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Understanding ultrasound induced sonoporation: definitions and underlying mechanisms

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Abstract

In the past two decades, research has underlined the potential of ultrasound and microbubbles to enhance drug delivery. However, there is less consensus on the biophysical and biological mechanisms leading to this enhanced delivery. Sonoporation, i.e. the formation of temporary pores in the cell membrane, as well as enhanced endocytosis is reported. Because of the variety of ultrasound settings used - and corresponding microbubble behavior, a clear overview is missing. Therefore, in this review, the mechanisms contributing to sonoporation are categorized according to three ultrasound settings: i) low intensity ultrasound leading to stable cavitation of microbubbles, ii) high intensity ultrasound leading to inertial cavitation with microbubble collapse, and iii) ultrasound application in the absence of microbubbles. Using low intensity ultrasound, the endocytotic uptake of several drugs could be stimulated, while short but intense ultrasound pulses can be applied to induce pore formation and the direct cytoplasmic uptake of drugs. Ultrasound intensities may be adapted to create pore sizes correlating with drug size. Small molecules are able to diffuse passively through small pores created by low intensity ultrasound treatment. However, delivery of larger drugs such as nanoparticles and gene complexes, will require higher ultrasound intensities in order to allow direct cytoplasmic entry.

Sonoporation, cavitation, ultrasound, microbubbles, endocytosis

Abbreviations

AFM	Atomic Force Microscopy
FITC	Fluorescein isothiocyanat
IC	Inertial Cavitation
PI	Propidium Iodide
PNP	Peak Negative Pressure
PRF	Pulse Repetition Frequency
ROS	Reactive Oxygen Species
SEM	Scanning Electron Microscopy
TEM	Transmission Electron Microscopy

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1. Introduction

Starting from the mid 90's several papers were published showing that ultrasound can facilitate the transport of membrane impermeable compounds into living cells. This includes several reports showing the ultrasound induced uptake of low molecular weight drugs, genetic drugs (pDNA, siRNA, mRNA), peptides and proteins [1-13]. In general, the uptake of these drugs or model-drugs is attributed to ultrasound mediated transient permeabilization of the cell membrane.

The first studies on ultrasound induced cell permeabilization introduced the term "sonoporation" to describe the temporal cell membrane openings that can arise after ultrasound exposure [12, 14, 15]. Several research papers investigating sonoporation reported the use of microbubbles to amplify the biophysical effects of ultrasound. These microbubbles are gas-filled structures stabilized by a lipid, protein or polymer shell and some of them have been clinically approved as ultrasound contrast agents [16, 17]. Due to their gas-filled, and hence compressible, core they can respond to the ultrasound pressure waves. This process of alternate growing and shrinking is called cavitation and can be divided into (i) stable cavitation, mainly occurring at lower ultrasound intensities and (ii) inertial cavitation, occurring at higher ultrasound intensities (Figure 1). The latter cavitation event may finally lead to microbubble implosion which will result in much stronger biophysical effects.

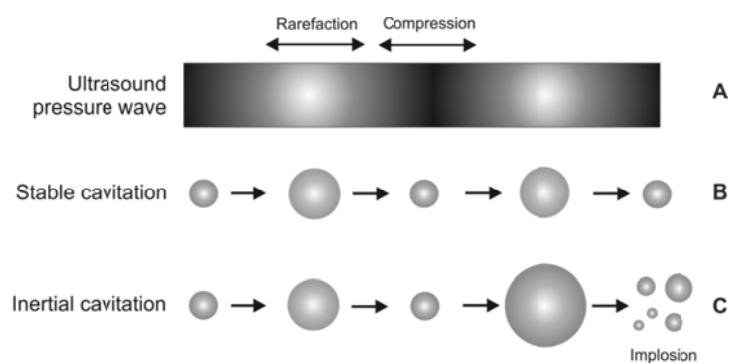


Figure 1. Stable and inertial cavitation. (A) Schematic representation of an acoustic pressure wave. **(B)** and **(C)** show, respectively, stable and inertial cavitation of microbubbles. Adapted from reference [118] with permission from the Royal Society of Chemistry.

Although several in depth reports have been published, it remains extremely difficult to quantitatively characterize the effects of different physiologic processes contributing to ultrasound induced drug uptake. This is mainly due to the plethora of different ultrasound settings and methods used to study sonoporation. For this reason we have defined the following three main ultrasound conditions: i) low intensity ultrasound leading to stable cavitation of microbubbles, ii) high intensity ultrasound leading to inertial cavitation with bubble collapse, and iii) ultrasound application in the absence of microbubbles. In this review, we give an overview of the state-of-the-art knowledge on ultrasound induced biophysical effects for each condition and the related physiological reactions of the sonicated tissue. It is important to note that the interaction of ultrasound with tissue can induce (i) mechanical effects, (ii) chemical effects and (iii) thermal effects, depending on the ultrasound setting, which in turn can lead to several bio-effects. We will limit the scope of this review to the mechanical and chemical aspects of ultrasound induced drug delivery. However, it cannot be ruled out that thermal mechanisms are contributing as well. In this regard, it is indeed important to mention that any temperature increase, provoked by ultrasound exposure, could change the physicochemical state of the cell membranes and could render them more sensitive to membrane deformation. Besides the sonoporation mechanisms, recent literature suggests that other mechanisms like endocytosis might be involved as well in ultrasound triggered drug delivery. Therefore, we focused in the last paragraph on recent contributions to elucidate the role of endocytosis in ultrasound triggered drug delivery.

To the best of our knowledge, this is the first extensive review categorizing and discussing the different cellular mechanisms which have been reported to contribute to ultrasound enhanced drug internalization. We believe that the understanding of sonoporation mechanisms and their relation to different biophysical processes are crucial steps to optimize and fully explore ultrasound induced drug delivery.

2. Mechanisms contributing to ultrasound induced sonoporation

2.1. Cell membrane permeabilization by stably cavitating microbubbles

2.1.1. Biophysical aspects of stable cavitation.

At very low acoustic pressures, microbubbles oscillate in a symmetrical, linear way. This means that their expansion and compression is inversely proportional to the local ultrasound pressure [18]. At higher ultrasound intensities, microbubbles behave non-linearly with a lengthening of the expansion phase of the microbubbles, as the microbubbles are more resistant to compression than to expansion [16, 19]. This phenomenon is also known as stable cavitation or non-inertial cavitation. During cavitation of the microbubble, there is gas influx (during expansion) and gas efflux (during compression). In the case of symmetrical oscillations, the netto gas influx over one expansion/compression cycle is zero. However, when the expansion phase extends, there is a net gas influx into the microbubble. For this reason, the microbubble grows until it reaches its resonant size, whereupon it demonstrates stable, low amplitude oscillation (Figure 1). Such stable oscillations create a liquid flow around the microbubbles, the so-called microstreams [20] (Figure 2). When these oscillating microbubbles are in close vicinity of cells, these cells will experience shear stress. The level of shear stress is largely dependent on the ultrasound parameters and can, according to simulations, range between 100 Pa and 1000 Pa [21]. The shear stress related to micro-streaming is relatively high compared to the shear stress associated with blood flow (0.1-4 Pa) [22]. Consequently, these US induced elevated shear stress levels may induce a large spectrum of biological effects [23, 24].

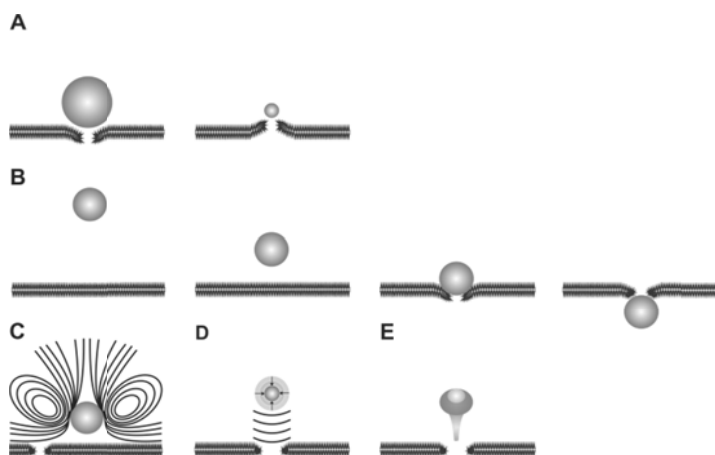


Figure 2. Biophysical effects of stably and inertially cavitating microbubbles. (A), (B) and (C) refer to biophysical effects caused by stable cavitation, while (D) and (E) depict effects of inertial cavitation. **(A)** Pushing (left) and pulling (right) effects during the expansion and compression phase, respectively, of a stably oscillating microbubble, thereby disturbing the membrane integrity. **(B)** Acoustic radiation force causes microbubble displacement and compresses the microbubble against the cell membrane resulting in membrane disruption. The microbubble may even be pushed through the lipid bilayer to enter the cell. **(C)** Stable oscillation of a microbubble creates microstreamings in the surrounding fluid, which exert mechanical stress on the cell membrane, causing pore formation. **(D)** Shock waves produced by microbubble collapse generate high stresses on cell membranes, which results in membrane disruption. **(E)** When a microbubble collapses near a surface, the collapse is asymmetrical, leading to the formation of a liquid jet towards the surface. This microjet punctures the cell membrane, thereby creating a pore. Adapted from reference [46] with permission from Elsevier.

2.1.2. Biological effects provoked by stable cavitation.

Although it was initially believed that inertial cavitation of microbubbles was required to enhance uptake of low molecular weight drugs, there is now mounting evidence that these stable oscillations may also stimulate the intracellular delivery of macromolecular drugs and nanoparticles. For example, Forbes et al. showed significant fluorescein isothiocyanate (FITC) labeled dextran uptake below microbubble collapse thresholds [25, 26]. These results confirm the early work of Guzman et al. who already found in 2001 that fluorescently labeled dextrans were taken up by ultrasound exposed cells even at low acoustic pressures [7].

At these lower ultrasound intensities, two mechanisms have been postulated to contribute to the uptake of cell impermeable molecules, namely (i) the formation of small pores [27] and (ii)

endocytosis [28] (Figure 3). Meijering et al. investigated the contribution of endocytosis and pore formation to the uptake of FITC-dextrans with molecular weights ranging from 4.4 to 500 kDa [29]. They demonstrated that the involvement of endocytosis was more extensive for larger molecules, while pore formation was the main mechanism for smaller dextrans. Endocytosis will be extensively discussed in section 3 of this review. The uptake of propidium iodide (PI) and loss of enhanced green fluorescent protein (eGFP) from cells stably transfected with eGFP has been attributed to pore formation [30-32]. Pore formation was also investigated in *Xenopus* oocytes by measuring cell membrane potential changes using a voltage clamp technique [33]. The changes in cell transmembrane current can be attributed to Ca^{2+} influx during pore formation [34-37], and are a clear indication of cell membrane disruption.

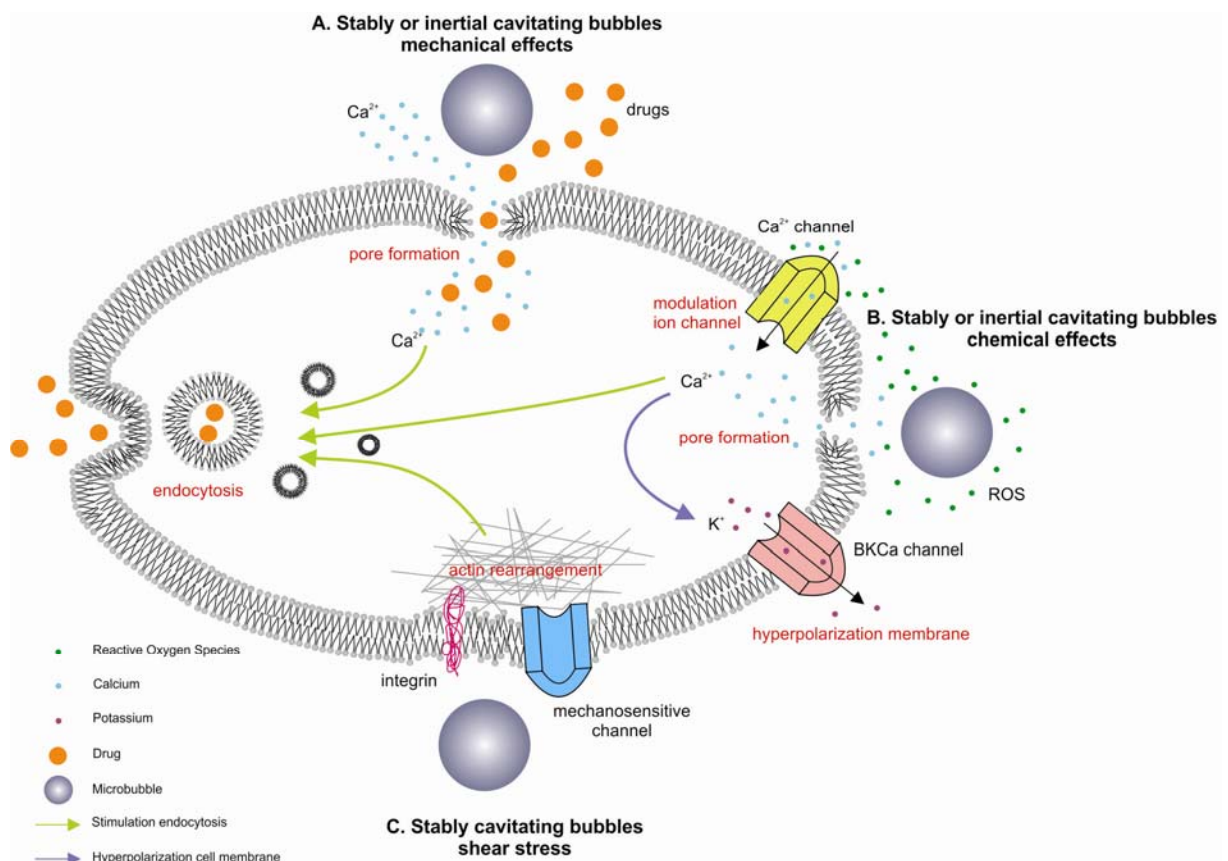


Figure 3. Biological effects of stably and inertly cavitating microbubbles. (A) The mechanical effects of stably and inertly cavitating bubbles, depicted in figure 2, cause the formation of pores in the cell membrane. This allows molecules such as drugs and calcium, to enter the cell via passive diffusion [6, 7, 33]. (B) During microbubble cavitation, ROS are produced [20, 78, 80]. These ROS can modulate

ion channels [53] or can lead to membrane disruption via lipid peroxidation [54]. The calcium influx mediated by those ROS is overcompensated by potassium efflux via BKCa channels, resulting in hyperpolarization of the cell membrane [57]. Moreover, calcium influx is shown to stimulate endocytosis [149]. **(C)** The microstreamings generated by stably cavitating bubbles and the corresponding shear stresses can deform the cell membrane. This leads to cytoskeletal rearrangements and differences in membrane tension, which is sensed by mechanosensors [130]. These sensors can initiate a signaling cascade that influences endocytosis/exocytosis processes [122].

Both ultrasound induced mechanical stress and chemical effects have been reported to be responsible for this pore formation. Ultrafast, real-time imaging techniques at single cell level have been crucial to study the mechanical mechanisms which are responsible for pore formation. In 2006, the research group of Prof. de Jong was the first to disclose with real-time, ultrafast transmission microscopy that a direct interaction between microbubbles and the cell membrane was required for membrane poration [31]. They attributed pore formation to stably cavitating microbubbles, closely located to the cell membrane, which were able to gently push and pull the cell membrane and in this way disturb the cellular membrane as a result of mechanical stress (Figure 2A and 4). This may also clarify that, upon using cell targeted microbubbles, much lower ultrasound intensities are required for cell membrane poration [38]. It was also demonstrated that pore formation correlates with the oscillation amplitude of the microbubbles. Given the close adherence of targeted microbubbles, the relatively small oscillation amplitude at lower ultrasound intensities can have a higher impact on the cell membrane, compared to non-adhered microbubbles.

In relation to these experiments, it is interesting to mention acoustic radiation force which can lead to translational movement of microbubbles. When microbubbles are cavitating, part of the ultrasound energy is absorbed resulting in a pressure gradient. Consequently, microbubbles can experience acoustic radiation force and are displaced in the direction of the ultrasound beam. It has been shown that oscillating microbubbles can be displaced several micrometers when exposed to successive ultrasound cycles [39, 40]. Apart from the fact that this can aid in microbubble adherence to specific targets, [41] acoustic radiation force could be applied to push microbubbles towards the

cell surface and in this way stimulate interaction with the cell membrane and promote drug delivery to specific cells [42-44].

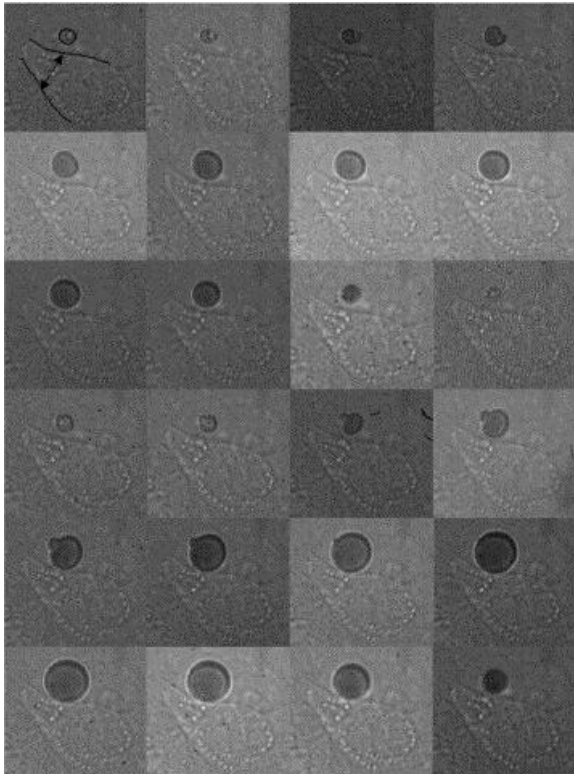


Figure 4. Example of pushing and pulling effects of stably cavitating microbubbles on the cell membrane. The effects of an oscillating microbubble on the cell membrane are imaged by ultrafast, real-time transmission microscopy. The initial microbubble is marked by a circle. Adapted from reference [151] with permission from Elsevier.

Moreover, Zhou et al. demonstrated that acoustic radiation force may be used to displace the microbubble and eventually compress the microbubble against the cell membrane resulting in cell membrane disruption as evidenced by membrane voltage changes [45] (Figure 2B and 5). As suggested by the authors, this mechanism could explain the outcome of several drug delivery studies in which longer ultrasound pulses and lower ultrasound intensities are used to prevent excessive cellular damage due to microbubble collapse [45].

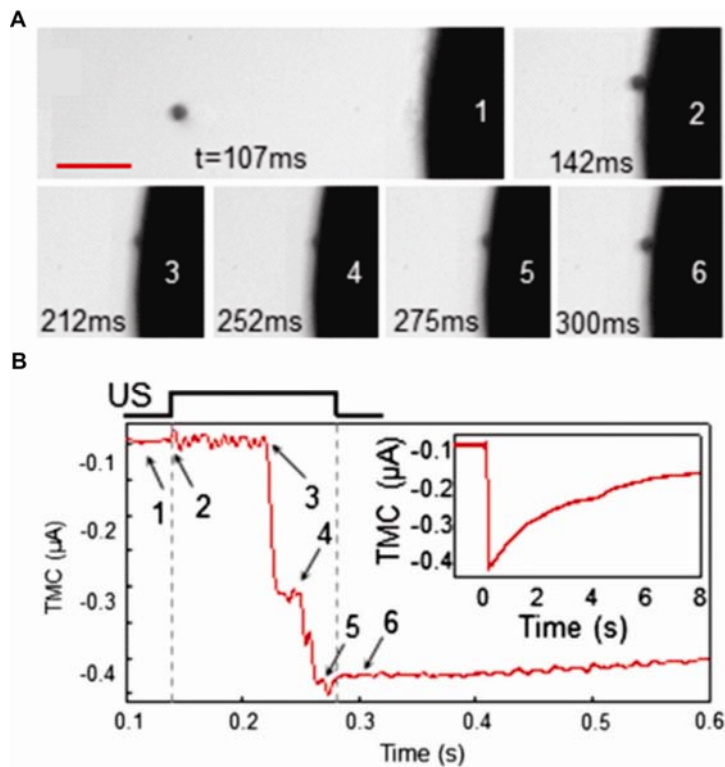


Figure 5. Example of a microbubble which is displaced and pushed against the cell membrane by acoustic radiation force. (A) Time-resolved optical images of a bubble moving toward the membrane and then pushed against the membrane. Ultrasound was on from frame 2 to 5. **(B)** The decrease in transmembrane current (TMC) indicates that the compression of the bubble against the cell results in membrane disruption. The numbers 1 to 6 are time points corresponding to the labeled frames in the optical images of (A). Inset: Recovery of the TMC. Adapted from reference [45] with permission from Elsevier.

Following sonoporation, even labeled microbubble shell components have been found intracellularly [37]. The concept of microbubble internalization could be particularly interesting for lipid-based drug loaded microbubbles that might fuse with the cell membrane resulting in increased drug delivery. Delalande et al. recently provided microscopy data of fluorescently labeled microbubbles entering inside HeLa cells [43, 46] (Figure 6). However, based on these images, it remains difficult to conclude whether microbubble shell fragments fuse with the cellular membrane or are internalized by the cells. Anyhow, they illustrate the potential of drug-loaded microbubbles for triggered drug delivery.

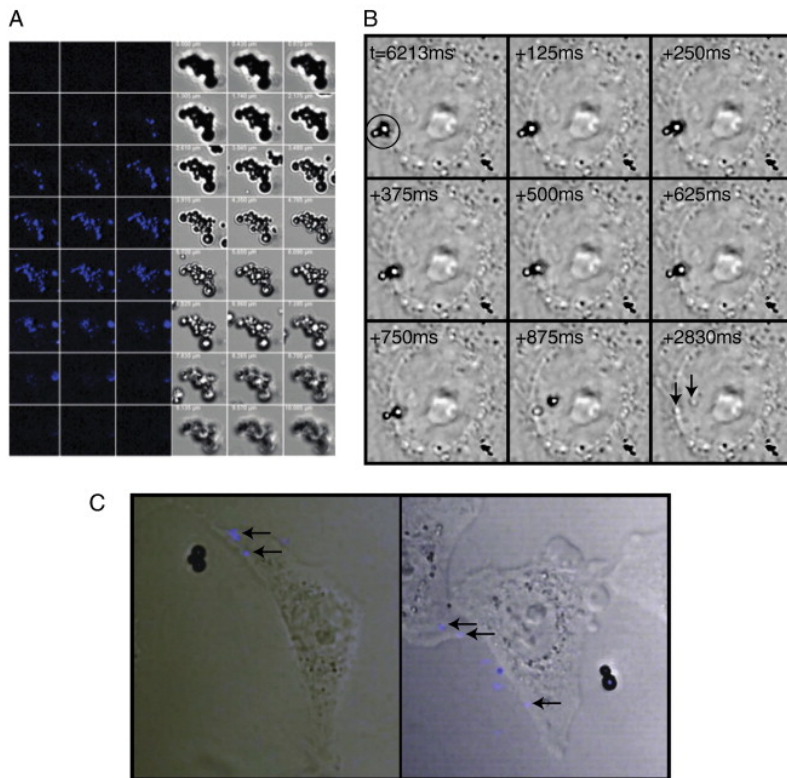


Figure 6. Microbubble internalization. (A) Z-stack of fluorescently labeled microbubbles observed by confocal microscopy. (B) High-speed imaging of two microbubbles (circle) during ultrasound application. The microbubbles were seen to push the plasma membrane and entered (frame +875 ms) in the cell until their disappearance (arrows). (C) Images after ultrasound application demonstrate intracellular fluorescence due to the internalization of fluorescent microbubbles. Adapted from reference [46] with permission from Elsevier.

Besides direct microbubble contact, microstreaming around cavitating microbubbles provides a second possible origin of mechanical stress on the cellular membrane (Figure 2C). As discussed above, this could lead to cell membrane disruption by tearing the lipid membrane open [24]. In this regard, it has been shown that microstreaming, generated by oscillations at low acoustic pressures, was responsible for the rupture of lipid vesicles [47] (Figure 7). This suggestion was also confirmed by the observations of Moosavi et al. who used real-time microscopy to study the interaction of moving, cavitating microbubbles with cells in suspension (Figure 8). They showed that only microbubbles moving in close vicinity with the cells could deform a small cell surface area caused by microstreaming induced shear stress. Moreover, when the microbubble repelled, the cell membrane

protrusion was retracted and the intracellular PI was expelled from the sonoporated cell, indicating cell survival [48].

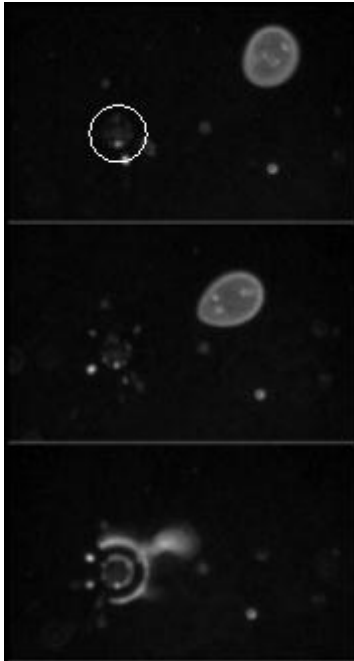


Figure 7. Vesicle deformation and rupture due to microstreamings generated by an oscillating microbubble. A fluorescently labeled vesicle approaches a bubble, marked by the white circle. The vesicle deforms and fragments. Adapted from reference [47] with permission from Nature.

In conclusion, all experiments performed at single cell level indicate that a direct contact between microbubble and cell membrane is required to induce pore formation by stable cavitation [27, 31, 45]. Larger distances would (i) hamper direct mechanical contact between cavitating microbubble and the cell membrane, and (ii) decrease the influence of microstreaming on the cell membrane. This hypothesis was already posed by Ward in 2000 when he noticed the clear relationship between cell-microbubble distance and sonoporation efficiency [49].

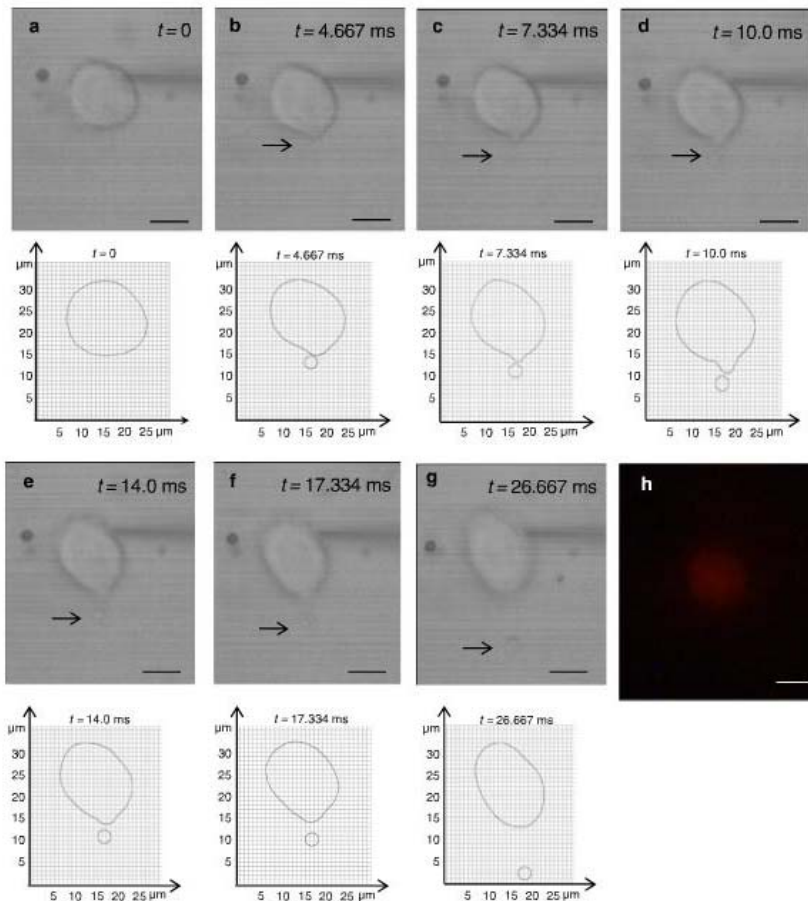


Figure 8. Moving cavitating microbubbles cause membrane deformation due to microstreaming induced shear stress. Top panels (A)–(G). High-speed time-resolved images of a microbubble motion in vicinity of the cell show local deformation of the cell membrane. The microbubble is indicated by the arrow. The cell membrane pulls outward as the microbubble approaches the cell. It gradually retracts back as the bubble repels. **Bottom panels (A)–(G).** Traced images of the real-time cell membrane deformation. **(H)** Fluorescence image of the cell immediately after ultrasound exposure reveals the increased cell membrane permeability and influx of the extracellular PI into the cell cytoplasm. Adapted from reference [48] with permission from Elsevier.

Finally, stable microbubble oscillations can lead to chemical stress by inducing the formation of free radicals. It was shown that free radicals play an important role in the increased cell membrane permeability for Ca^{2+} in cardiomyocytes [50] and primary endothelial cells [34, 51]. Particularly interesting is the fact that these studies were performed with two different ultrasound intensities (100kPa and 500kPa peak-to-peak or respectively 50 and 250kPa peak negative pressure (PNP)). Despite the fact that both intensities are inducing stable microbubble cavitation, the authors found

that catalase, a free radical scavenger, completely prevented Ca^{2+} influx at 50kPa PNP, while only a partial inhibition of 50% was detected at 250kPa PNP. These data suggest a more pronounced role for free radicals at lower acoustic pressures. It is known that shear stress associated with acoustic streaming or microstreaming can potentially lead to the formation of superoxide and H_2O_2 [20, 24, 52]. A first possibility is that the free radicals modulate existing ion channels like voltage gated Ca^{2+} channels [53]. The second possibility is that reactive oxygen species (ROS) induce cellular injury via lipid peroxidation which can result in lipid bilayer rearrangement and membrane disruption [54] (Figure 3).

To investigate the role of these ion channels in Ca^{2+} entry, the authors evaluated the influence of verapamil, a specific L-type Ca^{2+} channel blocker. They demonstrated that the influx of Ca^{2+} did not occur via these channels when cells were exposed to the highest pressure (250 kPa PNP), in agreement with earlier reports [55, 56]. Indeed, it can be expected that the larger vibration amplitudes associated with higher ultrasound intensities affect cellular permeability to a larger extent as a result of both chemical and mechanical stress. Consequently, the aspecific pores created by mechanical stress may lead to an additional Ca^{2+} influx which cannot be prevented by free radical scavengers [50, 51] or ion channel blockers [50, 56]. Unfortunately, they did not investigate the influence of the same inhibitor at the lowest pressure (50kPa PNP) applied. Therefore, it cannot be excluded that these ion channels do play a role at very low ultrasound intensities.

The same group showed that contact with an oscillating microbubble results in a local hyperpolarization of the cell membrane via the activation of BK_{Ca} channels [57]. The influx of Ca^{2+} ions is most likely overcompensated by a massive efflux of K^+ ions via these BK_{Ca} channels [57, 58]. Inhibition of BK_{Ca} channels with iberiotoxin will block K^+ efflux, resulting in a very slight depolarization of the cell membrane potential at very low acoustic pressures (50kPa PNP) due to a limited amount of Ca^{2+} entering the cell. In contrast, at higher acoustic pressure (250kPa PNP) blockage of the BK_{Ca} channels leads to a high cell membrane depolarization because of massive Ca^{2+} influx [57, 58]. The simultaneous influx of Ca^{2+} and PI is a clear indication that the acoustic pressures, applied in the

majority of research papers to induce stable cavitation, results in a diffusion driven influx of Ca^{2+} via cell membrane pores [59]. The fact that i) intracellular Ca^{2+} rise depends on the extracellular Ca^{2+} concentration and ii) is accompanied by the loss of fluorescence from fluorescently labeled cells seems to confirm this [28, 30, 35, 50, 55, 56].

2.1.3. Spatiotemporal aspects of stable cavitation.

Since the pores act as a filter for the internalization of drugs, several research groups have estimated the size of the cell membrane pores arising during sonoporation. Generally, pore sizes obtained with rather modest acoustic pressures were reported from several tens of nanometers to a few hundreds of nanometer. The uptake of fluorescently labeled marker molecules of different sizes has been studied by flow cytometry [9, 28, 52]. Using different acoustic pressures (125kPa, 246kPa and 570kPa) and fluorescently labeled dextrans with various molecular weight (up to 2 MDa), it was shown that pore openings as large as 56nm were formed independent of the pressure. Several other authors came to the same conclusion, although it remains difficult to compare the results because of the use of higher ultrasound intensities most likely resulting in microbubble implosion [8, 52, 60]. In contrast, Meijering et al. reported that molecules exceeding 155kDa are too large to be taken up via cell membrane pores but are mainly endocytosed [28]. At higher acoustic intensities, microbubbles will cavitate more extensively leading to larger cell membrane deformations and larger cell membrane disruptions [31, 38, 61].

Fast resealing of cell membrane porations are reported to occur in the order of milliseconds to seconds [55, 62, 63] after switching off the ultrasound. The fact that cell membrane permeabilization is rapidly decaying indicates that pores exist as long as the oscillating microbubbles are present [31]. The fast resealing of the cell membrane poration was demonstrated by rapid decline of intracellular calcium levels [50, 57] and restoration of cell membrane potential [55, 62-64].

2.2. Cell membrane permeabilization by inertial cavitation

2.2.1. Biophysical aspects of inertial cavitation.

At higher ultrasound intensities, the oscillation amplitude of the microbubbles can grow rapidly during the low pressure phase, until the microbubbles collapse due to the inertia of the intruding fluid. This results in the fragmentation of the microbubbles into many smaller microbubbles (Figure 1). This type of cavitation is called inertial cavitation. During the collapse of the microbubbles, shock waves (Figure 2D) can be generated in the fluid and jet formation (Figure 2E and 9) can occur. When a collapsing microbubble is located close to a surface like a cell membrane, an asymmetrical collapse takes place, and results in the formation of a liquid jet towards the nearby surface. It has been shown that shock waves and microjets create very high forces that can perforate cell membranes and even permeabilize blood vessels [65-67]. Dijkink and Ohl generated laser induced unshelled microbubbles to show that jetting occurred towards a cell layer [68, 69]. This was also the case for shelled microbubbles as demonstrated later on by Prentice [70] showing that liquid jets have the capacity to puncture cell surfaces and create cell membrane pores. It has been suggested that liquid microjets might act as microsyringes, delivering drugs to cells [65, 66]. This hypothesis was questioned in a later publication in which only sporadic jetting was reported [71].

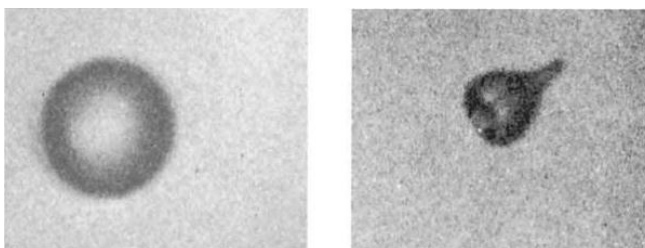


Figure 9. Ultrasound induced jet formation. Adapted from reference [65] with permission from Elsevier.

2.2.2. Biological effects provoked by inertial cavitation.

Higher ultrasound pressures have been used to stimulate microbubble collapse and subsequent cell membrane poration to induce the uptake of small and high molecular weight drugs [72] (Figure 3). This has mainly been attributed to the existence of cell membrane porations visualized by scanning electron microscopy (SEM) or atomic force microscopy (AFM) imaging [37, 60, 73, 74]. Cell size was shown to decrease after ultrasound radiation [60, 75, 76], and a smoother and flatter cell surface was observed [77]. Moreover, clear cell membrane invaginations were detected [1,3,4,5]. In a study of suspended MCF-7 cells after exposure to 1MHz ultrasound with increasing pressure (0,3 to 3 MPa PNP), treatment time and pulse repetition frequency (PRF) [73], it was noticed that the increase of the three parameters (either pressure, treatment time or PRF) had a substantial effect on the cell surface. Although the cell morphology was unchanged, larger sonoporation pores and rougher cell surface regions were observed (Figure 10). Although most research papers reported electron microscopy data to prove pore formation, it cannot completely be ruled out that these cell membrane invaginations are actually endocytic vesicles which are formed in response to sonoporation [28, 78].

When *Xenopus* oocytes were exposed to Optison™ microbubbles, transmembrane current (TMC) changes were larger when ultrasound pressures were increased (up to 600kPa PNP) [55]. Even more important was the fact that stepwise increases of the membrane potential were registered at lower ultrasound intensities, indicating successive microbubble collapses. They explained this by cavitating microbubbles, which grew until they reached their resonant diameter and finally collapsed. In contrast, higher ultrasound intensities resulted in faster and higher change of cell membrane potential which could be probably attributed to the fact that more microbubbles collapsed at the same time. This was one of the early indications that collapsing bubbles were responsible for the cell membrane disruptions. Subsequently, transmembrane current changes [64] and Ca^{2+} influxes [35] were investigated to further prove pore formation during microbubble collapse. The relation between microbubble implosion and sonoporation has been shown upon the passive detection of

inertial cavitation (IC) [73, 79]. DNA transfection of DNA containing nanoparticles was substantially enhanced by IC activity through the generation of cell membrane disruptions but only up to a certain threshold. When IC activity was further increased, cell viability substantially decreased and prevented the expression of the encoded protein[73].

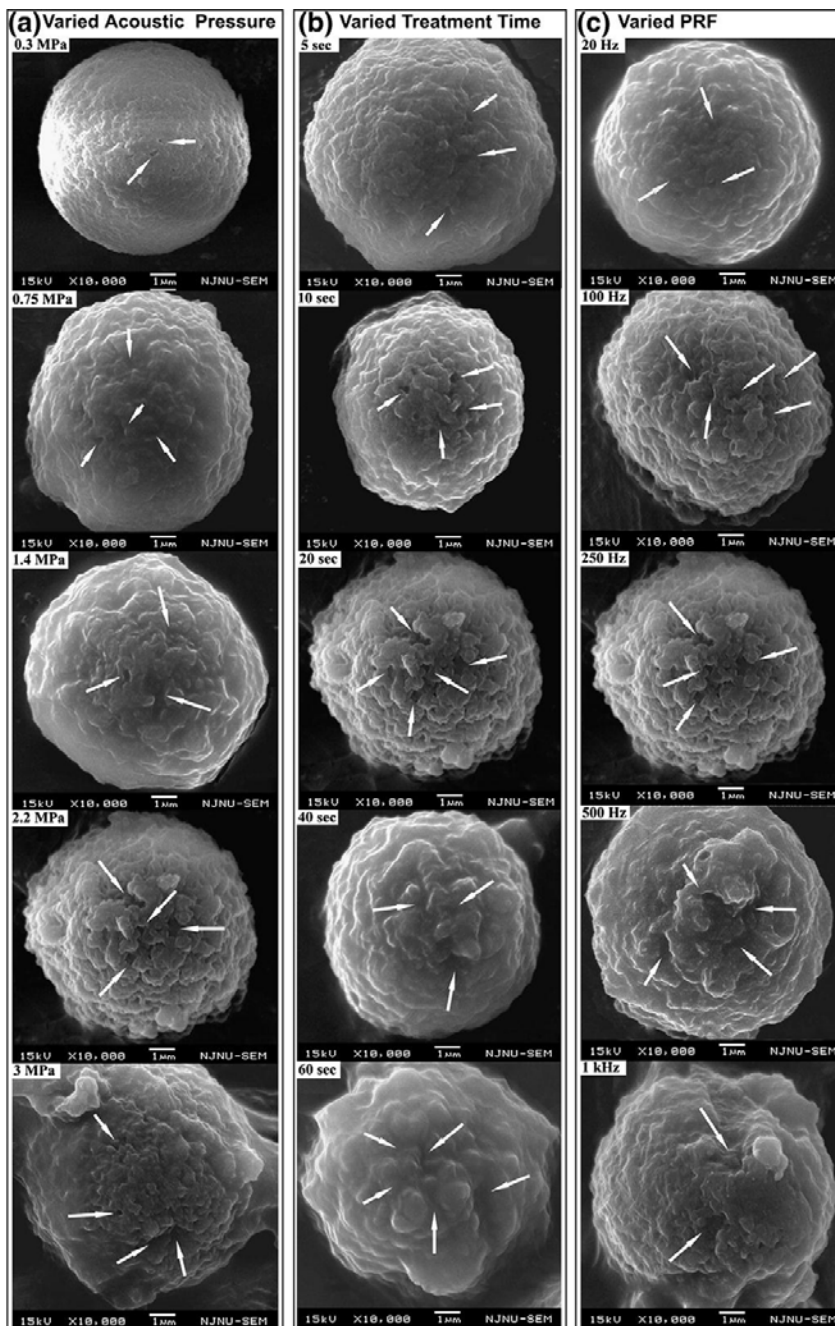


Figure 10. SEM-images of MCF-7 cells exposed to ultrasound. The **(A)** acoustic pressure (PNP), **(B)** US treatment time, and **(C)** PRF were varied. The white arrows point out some sonoporation pores. Adapted from reference [73] with permission from Elsevier.

Apart from the work published by Prentice et al., who showed that microbubble jets were able to perforate cell membranes, few studies have been reported on the direct impact of imploding microbubbles on the cellular membrane [70]. The data reported in literature only provide indirect evidence that collapse cavitation is causing cell membrane poration. For example, PI uptake was shown in prostate cancer cells during sonoporation [80]. The authors noticed that PI influx originated where microbubble implosion occurred and that cells were able to survive this poration as indicated by PI efflux. Recent real-time images demonstrated that microbubble implosion was directly linked with cell membrane poration and PI uptake [59, 61]. Moreover, the simultaneous influx of propidium iodide clearly demonstrated the existence of cell membrane porations or disruptions through which Ca^{2+} ions entered the cell [59, 61].

While stably cavitating microbubbles need to have direct contact with the cell to affect the membrane, the effects of inertial cavitating microbubbles reach over a larger distance. However, it was calculated that the maximal distance between microbubble and cell membrane should not exceed the microbubble diameter to have an effective impact on the cellular membrane [45].

2.2.3. Spatio-temporal aspects of inertial cavitation.

The analysis of transmission electron microscopy (TEM) images of sonoporated cells provided information regarding pore size. The technique has been mainly applied to study larger cell membrane disruptions arising after microbubble collapse [37, 60, 70, 73, 81, 82]. Pore sizes in the 100nm range were reported based on uptake of fluorescently labeled dextrans and beads [60]. It was hypothesized that smaller pores were probably present to a larger extent given the fact that the number of internalized dextrans or beads decreased with increasing size. AFM was also used to study the size of cell membrane pores. The reported sizes were in the order of 500nm to several μm [70, 74]. Although these microscopy images are useful to investigate cell morphology after sonoporation, it remains difficult to obtain quantitative information [45, 60]. Small pores might have resealed by the time the sample preparation is completed, and the sample preparation itself can also lead to artifacts in the image [60, 81].

Generally speaking, pore sizes which have been reported as a consequence of inertial cavitation (hundreds of nanometer to micrometer range) are larger than pores reported during stable cavitation (few nm to hundreds of nanometers). Moreover, pore size has been shown to correlate with acoustic pressure; higher acoustic pressures result in larger microbubble oscillations and larger pores [61, 73]. The effect of sonoporation on the cell surface of MCF-7 cells was studied with TEM. Only in the presence of ultrasound and microbubbles dimple-like craters of various sizes became visible on the cell surface. Based on these images pore size was estimated between 1-90 nm, 10-500 nm and 800 nm-1 μm corresponding with acoustic pressures of 190, 250 and 380 kPa, respectively [37], This correlation is in agreement with data showing an increase in uptake of all molecule sizes with increasing acoustic pressures [9, 52, 83].

In addition, pore size was estimated around 110 ± 40 nm by measuring the maximal transmembrane current (TMC) change via patch-clamp techniques [63]. Changes in the TMC were supposed to relate directly to the total area of pores since the total change of TMC was assigned to ion flows through the transient pores in the cell membrane. Very low microbubble concentrations were used to assure that only one pore was formed per cell, which was essential to calculate the average pore size.

By analogue with the data obtained with stably cavitating microbubbles, the patch-clamp technique has revealed that pores close within seconds after ultrasound is turned off [45, 61]. The intracellular fluorescence originating from PI internalization increased for a longer time, up to one minute [61]. This could be due to the internalized PI which became more fluorescent after nuclear uptake and complexation with genetic material. In contrast, Yudina et al. demonstrated that the cellular uptake of cell-impermeable small compounds persisted up to 24 hours with a half-life of 8 hours [84]. Since the internalization of these compounds persist beyond the pore lifetimes, other phenomena beyond the pores themselves must contribute to the drug uptake.

2.3. Ultrasound without microbubbles

Usually, cells are exposed to ultrasound in the presence of microbubbles, while application of ultrasound alone is only occasionally reported. However, ultrasound alone has also been shown to enhance delivery of DNA [85], proteins [83, 86] and drugs [87-89] into cells and tissue, though to a lesser extent. Indeed, it is well documented that ultrasound alone can also exert biophysical effects including thermal and non-thermal effects, such as cavitation and acoustic streaming ([90-92]. Cavitation in the absence of external microbubbles preferentially occurs at low frequencies and high intensities in order to generate and activate gas bodies in the medium, which can serve as cavitation nuclei [93, 94]. Acoustic streaming results from attenuation of the propagating ultrasound beam and resulting shear forces. Indeed, as an ultrasound wave travels through medium, part of its energy is absorbed, leading to an energy and pressure gradient. In fluids, this gradient creates a flow, called acoustic streaming [91, 95, 96], which in turn exerts shear stresses on cell membranes. Although acoustic streaming is not as strong as microstreaming generated by oscillating microbubbles, the corresponding shear stress leads to biological effects [97].

As regards the biological effects of ultrasound alone, pore formation as well as enhanced endocytosis have been reported. Tachibana et al. [98] were the first to image cells with pores by SEM after exposure to low frequency ultrasound of 255 kHz. Micron-scaled patches removed from the cell membrane have been reported following exposure of cells to 24 kHz high intensity ultrasound [99]. In addition, they found no involvement of endocytosis in the uptake of FITC-dextran. Furthermore, a correlation between the uptake of a marker compound and broadband detection upon low frequency ultrasound without microbubbles was reported, indicating cavitation [100]. As mentioned above, the low frequency ultrasound in the kHz range used in these studies promotes the generation of gas nuclei via rectified diffusion, which can subsequently implode and provoke the same bio-effects as imploding microbubbles. In contrast to these publications, other authors have suggested endocytosis as mechanism when using ultrasound in the absence of microbubbles [101-103]. However, in these cases higher frequencies and lower intensities were applied, so that cavitation was

less likely to occur and acoustic streaming may have been the dominating phenomenon. In section 3, it is discussed how this streaming and corresponding shear stress can affect endocytotic processes.

2.4. Influence of other ultrasound settings, and microbubble- and drug related parameters.

In the sections above we mainly looked at ultrasound pressure amplitudes to sort publications into those using stable or inertial cavitation. Obviously, it is rather difficult to make a clear distinction based on ultrasound pressure solely. The cavitation behavior of microbubbles at a certain frequency will mainly depend on their size, as microbubble response will be higher around their resonant radius [94]. This was also demonstrated by Deng who attributed the stepwise increase in transmembrane current to successive microbubble implosions which reached their resonant radius [55]. In agreement, Fan demonstrated successive microbubble implosions when acoustic pressure was increased [61]. Based on these observations, it is clear that the use of monodisperse microbubbles for further sonoporation studies is very attractive since all microbubbles would respond evenly at certain ultrasound setting allowing a better analysis of the influence of ultrasound conditions. From this point of view, it would be really interesting to make use of relatively new microbubble preparation techniques like microfluidic-based [104, 105] or ink-jet printed microbubbles [106]. Even the preparation of drug-loaded microbubbles has been optimized via microfluidic devices and could hence offer an interesting solution to fully optimize ultrasound induced drug delivery [107].

Although we mainly focused on ultrasound pressure in this review, it is clear that the ultrasound pulse length can have a major impact as well. Microbubble behavior of targeted microbubbles was investigated at two different pulse lengths: one of 10ms and one of 10 μ s, both with an acoustic pressure of 400kPa[108]. Simultaneously the authors evaluated the uptake of fluorescently labeled pDNA in the ultrasound treated cells. The short ultrasonic pulses resulted in localized microbubble implosion followed by relatively high delivery rates (30%) and higher cell viabilities (50%), while the longer pulses led to translational movements of the microbubbles causing large cell membrane

disruptions resulting in massive cell death (90%). It is known that microbubbles move towards each other as a consequence of secondary acoustic radiation forces or Bjerkness forces causing microbubble aggregation and displacement. In contrast to when using short pulses, this leads to very low pDNA delivery rates (10%). Additionally the authors evaluated the use of a ramped pulse scheme which consists of two successive, short ultrasound pulses with a more intense secondary ultrasound pulse. As suggested this resulted in larger cell membrane pores and consequently higher delivery rates since more microbubbles responded to the secondary pulse.

Although in the major part of drug delivery studies long ultrasound pulses (ms to s) are applied [77], it seems that very short pulses (few μ s) might be more efficient in combination with high acoustic pressures [9, 108] This is particularly the case when microbubbles are present with a resonant size corresponding to the ultrasound frequency, as they will rapidly implode when exposed to ultrasound [108]. This is in contrast to stable microbubble oscillations affecting probably cell membrane integrity when microbubbles are exposed to longer ultrasound pulses and vibration amplitudes are large enough [27, 31, 38]. Moreover, as already mentioned above, longer pulses can lead to acoustic radiation force, pushing intact microbubbles inside the cell membrane [45]. Indeed, this could explain why acoustic pulses of 0,25s were required to induce changes in cell membrane current at 230kPa PNP while much shorter acoustic pulses (0.02s) were sufficient to obtain cell membrane permeabilization at 600kPa [55].

Another parameter which has to be taken into account is the microbubble shell. Microbubbles can be categorized in different types according to their shell, i.e. lipid, polymer or protein shell. Lipid-shelled microbubbles are thinner and more flexible, while polymer- and protein-shelled microbubbles have a thicker and more rigid shell [109]. Although their cavitation behavior has been studied extensively, it remains a crucial question which microbubble type is most suited for drug delivery purposes. It was reported that the lipid-shelled contrast agent Definity[®] is more efficient in inducing drug delivery than the protein-shelled microbubble Optison[™] [110]. However, the studies reviewed in his paper used different bubble concentrations and different ultrasound parameters,

making an accurate comparison difficult. Therefore, the authors performed an experiment using these two types of microbubbles at equal microbubble concentrations and at the same ultrasound settings. Nevertheless, they also found Definity[®] to be superior to Optison[™] for delivery of 70 kDa FITC-dextran. Their findings are in agreement with the data of Karshafian et al. [9, 111], who performed similar experiments and concluded a better therapeutic ratio for Definity[®] microbubbles compared to Optison[™] microbubbles. In contrast, Mehier-Humbert et al. reported higher fluorescence intensities of GFP-DNA per cell, indicating a higher delivery, when using polymer- compared to lipid-shelled microbubbles [112]. Though, it must be noted that they insonated the microbubbles at higher acoustic pressures than the studies of Liu et al. and Karshafian et al.; and they used a larger molecule, which requires larger pores for delivery. These observations may be explained by the bubble behavior, which is highly dependent on the acoustic pressure. The higher flexibility of lipid-shelled microbubbles allows them to oscillate at low acoustic pressures. Upon rupture, they fragment into smaller bubbles which stay centered around the initial bubble [113]. In contrast, response of hard-shelled microbubbles requires higher acoustic pressures. Furthermore, rupture of these microbubbles is caused by a process called sonic cracking. This occurs through the formation of a small defect in the shell, thereby releasing a violent stream of gas which can be propelled for a few microns [113-115]. Therefore, lipid-shelled bubbles may be preferred when low acoustic pressures and small pores are required, while polymer- and protein-shelled bubbles may produce stronger effects and larger pores, though only above a certain threshold. This also indicates that quantitative measurements of the cavitation activity during sonoporation might be a good solution to compare the sonoporation events of different studies. Cellular bio-effects correlate directly to the cavitation dose, while this depends on the interaction of a broad range of ultrasound and microbubble parameters like ultrasound pressure, exposure time, microbubble type and concentration [8, 73, 79, 100].

The microbubble shell might also influence the interaction between microbubbles and cell membranes and this might have a substantial impact on sonoporation efficiency [46]. In this regard,

it has been demonstrated that hard-shelled albumin microbubbles like Quantison® might be less efficient for drug delivery purposes since they seem to interact to a substantially lower degree with biological membranes [43]. It seems logic that based on these data, lipid microbubbles might be first choice contrast agents for drug delivery studies. Given the fact that ultrasound radiation has been shown to stimulate mixing of lipid monolayers and cell membrane lipids, it cannot be excluded that microbubble lipid shell could also fuse with the cell membrane [116, 117].

Additionally, drug size can have a substantial impact on delivery rates. Regarding pore formation, it is evident that pores are acting as sieves, only allowing molecules to pass if their size is below the pore diameter. Furthermore, molecules pass through pores via passive diffusion, which also favors small molecules. These hypotheses are supported by results obtained by several authors reporting the amount of internalized molecules to be inversely proportional to the molecule size.

Finally, the use of targeted microbubbles, as already used by Kooiman et al., could decrease the distance between microbubbles and cell membranes thereby increasing the chance of pore formation [38]. Generally speaking we can conclude that both phenomena, stable and inertial cavitation, can result in pore formation although pore sizes created by imploding microbubbles are generally larger [108].

Besides visualizing the uptake of marker molecules it might be interesting as well to consider the uptake of microbubble shell fragments. Increasing evidence suggest that drug-loading of microbubbles can aid in the drug delivery process [46, 118]. Not at least this might be provoked by a direct contact between microbubbles and the cell membrane and even the internalization of intact microbubbles was already suggested [43, 45, 119]. Moreover, we should be able to combine real-time imaging of sonoporation at a single cell level [27, 31, 45, 59, 61, 64] with more quantitative methods like flow cytometry [7-9, 120]. Only this combination will give us the crucial information we need to fully understand and optimize ultrasound triggered drug and gene delivery.

3. Sonoporation induced endocytosis or exocytosis?

Several endocytotic processes are taking place in mammalian cells. In general the two main endocytotic processes are clathrin-dependent (CDE) or receptor-mediated endocytosis and clathrin-independent endocytosis (CIE), comprising lipid raft endocytosis like caveolin-mediated endocytosis [121]. It was shown that caveolae-mediated endocytotic activity in endothelial cells of fluorescently labeled proteins was increased after pulsed diagnostic ultrasound exposure at elevated pressure levels (corresponding to 1.5 MPa PNP) in the absence of microbubbles [122]. The identical ultrasound settings did not influence clathrin-mediated endocytosis of fluorescently labeled transferrin. In contrast, receptor-mediated endocytosis of fluorescent markers was demonstrated in fibroblasts exposed to low intensity (0.1 MPa) pulsed ultrasound in the absence of microbubbles [123].

It has also been shown that stably cavitating microbubbles could enhance the uptake of fluorescently labeled dextrans by the formation of small membrane pores and/or endocytosis [28]. After sonication (220kPa PNP) the smaller dextran molecules (4.4 and 70 kDa) were homogeneously distributed throughout the cytosol, whereas the larger dextran molecules (155 and 500 kDa) were mainly localized in vesicle-like structures, indicating that endocytosis was involved in the uptake of the larger dextrans (Figure 11). This first suggestion was confirmed by measuring the ultrasound mediated uptake of dextran molecules while inhibiting one of the three main endocytotic pathways (i.e. clathrin-mediated endocytosis, caveolae-mediated endocytosis and macropinocytosis). The authors demonstrated that all three main routes of endocytosis were involved in the ultrasound mediated uptake of all sizes of dextran molecules. Though, mainly a clathrin-dependent mechanism was contributing to the uptake of 500 kDa dextran. This is also in agreement with the paper from Paula et al. who also attributed the transfection efficiency of naked plasmid DNA to an increased clathrin-dependent uptake of the plasmid, although they did not use any microbubbles but only ultrasound [124]. Consistent with these data, there is experimental evidence that also the enhanced and long-lasting transfection of naked pDNA in the presence of microbubbles and ultrasound could be attributed to enhanced endocytosis of naked pDNA [46, 125]. This is in contrast with publications

from our group in which increased gene transfection efficiency of PEGylated lipoplexes has been attributed to direct cytoplasmic entry, as evidenced by the use of endocytotic inhibitors and confocal microscopy images[126-128]. It is however difficult to compare these studies given the fact that the latter study made use of microbubbles loaded with gene complexes and did not depend on co-administration of gene complexes and microbubbles. Moreover, the relatively long pulses which were used in this study can result in translational movements of the gene-loaded microbubbles towards the cellular membrane. This could result in a direct deposition of the gene complexes in the cytoplasm, thereby avoiding endocytosis.

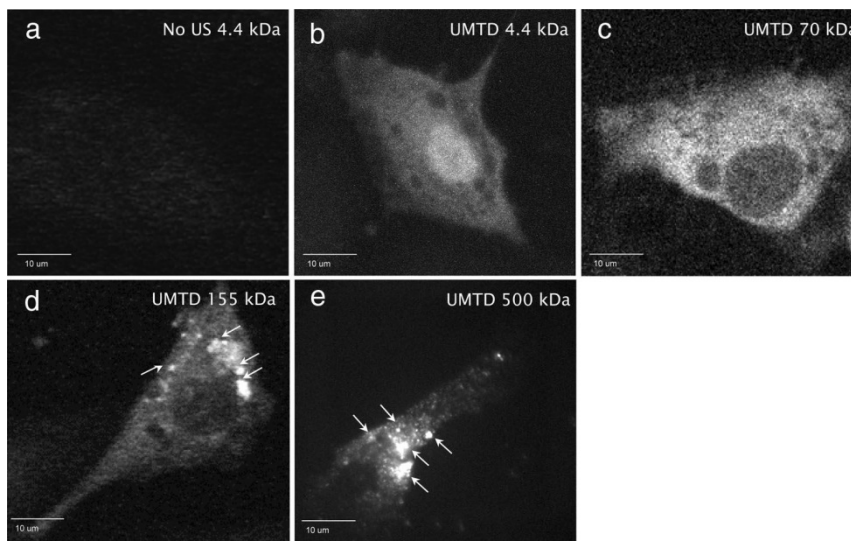


Figure 11. Ultrasound induced endocytosis of fluorescent dextrans with different molecular weights. (A) No uptake of 4.4 kDa dextran in the absence of ultrasound. **(B)** Homogeneous distribution in the cytosol and nucleus of 4.4 kDa dextran after ultrasound application. **(C)** Homogeneous distribution in the cytosol of 70 kDa dextran but absence of nuclear localization. **(D)** and **(E)** Localization of 155 kDa and 500 kDa dextran, respectively, in vesicle-like structures (arrows). Adapted from reference [28] with permission from Lippincot-Williams & Wilkins.

The mechanisms which are responsible for the ultrasound induced endocytosis have been the subject of debate and have not been completely elucidated up till now. A first possible explanation is that microstreamings or acoustic streaming induces endocytosis (Figure 3). In this regard, it has been demonstrated that shear stress can stimulate the endocytic uptake of fluid-phase markers in

endothelial cells [22, 129]. The ultrasound induced mechanical forces can lead to plasma membrane deformation which is accompanied by cytoskeletal rearrangements as a result of changes in cell membrane tension [130]. Indeed, several reports have been published indicating that ultrasound or oscillating microbubbles can induce cytoskeletal rearrangements [34, 44, 131-133]. Mechanosensors, such as integrins and stretch-activated ion channels, are able to sense these changes and transduce these signals into downstream cellular processes such as endocytosis and exocytosis. It is believed that these processes add (exocytosis) or remove (endocytosis) plasma membrane and hence restore plasma membrane tension. A first type of mechanosensors are integrins which link extracellular matrix molecules to the intracellular actin cytoskeleton and can indirectly influence endocytotic or exocytotic processes via a cascade of intracellular signaling pathways [130]. Secondly, mechanosensitive channels have been identified that mediate direct Ca^{2+} -influx and/or promote Ca^{2+} release from internal stores [118]. A detailed overview of these interactions is however beyond the scope of this review.

Clearly the influx of Ca^{2+} plays a pivotal role in the ultrasound enhanced endocytotic activity [134]. As already extensively discussed, higher shear forces can result in a physical disruption of the cell membrane which will also lead to elevated intracellular Ca^{2+} levels due to a concentration driven passive diffusion of Ca^{2+} . In this context, Ca^{2+} -influx has also been shown to play a key-role in membrane repair processes [135] and more specifically in the closure of cell membrane porations after sonoporation [45, 55]. Initially, cell membrane wounds were believed to be repaired by self-sealing of the phospholipid bilayer, driven by the energetically favored outcome [136]. However, the cell membrane is supported by the cytoskeleton, thereby creating a membrane tension which opposes spontaneous resealing. Therefore, only repair of small pores ($<0.2 \mu\text{m}$) may be explained by self-sealing [137]. Later on, research has demonstrated that influx of Ca^{2+} through membrane wounds triggers exocytosis, with recruitment of intracellular vesicles such as lysosomes to the site of injury [138, 139]. Although exocytosis is clearly involved in repair, how exocytosis contributes to

resealing of the membrane wound is less understood. Two mechanisms are proposed. Firstly, exocytosis lowers the membrane tension in order to facilitate spontaneous resealing [140]. Secondly, the intracellular vesicles recruited by exocytosis fuse with each other and form a giant patch that subsequently fuses with the damaged plasma membrane and reseals the membrane disruption [87, 141] (Figure 12A). These theories could explain why Schlichler et al. [99] observed vesicles associated with wound sites after ultrasound exposure on TEM-images. Moreover, when fluorescently labeling the intracellular vesicle pool, they reported a decrease in fluorescence, indicating vesicle trafficking to the cellular membrane. These findings indicate that exocytosis is also triggered by ultrasound created cell membrane disruptions.

While patching seems to be the predominant mechanism for repair of mechanically induced lesions, smaller membrane pores can be physically removed via endocytosis [142] (Figure 12B). This could explain the contradictory findings of different groups which reported either ultrasound induced exocytosis [37, 82] and no involvement of endocytosis in the uptake of model-drugs [8] or nanoparticles [126, 128] or ultrasound induced endocytosis [28, 52]. The rather harsh ultrasound conditions which were applied in the first category provoke inertial cavitation and will lead to rather large cell membrane disruptions in the μm range which probably requires the pre-formation of a lysosomal patch to repair the injured cell membrane. In contrast, stable microbubbles oscillations will create only smaller cell membrane disruptions (nm scale) and might be as well removed from the cell membrane via endocytosis. Since the Ca^{2+} influx is known to be a dominant trigger for cell repair, it is very likely that intracellular Ca^{2+} concentrations will determine which repair mechanism becomes active or whether cellular apoptosis proceeds when the Ca^{2+} influx exceeds a certain threshold [134, 143].

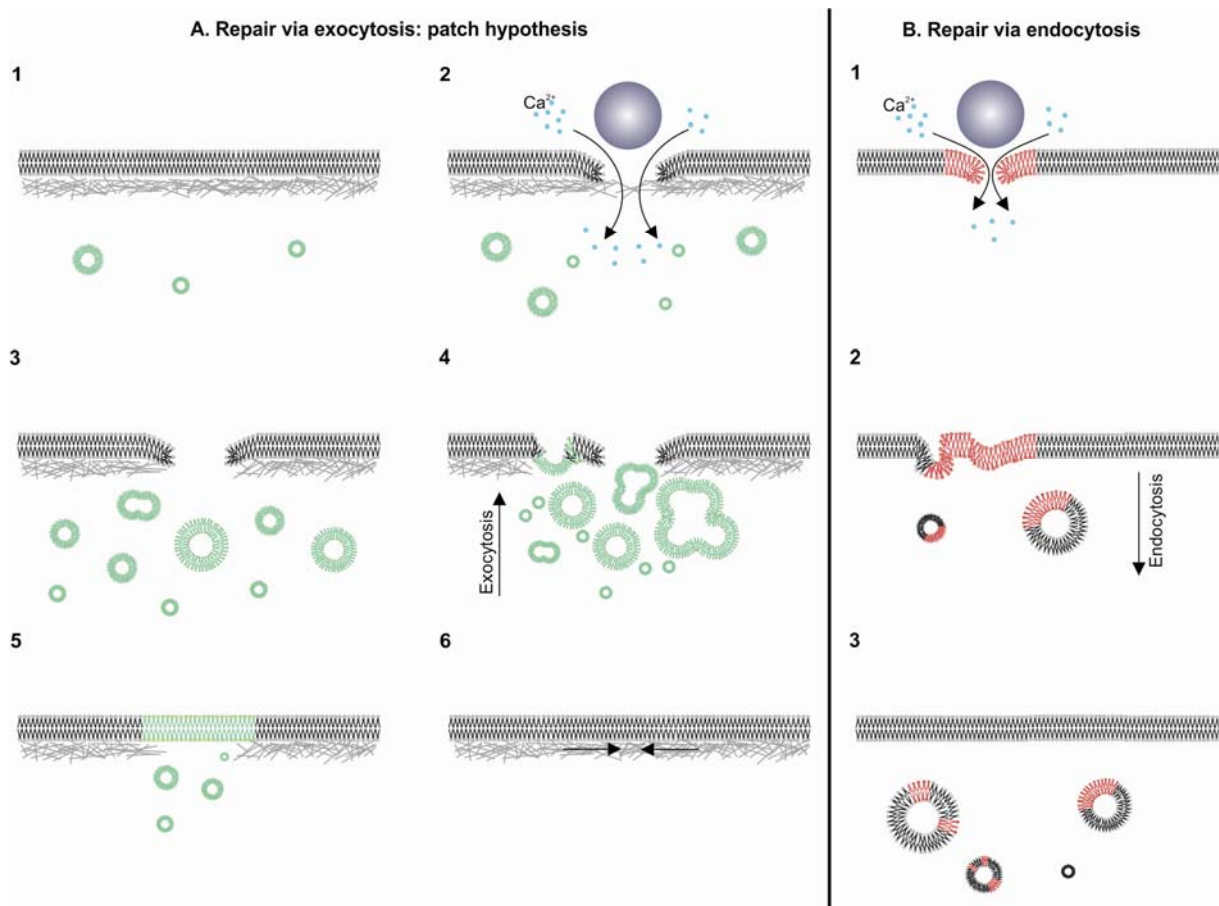


Figure 12. Pore repair mechanisms. (A) Large membrane disruptions are suggested to be repaired via exocytosis of a patch of intracellular vesicles. (1) A cell with a continuous lipid bilayer, underlying actin skeleton (grey) and intracellular vesicles (green) is depicted. (2) Microbubble cavitation causes a large disruption in the cell membrane. Ca^{2+} entering through the disruption initiates depolymerization of the actin skeleton and triggers the accumulation of intracellular vesicles. (3) The accumulated vesicles fuse with each other to create large patch vesicles. Local dissolution of the actin skeleton allows recruitment of the vesicles to the cell membrane. (4) Increasing vesicle-vesicle fusion leads to the formation of a large patch. Vesicle-membrane fusion adds new membrane to the disruption site. (5) The patch of internal vesicles completely reseals the disruption and the integrity of the lipid bilayer is restored. (6) Post-resealing polymerization of actin reestablishes cytoskeleton continuity [87]. **(B)** Smaller membrane pores may be resealed via endocytosis. Ca^{2+} influx via the pores induces endocytosis, thereby removing the pores from the cell membrane.

Recent insights from Idone and colleagues suggest that both mechanism are taking place in membrane resealing [144]. They observed a rapid Ca^{2+} -dependent endocytosis of fluorescent dextrans when creating small pores in the cell membrane by SLO-toxines (Streptolysin O) or by mechanical injury. Therefore, they hypothesized that endocytosis compensates the lysosomal exocytosis triggered by membrane pores. In a later study, this compensatory endocytotic process

was shown to be mediated by the exocytotic release of lysosomal acid sphingomyelinase (ASM). ASM converts sphingomyeline in the cell membrane to ceramide, which promotes inwards budding and vesicle formation [145]. Accordingly, the observed ultrasound enhanced endocytosis may be a consequence of exocytosis.

The cell membrane repair processes discussed above regard cells treated with pore-forming toxins or cells mechanically injured by scraping to mimic physiological processes in the gut, endothelium, skin or muscle [146]. However, there are strong indications that the same repair processes occur in ultrasound treated cells. Ultrasound and microbubbles also create aspecific pores leading to Ca^{2+} -influx [147]. This Ca^{2+} -influx is also shown to be required for resealing [148]. Therefore, it is reasonable that also in ultrasound treated cells a compensatory endocytotic process will be initiated, which may contribute to the observed ultrasound enhanced endocytosis (Figure 3). In this regard it would be very interesting to investigate whether cells exposed to inertial cavitation have an increased endocytotic activity at a later time point.

Besides the role of Ca^{2+} in repair mechanism, recent experimental results suggest that Ca^{2+} can also directly stimulate endocytosis independently of ASM release but via interaction with cholesterol rich cell membrane areas. These cell membrane domains can spontaneously vesiculate and form endocytic vesicles under the influence of Ca^{2+} [149]. This mechanism might be important in reference to the calcium waves which move from the sonoporated cells to adjacent cells [35, 36, 59, 150]. The delay in Ca^{2+} influx in these cells indicates the involvement of a secondary messenger which travels through gap junctions from one cell to another. This could mean that in contrast to cell membrane poration, which depends on direct microbubble contact, endocytosis might be enhanced in a larger fraction of ultrasound treated cells. Zhou et al. studied the influence of extracellular Ca^{2+} levels on membrane resealing and defined two different membrane resealing processes [45, 64]. An early stage recovery phase was found to be upregulated in the presence of higher extracellular Ca^{2+} concentrations, while a much slower, secondary repair process accelerated when more Ca^{2+} was

available. According to the authors this strokes with the idea that a Ca^{2+} dependent, active repair mechanism like cell membrane patching is required to restore cell membrane damage [35].

4. Implications for drug delivery and concluding remarks

The aim of this review was to provide an in depth overview of recent attempts which have been published to understand the impact of ultrasound and more specifically microbubble cavitation on cellular integrity and to provide an overview of different mechanisms which have been reported to contribute to ultrasound induced drug delivery up till now. It is without doubt that a complete understanding of these processes is a crucial step to maximize the efficiency and safety of ultrasound induced drug delivery. In the past, many post ultrasound assays have been performed to gain information on the mechanisms involved in ultrasound induced drug delivery. Although they were useful to demonstrate the uptake of several (marker) drugs, they did not take into account the actual transient process of sonoporation and the subsequent very fast resealing of cell membrane perforations [112]. As such, these techniques inevitably lacked crucial information. Moreover, their outcome might have been influenced by the individual experimental conditions after ultrasound exposure. Later on, transmembrane current studies were used to monitor the dynamics of ultrasound and microbubble induced pore formation. Those studies have provided us with a more detailed view on pore size and resealing kinetics. However, only recently, these methods were combined with fast, real-time microscopy techniques allowing the investigation of the direct impact of microbubble behavior on individual cells [45, 59, 61]. Such real-time assays make it possible to estimate the impact of ultrasound settings on the physiologic process involved in ultrasound induced drug delivery. It is striking how these responses could be possibly tailored by changing ultrasound conditions [9, 108]. Needless to say that dependent on the drug type, different internalization routes are preferentially upregulated. Using low intensity ultrasound, the endocytotic uptake of several drugs could be stimulated, while short but intense ultrasound pulses can be applied to induce pore

formation and the direct cytoplasmic uptake of drugs which are sensitive to lysosomal degradation. Additionally, ultrasound intensities may be adapted to create pore sizes which correlate with drug size. It is obvious that small marker molecules or drugs such as low molecular weight dextrans, propidium iodide and doxorubicine, are able to diffuse passively through small pores created by low intensity ultrasound treatment. However, delivery of larger drugs such as nanoparticles and gene complexes, will require higher ultrasound intensities in order to allow direct cytoplasmic entry.

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