic impedance than the surrounding medium, this membrane will act as an ultrasound wave reflector and causes the creation of a virtual image bubble oscillating in phase with the real microbubble.^{5,36} Due to secondary radiation forces, the microbubble will be attracted to this image bubble and will move toward the membrane, as visualized in several in vitro experiments.^{62,66} This can affect the outcome of several in vitro drug delivery studies that are often performed with membrane-adherent cells.

Finally, numerical modeling and experimental studies have been performed to investigate microbubble shell shedding when a microbubble is oscillating asymmetrically in the presence of a nearby membrane.³⁶ Microbubbles that oscillate near a membrane will create axisymmetric microstreaming patterns since their vibration will be affected by the presence of that membrane.^{31,36,91} Upon insonation, oversaturation of the lipids occurs in the shell region near the wall, where the lipids finally detach and are transported away from the membrane via microstreaming, as represented in Figure 5.³⁶ This directional shedding can be important in drug delivery studies because it actually transports the drugs away from the cell-carrying membrane. However, the impact on an in vivo situation remains unclear as these microstreaming patterns will depend on the mechanical properties of the nearby membrane, which

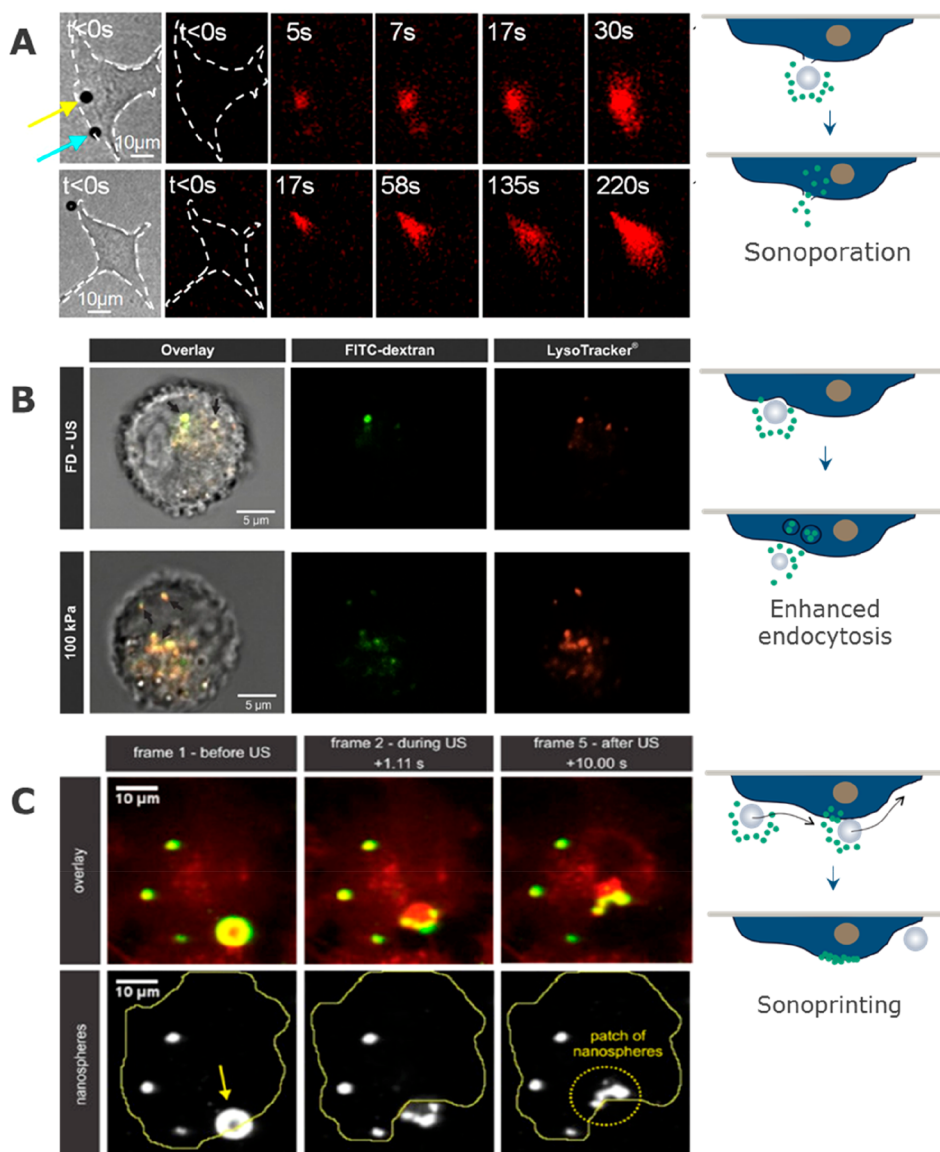


Figure 6. Mechanisms of microbubble-induced drug delivery at the single-cell level. (A) Sonoporation. Upon insonation with a 1.25 MHz frequency, 170 kPa acoustic pressure ultrasound wave for a total ultrasound duration of 8 μ s, the microbubbles create small pores in the cell membrane allowing the otherwise impermeable PI to leak into the cells. In the top image, two microbubbles create side-by-side pores, of which the larger of the two microbubbles (indicated by the yellow arrow) leads to more PI influx, presumably due to the creation of a larger pore. (B) Enhanced endocytosis. FITC dextran is taken up through endocytosis, as evident from the colocalization with lysotracker (i.e., an endolysosomal marker), both with and without ultrasound. However, applying ultrasound at a center frequency of 1 MHz, a pulse length of 2000 cycles, a driving pressure of 100 kPa, and a repetition rate of 125 Hz for a total duration of 5 s increases the quantity of FITC dextran inside the lysosomes, indicative of microbubble-induced enhanced endocytosis. (C) Sonoprinting. Sonoprinting can be seen when fluorescently labeled nanospheres are coupled onto microbubbles through chemical linking. After ultrasound radiation with a 1 MHz center frequency, an acoustic pressure of 300 kPa, a pulse length of 1000 cycles, and a repetition rate of 100 Hz for a total duration of 5 s, a patch of nanospheres is left on the surface of the cell. Adapted from (A) Fan et al.,¹⁰⁶ (B) De Cock et al.,¹²¹ and (C) De Cock et al.⁹² with permission from Elsevier.

differ greatly between in vitro studies and the more compliant walls present in vivo.

■ PART 2: MICROBUBBLE–CELL INTERACTIONS TO RELEASE AND DELIVER DRUGS TO TISSUE

Methods Used to Unravel Microbubble–Cell Interactions. Studying the microbubble–cell interactions that lead to intracellular delivery of drugs is challenging because they occur on various time scales and length scales and involve numerous physical, chemical, and biological parameters.⁴ Therefore, the simultaneous use of multiple techniques is required to obtain a complete overview. Microscopy-based

techniques are ideal for managing multiple time scales since imaging rates can vary from real-time imaging (a few frames per second) allowing the evaluation of drug influx and cellular responses⁹² to high-speed (150 000 fps) imaging which can resolve the release of model drugs from microbubbles and streaming effects⁷² and even to ultra-high-speed imaging (up to 25 million fps), making it possible to resolve the microbubble oscillations during ultrasound exposure.^{58,73} Other methods can be used to evaluate the consequences of microbubble–cell interactions during or after ultrasound treatment.⁴ For example, the integrity of cell membranes and intercellular junctions can be measured using electrical sensing techniques

(TMC and TEER).^{93,94} The proteomic and transcriptomic changes in the exposed tissue can be analyzed using Western blots,^{95–97} ELISA assays,⁹⁷ and PCR analysis.^{96,97} The effects can be studied in further detail quantitatively using flow cytometry and qualitatively using electron and atomic force microscopy. Nevertheless, owing to this inherent complexity, the interpretation and integration of these different techniques remains a significant challenge.

Mechanisms of Ultrasound- and Microbubble-Induced Drug Delivery at the Single-Cell Level. The best-known and most-studied microbubble-associated drug uptake mechanism is sonoporation^{24,26,27,69,98,99} (Figure 6A). Sonoporation can be defined as the formation of transient openings in the cell membrane induced by microbubble oscillations, through which molecules can passively diffuse in and out of the intracellular space. These pores can range from 10 nm to several hundred nanometers and even a few micrometers in size.^{24,100,101} However, for a cell to remain viable, the pores should close within a reasonable time frame and should therefore be sufficiently small to completely reseal during that time. Studies that have looked into these aspects have found that pores should be smaller than $100 \mu\text{m}^2$ and ideally even smaller than $30 \mu\text{m}^2$ to reseal quickly and successfully,²⁶ while resealing times were reported to be anywhere between a few seconds and 120 s.^{26,69,94,102}

The disruption of the plasma membrane during sonoporation will have several bioeffects because this membrane is crucial in maintaining the balance between the intracellular and extracellular environments, which is indispensable to the normal function of the cell. These bioeffects may be caused by a direct mechanical effect of the microbubble oscillations but can also be triggered by chemical effects caused by oscillating microbubbles. These include the formation of reactive oxygen species that can alter the cell membrane integrity and affect ion channels.^{100,103} The direct connection between the intracellular and extracellular space during sonoporation allows molecules to leak into the cells but will also result in the leakage of intracellular constituents. As a consequence, sonoporation can be detected through the influx of otherwise impermeable agents such as propidium iodide^{69,102} as well as through the loss of intracellular agents such as calcein or GFP from stably transfected GFP cell lines.¹⁰⁴ Moreover, it can lead to the influx and outflux of ions, which results in a disruption of the transmembrane potential.^{105,106} One ion that has been of particular interest is calcium. Because the calcium concentration is higher in the cell surroundings than in the intracellular environment, sonoporation will lead to an influx of calcium, which can influence several intracellular processes and was found to play an important role in membrane recovery after sonoporation.^{24,107–109} Moreover, both transmembrane potential variations and calcium concentration changes are not limited to the cells that experienced pore formation but are passed on to neighboring cells in waves propagating from the initially affected cells.^{105,106,108} This indicates that even though the pores quickly reseal, cellular effects persist over longer times and over larger areas. Sonoporation can additionally lead to structural modifications such as the shrinking of the cell,¹¹⁰ the formation of blebs,¹¹¹ changes in cytoskeletal orientation,¹¹² and changes in cellular proliferation.^{105,113} Therefore, even though cells remain viable after successful resealing, they may still be altered over a longer term and could progress toward apoptosis later on.^{100,105}

In some studies, enhanced delivery was seen to last for several hours.^{114,115} However, membrane pores that stay open during such extended periods of time will surely result in cell death, which is why other mechanisms must be at play here. One potential mechanism is the opening of cellular tight junctions in between cells.^{27,116,117} These temporal openings make the cell layer more permeable without affecting the cellular integrity and have been proven to prolong the transport over a confluent cell layer for up to 30 to 60 min.²⁷ They are thought to arise from changes in the cytoskeleton as a result of the shear stress induced by oscillating microbubbles and could be an indirect result of microbubble sonoporation.^{27,117} Alternatively, protein-based studies have suggested that ultrasound is able to reduce the protein expression of efflux pumps such as P-glycoprotein, which is normally expressed by the cells that make up the blood–brain barrier and by certain types of tumor cells.^{118,119} Downregulation of P-glycoprotein due to ultrasound exposure will ultimately result in a prolonged intercellular presence of drugs as well.

Another mechanism that enhances drug delivery using microbubbles and ultrasound and that could sustain drug influx over longer periods of time is the increased incidence of endocytosis^{115,120–122} (Figure 6B). Endocytosis is a process that occurs naturally and allows cells to take up important macromolecules from their surroundings. Microbubble oscillations have been shown to promote this process as a result of cell membrane deformation that these oscillations can cause.^{24,103,121} Enhanced endocytosis has been shown to be more involved in the uptake of larger molecules (>155 kDa) that would have trouble entering the cell through sonoporation as a result of their low diffusivity.¹²⁰ However, it has also been proposed that enhanced endocytosis could, in part, be due to membrane disruption by sonoporation and that both phenomena are actually linked.^{103,123} Indeed, studies have reported that small pores created in the cell membrane are often sealed through endocytosis.^{24,123,124} Moreover, the calcium influx resulting from sonoporation induced the formation of endocytic vesicles as well.¹²⁵

Recently, a novel mechanism called sonoprinting was proposed⁹² (Figure 6C). Sonoprinting is defined as the direct deposition of nanoparticles along with parts of the bubble shell onto cell membranes upon applying ultrasound to nanoparticle-loaded microbubbles. It was shown that these patches remain associated with the cell membrane for several hours before finally being internalized. This phenomenon was seen only when nanoparticles were physically coupled onto the bubble surface, either via linking drug-loaded nanoparticles to the microbubble shell or via direct incorporation into that shell, and not simply coadministered. Various other studies have reported similar phenomena where they saw fragments of the bubble shell and attached nanoparticles remaining on cell surfaces.^{30,85,126} In a follow-up study, high-speed imaging was used to elucidate this sonoprinting phenomenon further, and it was found that the occurrence of these patches on the cell membrane are most likely due to secondary radiation forces between microbubbles that drag nanoparticles along in their wake.¹²⁷ This mechanism can be useful for providing a local reservoir of drug-loaded nanoparticles in the vicinity of the target tissue from where the drugs can leak out and can be taken up by surrounding cells.

The potential of each of the above-mentioned mechanisms to enhance the delivery of a therapeutic agent will rely at first

instance on the drug itself. Sonoporation can provide direct access to the cellular cytoplasm and, as such, circumvent several potential biophysical barriers. Nevertheless, because it relies on passive diffusion, this approach will be suitable only for drugs with a relatively small molecular weight since it is unlikely that larger drugs such as nanoparticles, proteins, and genetic drugs will be able to sufficiently diffuse into the cell before the pores have resealed. This was confirmed by studying the contribution of pore formation and enhanced endocytosis on the uptake of molecules of various sizes.^{120,121} Indeed, endocytic uptake can theoretically occur for small and large molecules and even for nanoparticles. Larger drug constructs such as nanoparticles can also be efficiently delivered via sonoprinting after coupling them to the microbubble surface. Uptake into the cells occurs through internalization of the local patch of nanoparticles. Alternatively, the drugs could leak out of the nanoparticles themselves before endocytosis of the drug-carrier occurs. At this point, the drug should have the right physicochemical characteristics to penetrate into the deeper layers of the tissue and through the cell membranes to exert its function. This approach could be particularly interesting for chemotherapeutic drugs that have sufficient potency but have dose constraints due to off-target effects.

Acoustic Settings Influencing Microbubble–Cell Interactions and Drug Uptake Mechanisms. Which of the above-mentioned mechanism will be primarily at play will depend on the acoustic settings used (i.e., on the ultrasound frequency, the acoustic pressure, the pulse duration, the pulse repetition frequency, and the duration of insonation). Any one of this large number of ultrasound parameters will largely affect these drug delivery processes by influencing microbubble–cell interactions. Most studies report an ultrasound center frequency of around 1 MHz because it roughly matches the resonance frequency of the majority of bubbles (volume-weighted) in standard microbubble formulations ($\pm 3 \mu\text{m}$ in size).

However, in terms of acoustic pressures, pressures ranging from 80 kPa⁷¹ to 1.1 MPa¹⁰¹ are used to enhance drug uptake. These pressures span both stable and inertial cavitation regimes. Although the frequency and acoustic pressure determine which cavitation regime will occur, the pulse duration controls the time period over which microbubbles can exert an effect. Understanding the interplay between both parameters is essential to tuning the desired drug delivery mechanism. Rather short pulses and high pressures are usually employed for sonoporation purposes.^{27,109,128} Upon utilizing targeted microbubbles to ensure close microbubble–cell contact, Kooiman et al.⁷¹ demonstrated that sonoporation is induced whenever the relative microbubble vibration amplitude exceeds 0.5. They showed that this could already be achieved in the stable cavitation regime. The disruption (temporary or permanent) of the cell membrane during stable cavitation is believed to be due to pushing and pulling of the vibrating microbubble on a cell membrane and to the formation of microstreaming that can exert shear stress on the cell membrane.^{5,69} Larger shear stresses have been shown to correlate with higher propidium iodide uptake, which shows that the pores either stay open longer or are larger.²⁷ Likewise, larger holes,¹²⁹ higher sonoporation efficiency,⁴⁰ and spatio-temporal control over pore formation¹⁰⁶ can be achieved by using two successive sonoporation pulses with different pressures. Furthermore, the direct creation of holes in the cell membrane by shock waves and microjets was observed

when higher pressures resulting in microbubble fragmentation were used.^{66,70} To obtain a long-term drug delivery increase, an enhancement of the endocytic pathway can be induced.^{115,120,121} To elucidate which parameters lead to the most prominent endocytic uptake, De Cock et al.¹²¹ compared the contribution of endocytosis to that of sonoporation in the uptake of FITC dextrans under various acoustic pressures. They showed that when the acoustic pressure is rather low (100–200 kPa) enhanced endocytosis is the dominant mechanism, whereas the fraction of sonoporated cells increases with increasing acoustic pressure. This was confirmed by studies using low acoustic pressures to promote endocytosis as well.^{115,130}

Whenever the pulse length is increased, radiation forces will come into play. The primary radiation force can push the microbubbles toward target cells and ensure a closer cell–microbubble contact, which stimulates both sonoporation and endocytosis. Secondary radiation forces result in attractive forces between microbubbles, leading to microbubble aggregation and coalescence.¹²⁹ The displacing forces can move only the microbubbles over a substantial distance when the microbubbles are under the influence of ultrasound for a sufficient amount of time, which is why this phenomenon is mostly observed during long pulses (>100 cycles).²⁵ Secondary acoustic radiation forces have proven to be essential to achieving sonoprinting.¹²⁷ In an attempt to elucidate the impact of acoustic settings on sonoprinting, it was found that increasing the acoustic pressure (>300 kPa) and number of cycles (>100 cycles) will result in higher sonoprinting rates. This can be explained by the fact that the released nanoparticles are dragged along with translating microbubbles, resulting in nanoparticle accumulation on the cell membranes. However, such translating microbubbles have previously been related to cell death in *in vitro* studies.^{102,129} In fact, increases in both acoustic pressure and ultrasound pulse lengths have been positively correlated with cell death.^{98,128,129} It is therefore important to maintain a balance between toxicity and drug delivery efficacy and assess the risks and benefits for each treatment goal.

Finally, other ultrasound parameters such as the pulse repetition frequency and total exposure duration become increasingly important in the *in vivo* situation. The pulse repetition frequency will determine the time for microbubble replenishment in between pulses, while the total radiation time will determine which fraction of the microbubbles was able to reach the target site within the duration of the treatment.^{13,131} Due to the dependency on blood flow velocity, the pulse repetition frequency and the total exposure time should be fine-tuned to the characteristics of the target site to allow a maximal replenishment in between the ultrasound pulses and an optimal microbubble response in order to maximize therapy.

Impact of Microbubble Characteristics on Drug Delivery. Often, the effects of microbubble properties on the drug delivery mechanisms are largely overlooked. However, microbubble-related factors could be of equal importance as compared to the acoustic settings in the drug delivery outcome and are therefore also worth investigating. As discussed before, microbubble size is the crucial factor in determining the microbubble's resonance frequency. The therapeutic contribution of microbubbles that do not oscillate at the same amplitude as the resonant bubbles is hard to predict and might affect the balance between inducing therapeutic effects and

limiting tissue damage. This once again highlights the importance of more acoustically uniform microbubble formulations for drug delivery applications.

Additionally, the microbubble concentration will have a substantial impact, given that the complex interplay between microbubbles can influence the microbubble response. Indeed, higher microbubble concentrations lead to increased microbubble coalescence and aggregation under the influence of secondary radiation forces, while more microbubble translation in the direction of the traveling ultrasound wave due to primary acoustic radiation forces was seen at lower microbubble concentrations.^{5,81,87} Furthermore, higher microbubble concentrations yield acoustic shielding of microbubbles further away from the transducer, causing a variable microbubble response.¹³² Several studies, performed with pressures aiming at inertial cavitation (600 kPa), investigated the impact of the bubble-to-cell ratio on sonoporation and have found that the sonoporation efficiency increased with a higher microbubble-to-cell ratio with a limit of 4 microbubbles per cell to avoid irreversible sonoporation.^{129,133} Consequently, increasing the possibility for bubble–cell interactions augments the chances of efficient drug delivery, but there is a trade-off with cell viability at higher microbubble concentrations.⁹⁸ Probably even more important than the number of microbubbles per cell is the microbubble–cell distance because this will determine the magnitude of the effect a cavitating microbubble can have on a cell. In this respect, studies have tried to elucidate how close the microbubble and the cell need to be and have found that this distance should not exceed the microbubble radius.^{94,133,134} Nonetheless, shock waves that occur during inertial cavitation can travel over longer distances and affect cells from further away.^{24,135}

In *in vitro* setups, close microbubble–cell contact is often ensured by allowing the microbubbles to float against a cell-carrying substrate,^{14,71,136} which could explain why short ultrasound pulses are sufficient for intracellular uptake in these kinds of studies.^{71,100} Even so, in more complex, free-flowing systems, longer pulses might be needed to increase the possibility of close microbubble–cell interactions. To ensure such close microbubble–cell proximity *in vitro* as well as *in vivo*, molecularly targeted microbubbles are most often utilized.^{71,102,137} These microbubbles contain specific ligands on their shell that bind to receptor molecules on target cells.^{126,138} Note that, as discussed above, modifying the microbubble shell in this way may influence the microbubble's acoustic properties. For instance, it was seen that upon applying long pulses on targeted microbubbles that are bound to an underlying surface, secondary Bjerkness forces cause the microbubbles to deform to a prolate shape in the direction of neighboring bubbles at low pressures before being released from their target at higher pressures.¹³⁹ Other studies have confirmed that a smaller displacement occurred when targeted microbubbles are used.¹⁰² Therefore, drug delivery and associated cell death might be significantly different when targeted microbubbles are used instead of nontargeted microbubbles under the same ultrasound conditions.¹⁰²

■ PART 3: TRANSLATION TO PRECLINICAL AND CLINICAL STUDIES

Several *in vivo* studies have been utilizing microbubbles and ultrasound for drug delivery with promising results. However, the lack of optical transparency prevents the use of microscopy-based techniques that are commonly used to

study microbubble behavior and microbubble–cell interactions. It is therefore not straightforward to determine the behavior of the microbubbles and how to maximize drug release *in vivo*, which is reflected in the plethora of different ultrasound parameters being used in these kinds of studies. However, a better understanding of the microbubble dynamics in the complex *in vivo* environment is of vital importance to maximizing the therapeutic effects while minimizing adverse effects.

Gap between the *in Vitro* and *in Vivo* Situations.

Although a single-microbubble single-cell approach results in an increased understanding of the biophysical mechanisms involved, complex models are crucial to represent the *in vivo* environment better. It is therefore important to comprehend the inherent differences between *in vitro* and *in vivo* that will affect microbubble behavior.

Vascular Confinement. A major drug delivery barrier *in vivo* is the endothelial vessel wall because microbubbles are too large to extravasate through the endothelial fenestrae. Several studies have investigated the effects of vascular confinement on oscillating microbubbles *in vitro* and *in vivo*. Due to the damping effect of nearby vessel walls, the maximum oscillation amplitude of microbubbles is less than in free space under the same ultrasound driving conditions.^{140–143} Since the maximum amplitude of oscillation has a direct impact on drug release as well as on the various microbubble–cell interactions, it will affect the entire mechanism underlying the drug delivery process. Moreover, the viscosity of blood is approximately 3 to 4 times higher than the viscosity of water-based buffers. As a result, the pressure threshold for inertial cavitation was reported to be higher in blood.¹⁴⁴

Additionally, the blood flow velocity and viscosity vary greatly within differently sized vessels and even more in the strongly altered microcirculation of a tumor.¹⁴⁵ Even within a vessel, the flow velocity generally decreases from the center to the edges of the vessel, and branches within vessels will cause intricate flow patterns,¹⁴⁶ leading to regions of high and low probability of microbubble contact with the vessel wall and local microbubble accumulations. Consequently, the time a microbubble is exposed to ultrasound and therefore its potential to induce local bioeffects may be different for each microbubble. Similarly, the microbubble concentration will fluctuate within the target area. Therefore, drug delivery mechanisms that rely on the interaction between adjacent microbubbles might be affected as well. It remains to be seen if targeting strategies can ensure the close contact needed for these kinds of interactions to occur. Moreover, since nearby membranes create additional microbubble translation due to secondary radiation forces, the microbubbles' behavior may vary when a microbubble is oscillating within a large vessel as compared to when it is closed in by nearby capillary walls.

Furthermore, ultrasound-mediated microbubble cavitation is also able to locally alter tissue perfusion. Vasoconstriction and even a complete shutdown of microvascular blood flow have been reported when violent microbubble collapses are induced by relatively high ultrasound pressures of 1.6 MPa.¹⁴⁷ This temporary vascular shutdown can hamper drug influx and even lead to ischemic damage.¹⁴⁸ This has led to a growth delay in tumors caused by endothelial cell apoptosis and local inflammation, resulting in platelet activation and occlusion of the microvasculature.^{149–151} In contrast, increased perfusion after microbubble-assisted ultrasound treatment was also described in the literature.^{152–154} Rix et al.¹⁵³ reported an

augmented signal enhancement in tumors after repeated microbubble injections and mentioned the cavitation-assisted mechanical opening of closed microvessels as one of the possible reasons. Belcik et al.¹⁵² stated that microbubble cavitation could reverse tissue ischemia in the hind limb muscle of mice, yet it could not be completely ruled out that thermal ultrasound effects caused this enhanced perfusion. Likewise, since perfusion imaging was performed only 10 to 15 min after therapy, it is possible that the vasodilation resulted from a vascular rebound effect after an initial vasoconstriction.¹⁴⁸

Tissue Structure. Before reaching its *in vivo* target site, ultrasound will be subjected to attenuation and reflection due to tissue viscoelasticity and inhomogeneity. Therefore, microbubbles within this target site will not receive the same ultrasound energy as they would in an *in vitro* setting. Furthermore, this attenuation is highly dependent on individual tissue structures and can hence vary between patients.

Cells grown in a three-dimensional system instead of a two-dimensional monolayer will have altered phenotypes^{155–157} that can lead to variations in their response to ultrasound and microbubbles. Even on an *in vitro* level, it has already been shown that endothelial cells grown under flow condition are less responsive to the same ultrasound regime than cells grown under static conditions.¹⁰⁹ The authors attribute this finding to the alteration of the cell's phenotype, resulting in extended F-actin stress fibers, an increase in cytoplasm viscosity, and cell membrane rigidity that make the cell more resistant to external shear forces.

Another major difference is the lack of rigid membranes in an *in vivo* setting, which has been shown to alter the microbubbles' acoustic response significantly. For example, microjets have been reported to occur due to bubble collapse in the direction of the cell-carrying stiff wall and were able to puncture the cells.^{66,70,158} However, because soft tissue is much less elastic, some of the nonspherical effects seen *in vitro* may not be present to the same extent or not at all *in vivo*.⁵ Likewise, the shedding of shell material was seen to be directed away from the supporting membrane,³⁶ which might not be the case in the clinical situation.

In Vivo Reports of Drug Delivery Enhancement Using Microbubbles and Ultrasound. Since the *in vivo* situation is often regarded as a black box system, especially in comparison to the highly controlled environment of an *in vitro* study, most studies only report on the outcome of the treatment. Even so, promising therapeutic results have already led to a first clinical trial using gemcitabine (i.e., a small-molecule chemotherapeutic) in combination with microbubbles and ultrasound in 10 patients with inoperable pancreatic tumors.¹⁵⁹ The results of this study indicated that the treatment was well tolerated and that the tumor diameter decreased more than for gemcitabine treatment alone (based on results from historical patients). Other clinical trials reported on the opening of the blood–brain barrier to allow the chemotherapeutic treatment of brain tumors, and their preliminary findings have indicated that the procedure is safe and well tolerated in patients.^{160–162} Notwithstanding these encouraging results, they are only primary results on a selected group of patients, and there are many variables that remain undiscussed. Moreover, some results do not correlate well with what was previously established. For instance, the clinical study by Carpentier et al.¹⁶⁰ reports safe opening at pressures that

have been reported to cause extensive adverse effects in small laboratory animals and nonhuman primates.^{161,163–165} They attributed this discrepancy to the size of the human skull, which could limit the occurrence of standing waves, the nature of human microvessels, and an anesthesia-related difference under physiological conditions.¹⁶⁰ To obtain a conclusive understanding of the significance of these effects, additional research is necessary.

Nonetheless, (pre)clinical models provide a unique opportunity to study cellular interactions that are difficult to replicate *in vitro*. These models not only can confirm the mechanisms that are previously observed but also can reveal new mechanisms of action, yet different evaluation methods are often required, which complicates the interpretation of results. To increase the knowledge of microbubble-assisted drug delivery *in vivo*, methods to estimate the tissue distribution of the drug in small animals have been described, either on the entire animal using near-infrared fluorescence imaging^{15,166} or by harvesting the organs and extracting the drug separately.¹⁶⁷ MRI has been widely used to monitor the blood–brain barrier opening, with the aid of MR contrast agents.^{97,165,168–170} This has proven that even the tight junctions that make up the blood–brain barrier can be opened by microbubble-assisted ultrasound treatment, giving this approach a unique opportunity to channel drugs into the cerebral fluids across this otherwise impermeable barrier.^{68,161,171–173} A more detailed analysis of the physiological effects and drug uptake in different organs can be obtained via histology and immunohistochemistry. Using this approach, positive results have been obtained for numerous microbubble–drug formulations and ultrasound in comparison to the delivery of drugs or nanoparticles alone.^{10,15,167,174–176} Nevertheless, a number of adverse effects have also been reported, including hemorrhage,^{15,171,175} local burns,¹⁶ necrosis due to vascular shut down, the formation of thrombi,^{148,177} and drug accumulation in the lungs, liver, and kidneys. Furthermore, immunohistochemistry has confirmed the down-regulation of P-glycoprotein in endothelial cells and astrocytes that make up the blood–brain barrier due to microbubble-assisted ultrasound treatment.^{170,178}

Other studies have tried to retrieve the (model) drug or drug carrier inside the ultrasound-treated tissue using cryosectioning,^{15,171,175,179} fluorescence molecular tomography (FMT),^{116,180} or two-photon microscopy.¹⁷⁴ These techniques were used to investigate the drug distribution in the tissue and the ability of drugs and nanoparticles to reach more distant parts, beyond the blood vessels. For nanoparticles that are approximately 100 nm in size, average penetration distances of 15 μm ¹⁷⁵ to maximally 45–50 μm ^{174,175} have been reported. Rather large (177 nm) nanoparticles have also been found to penetrate the vessel wall in the brain but remained just outside the vasculature in this case.¹⁷¹ Furthermore, Burke et al.^{116,180} showed that the nanoparticles can be retrieved inside endothelial cells, indicative of sonoporation, as well as in the extracellular space, indicative of enhanced permeability of the endothelial lining. Different studies have also compared the delivery efficiency of nanoparticle-loaded microbubbles to the coadministration approach and have concluded that a higher nanoparticle delivery could be achieved when the nanoparticles are directly coupled to microbubbles.^{116,180} Furthermore, coupled nanoparticles were found to concentrate in discrete regions, as opposed to a more even distribution in the case of coadministered nanoparticles.¹⁸⁰ This indicates that the nanoparticles remained grouped in clusters, possibly delivered

via a sonoprinting-like phenomenon. Another study by Kilroy et al.¹⁸¹ used an intravascular ultrasound probe to promote acoustic radiation forces and found fluorescently labeled microbubble shell fragments deposited on the vessel wall, even without the subsequent administration of a “delivery pulse” (i.e., a shorter ultrasound pulse at higher pressure to induce sonoporation). This again indicates that phenomena other than pore formation through sonoporation might be at play in vivo as well.

Finally, the intricate intra- and intercellular signaling pathways that are often lacking in simple in vitro models can lead to the uncovering of additional therapy-related effects. For example, the potential induction of sterile inflammation in the brain after the blood–brain barrier opening was recently described.⁹⁷ Here, it was reported that although no microscopic tissue damage was observed, an increased presence of proinflammatory factors was present at the opening site. This acute inflammation was compatible with ischemia or mild traumatic brain injury and could potentially pose a safety risk. Conversely, it could also be linked to positive effects such as the augmented removal of amyloid plaques, which play an important role in certain neurodegenerative diseases. Another drug delivery approach that was primarily investigated in vivo is the injection of microbubble–microdroplet clusters that undergo a phase shift upon ultrasound radiation and create large bubbles that can temporarily block capillaries.¹⁸² This system was used in combination with numerous commercially available drug formulation and has shown enhanced therapeutic effects in small animal studies.^{183–185} However, the exact mechanism behind this approach and its safety in clinical translation has not yet been fully clarified. It is likely that a variety of other factors will be involved in the complex in vivo situation, which is why it was very recently suggested to refer to ultrasound and microbubble-mediated enhanced drug delivery in general, as “sonopermeation”.¹⁸⁶

Acoustic Parameters Used in in Vivo Studies. Similar to in vitro studies, a high variability in acoustic parameters can be found in in vivo studies. As compared to in vitro reports, in vivo studies are generally performed at higher acoustic pressures (hundreds of kPa to even 7 MPa,^{15,116,175} as measured in pure water) with longer pulse durations (hundreds to tens of thousands of cycles^{15,187}), rationalized by the higher level of attenuation and the lack of immediate microbubble–cell contact. Also, the overall exposure time is usually much longer (up to 10 min¹⁷⁴) to allow a maximum number of microbubbles to be affected during treatment. Authors have suggested that inertial cavitation is needed to provide adequate intratumoral drug delivery in vivo,^{175,188} but this has not yet been thoroughly examined. In contrast, stable cavitation is said to be sufficient for blood–brain barrier opening without tissue damage.^{163,165,189,190} Furthermore, while the use of longer cycles may be crucial in vivo to elicit acoustic radiation forces and sonoprinting effects needed for the delivery of larger drugs, short pulses (five cycles) emitted at high pulse repetition frequencies (1.25 MHz) have been shown to result in a more uniform distribution of small molecules taken up through tight junctions.¹⁸⁷ Recently, follow-up studies on the report of Kovacs et al.,⁹⁷ which claimed that microbubble-assisted opening of the blood–brain barrier could lead to a sterile inflammatory response, have pointed out that the outcome greatly depends on both the microbubble and the ultrasound parameters and that strict control over these factors is crucial.^{191–193} This highlights once again the importance of

control over these parameters and shows that variations herein may result in completely different interpretations of occurring phenomena. Again, the optimal parameters for each application will depend on the drug itself, on the microbubble response, and on the desired therapeutic application.

Most studies have used a trial-and-error approach to optimize the acoustic parameters needed for drug delivery,^{16,194} which did not always match the set of parameters found to be ideal in accompanying in vitro prescreening studies.^{10,194} This strategy is, a priori, ill-suited when dealing with such a large number of parameters. Additionally, many preclinical and clinical ultrasound devices do not allow the modification of a number of important ultrasound parameters. The frequency can be changed within the limited bandwidth of the ultrasonic probe and the maximum intensity output can be adjusted, but the pulse length is often fixed in clinical machines. Therefore, many in vivo studies report ultrasound output only in terms of the mechanical index (MI) or ultrasound intensity in W/cm²,¹⁶⁶ which does not reflect all characteristics of an ultrasonic wave. In fact, the MI is determined only by the frequency (f) and pressure (PNP) of the ultrasound wave ($MI = PNP / \sqrt{f}$), while the pulse length, pulse repetition frequency and total duration are disregarded despite the large impact they can have on the overall effect. Moreover, equal MI values do not necessarily result in equal microbubble behavior, as explained in section 2. Some studies report the pulse length (PL) and pulse repetition frequency (PRF) in terms of the duty cycle (DC, where $DC = PL * PRF$), but the same value for the duty cycle can be obtained by a combination of these terms, which will not affect the microbubble response in the same way as explained earlier. Therefore, a thorough characterization of the ultrasound system is mandatory, as is a detailed report of all acoustic settings (frequency, pressure, pulse length, pulse repetition frequency, and total duration of the treatment). This might allow a more straightforward comparison between research results from different groups, and it might make the in vitro to in vivo translation more straightforward.

As mentioned above, most clinical and preclinical ultrasound imaging systems have a few fixed settings, mostly in terms of the pulse length, which are not ideal for drug delivery purposes. In a study with the Vevo 2100, the power Doppler mode was employed to improve the vascular permeability.¹⁷⁴ However, this allows only very short pulses at a frequency far from the resonance frequency in this case. Another study by Luo et al.¹⁰ used a flash mode on the Philips iU22 with similar issues. Nowadays, programmable ultrasound research systems can be used to generate the longer pulses needed for drug delivery.¹⁹⁵ Clinical transducers often have a frequency range starting at 2 MHz, while for most microbubble formulations, 1 MHz ultrasound matches the overall resonance frequency the best. For preclinical small-animal scanners, this difference is even more profound because these types of transducers typically start only at a frequency of 10 MHz to allow sufficient resolution in ultrasound scans of small laboratory animals. It would be possible to formulate monodisperse microbubbles that are an ideal size for the higher frequencies of commercial clinical scanners, yet smaller microbubbles allow less loading of drug molecules and will be intrinsically less efficient as a result of reduced backscatter signals and higher damping.

Unified Approach to Evaluate Microbubble Behavior and Associated Drug Uptake in Vitro and in Vivo.

To monitor microbubble and ultrasound-assisted treatment in vivo, researchers have used conventional B-mode imaging to study the presence and destruction of microbubbles, thermocouples to evaluate temperature changes, MRI with MRI contrast agents to check the biodistribution, and CT and PET techniques to examine the biological effect.^{13,131} However, these techniques monitor either physical parameters or secondary biological effects, while techniques to study direct biophysical aspects in vivo are lacking. Moreover, these techniques often have rather poor resolution and cannot be used in real time.¹³¹

A more recent approach is to use passive cavitation detection (PCD) to monitor the microbubble cavitation behavior in real time, and this can be extended to form 2D and 3D cavitation maps.^{13,131,188,196,197} In PCD, a single-element transducer is used to detect scattered signals from the microbubbles, which provides information on the microbubble dynamics in the treated region. Therapy monitoring using PCD is based on the hypothesis that the cavitation behavior of microbubbles in the target area is directly related to the overall therapeutic outcome.¹⁹⁸ In this way, therapeutic bioeffects could be linked to a certain cavitation dose, and the system can be used to monitor whether, when, and where this dose is achieved. Because the microbubbles oscillate nonlinearly, their backscattered signal will contain not only the incident frequency but also harmonics of the insonifying frequency. A frequency spectrum can be derived from the acoustic signals received by the PCD transducer via a fast Fourier transform and can be used to determine the acoustic behavior of the microbubbles in the focus region. If the spectrum predominantly consists of harmonics and subharmonics, then microbubbles are mostly in the stable oscillation regime. In contrast, if broadband noise prevails, then microbubbles are cavitating inertially. As single-element transducers can provide only limited information on the location and number of cavitation events, new techniques making use of array transducers have recently been introduced.^{131,199} Array transducers allow the analysis of acoustic emissions from microbubbles in individual voxels within the ultrasound treatment zone independently and can be used to form a cavitation map of the region of interest. This technique is called passive acoustic mapping (PAM) and is especially useful to locate the regions of highest cavitation energy.^{13,131} One example where PAM can be beneficial is in assessing patient-specific attenuations in ultrasound radiation of the brain to ensure that the desired microbubble behavior is present within the treatment zone.¹⁹⁸

Several studies have already used passive cavitation detection or passive acoustic mapping to link the cavitation behavior of microbubbles to therapeutic bioeffects, both in vitro^{13,175} and in vivo.^{131,188,200} One study in particular showed that the therapeutic activity of oncolytic viruses can be detected only when the microbubble emission spectra displayed signs of inertial cavitation.²⁰⁰ Inertial cavitation was also required to stimulate liposome breakup of specific drug-releasing liposomes coadministered with microbubbles^{131,188} (Figure 7). It was also shown that therapeutic activity was mostly present at sites where the highest acoustic activity was measured, although this was not always at the focus of the transducer.¹³¹ This was assumed to result from a nonuniform distribution of the microbubbles in the tumor owing to its irregular

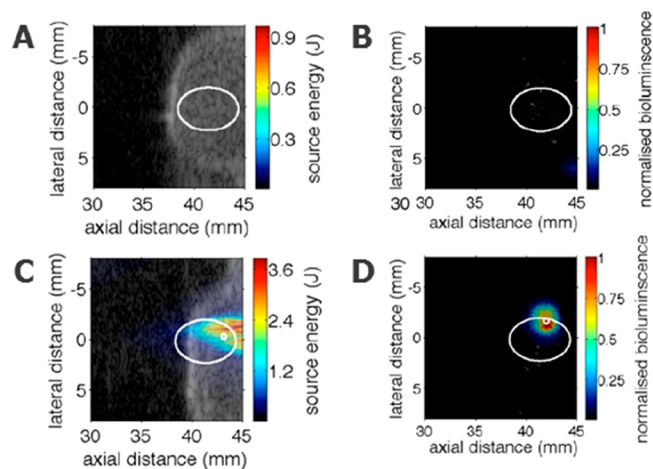


Figure 7. Murine tumors exposed to 80 pulses of 0.5 MHz ultrasound with a 100 ms pulse length, 1.2 MPa peak negative pressure, and 3 s pulse repetition period in the presence of systemically administered luciferin-containing liposomes, with (C and D) or without (A and B) Sonovue microbubbles. (A and C) Overlay between B-mode images and passive acoustic maps of the tumor volume showing the regions of cavitation. (B and D) Bioluminescence measurements at the same location, indicating luciferin release from the liposomes. From C and D it is clear that inertial cavitation of the microbubbles, as evident from the source energy of broadband noise, corresponded to luciferin release from the cavitation-sensitive liposomes. Adapted from Choi et al.¹³¹ with permission from IOP Publishing. Copyright Institute of Physics and Engineering in Medicine. All rights reserved.

microvasculature, which is represented in the maps as well. Studying cavitation activity over time allowed the authors to monitor the impact of different ultrasound parameters in vivo. This revealed that a sufficiently long pulse repetition period (i.e., on the order of seconds) was advantageous because it ensured microbubble replenishment in the target area.¹³¹ It was also shown that the cavitation lifetime of the microbubbles is strongly shortened when higher pressures are applied, which makes the use of longer pulse lengths redundant.^{131,188,198,201} Recently, a real-time feedback system was developed to control the opening of the blood–brain barrier in situ.^{189,190} With this loop control, acoustic emissions from microbubbles at the target site could be directly used to tune the ultrasound parameters to precisely control the magnitude of the blood–brain barrier opening and the resulting drug delivery.

The implementation of passive acoustic mapping is nonetheless not straightforward. Only a limited number of clinical systems allow the implementation of this technique.¹⁹⁶ High computational power and specially designed algorithms are required to process the data. As mentioned before, one would ideally use a second transducer to capture the echoes from the microbubbles at a higher frequency to study the harmonics and broadband content without interference from scatter at the fundamental frequency. Occasionally, a system of even three transducers is used, where one provides imaging, the second sends out the therapeutic ultrasound radiation, and the third records the passive cavitation signals.^{131,175} Synchronized passive recording using a single transducer should be possible, but only if the pulses are sufficiently short.¹⁹⁶ Only very short pulses guarantee that the same microbubble population is still present, which could form a problem for the typically longer pulses needed in drug delivery. Another difficulty of longer pulses is the relatively poor axial

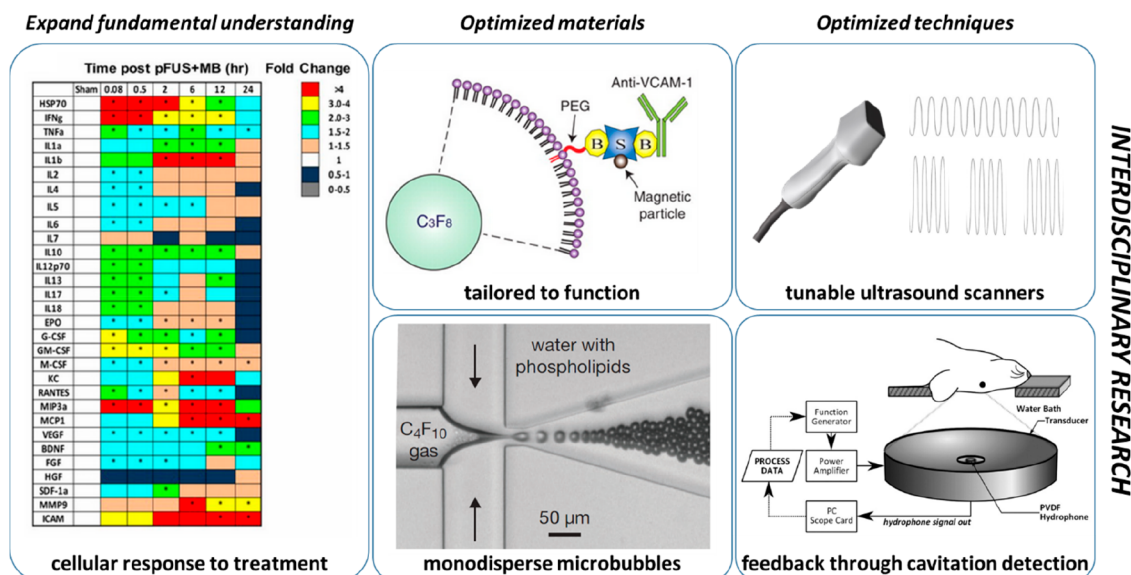


Figure 8. Future directions. (Left panel) Expanding the fundamental understanding of microbubble behavior and the cellular response to treatment in vitro as well as in vivo will remain crucial for future development. For example, protein analysis of several proinflammatory factors in rat brains following treatment with microbubbles and ultrasound by Kovacs et al. revealed that this treatment can lead to a sterile inflammatory response compatible with ischemia or mild traumatic brain injury, raising possible safety questions for clinical translation. (Middle panel) For therapeutic purposes, commercial microbubble preparations will be far outperformed by custom microbubbles specifically tailored for targeting, drug loading, multimodal imaging, etc. (top panel) and monodisperse microbubble preparations that result in more robust and predictable microbubble behavior (bottom panel). (Right panel) Progress in ultrasound equipment and the in situ monitoring of treatment may lead the way to more effective and controlled therapy. Figures were adapted from Kovacs et al.⁹⁷ (left panel); Wu et al.¹⁸ with permission from RSNA (middle panel, top); Segers et al.²⁰² with permission from The Royal Society of Chemistry (middle panel, bottom); and O'Reilly et al.¹⁹⁰ with permission from RSNA (right panel, bottom).

resolution which makes it difficult to avoid interference from regions of high bubble density with regions of low bubble density along this axis. For example, imaging cavitation in the myocardium proved to be nearly impossible as a result of signal smearing from the highly perfused nearby ventricle.¹⁹⁸ Even though there are still some issues to be resolved here, passive cavitation detection would allow researchers to directly link microbubble dynamics to therapeutic effects in real time and could help to bridge the gap between studies focusing only on the acoustic behavior of microbubbles on one hand and studies focusing only on the therapeutic outcome on the other hand.

CONCLUDING REMARKS

Microbubble and ultrasound-driven drug delivery has been extensively studied with a promising outcome, which even resulted in the first clinical trials. A critical issue remains the prediction and control of therapy in different models. Having a basic understanding of the physics of microbubble dynamics is necessary to understanding why acoustic settings and microbubble characteristics are crucial and how they can affect microbubble behavior. However, from the literature, it is obvious that a plethora of parameters are used, leading to an equal variety in bioeffects. Similarly, microbubble polydispersity can be the cause of inconsistent results since microbubbles that vary in size will respond differently to the same ultrasound driving pulse. The narrow bandwidth resulting from the long pulses needed for drug delivery makes this issue even more essential. Moreover, inherent features of in vitro setups may alter the microbubble behavior in ways that are not representative of the clinical situation. Hence, translating microbubble dynamics and the therapeutic outcome from in-

depth in vitro studies to in vivo results has proven to be very challenging.

Figure 8 shows recent developments and future directions that can help to overcome these issues and may eventually pave the way to more efficient and tailored microbubble-assisted ultrasound therapy. Expanding the fundamental understanding of microbubble behavior and the cellular response to treatment in vitro as well as in vivo will remain crucial in obtaining new perspectives on previously established concepts. For example, exploring functional effects of microbubble treatment such as changes in protein expression can provide additional insights into the interaction of cavitating microbubbles with cells and tissues. Furthermore, newer models and techniques such as monodisperse microbubble formulations, tunable ultrasound scanners, and cavitation detection techniques have provided us with different tools for obtaining more uniform microbubble responses and monitoring treatment. This could eventually lead to more controllable and predictable microbubble responses and thereby to more efficient and measurable therapy in the future. Despite these promising developments, implementing these new techniques requires specific knowledge of various fields of expertise, highlighting the need for interdisciplinary partnerships among biologists, chemists, engineers, physicists, and programmers. Future work should therefore focus on merging the knowledge from these subdomains to develop an ideal therapeutic agent, together with a clinical ultrasound system that allows simultaneous imaging, treatment, and monitoring within a single device usable by all research groups.

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Notes

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