Sonopermeation to Improve Drug Delivery to Tumors:

From fundamental understanding to clinical translation

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Abstract

Introduction

Ultrasound in combination with microbubbles can make cells and tissues more accessible for drugs and thereby achieve improved therapeutic activity. In this review we establish the term "sonopermeation", covering mechanisms such as pore formation (sonoporation), opening of tight junctions, stimulated endocytosis/transcytosis, altered perfusion and changes in stromal compartment. Sonopermeation has gained a lot of interest in the last decade, especially for delivering drugs through the otherwise impermeable blood-brain barrier, but also to tumors.

Areas covered

In this review we summarize various in vitro assays and in vivo setups that have been employed to unravel the fundamental mechanisms involved in ultrasound-enhanced drug delivery, as well as clinical trials that are ongoing in patients with brain, pancreatic, liver and breast cancer. We summarize the basic principles of sonopermeation, describe recent findings obtained in (pre-) clinical trials, and discuss future directions.

Expert Opinion

We suggest that an improved mechanistic understanding, and microbubbles and ultrasound equipment specialized for drug delivery (and not imaging) are key aspects to create more effective treatment regimens by sonopermeation. Real time feedback and tools to stratify which tumors will benefit from sonopermeation will be important for clinical success.

Keywords: Sonopermeation, sonoporation, ultrasound, microbubble, cancer, blood-brain barrier

Highlights:

- We suggest "sonopermeation" as a new term to describe increased drug delivery by ultrasound and microbubbles.

- Specialized microbubbles and ultrasound transducers are being developed for therapeutic applications in drug delivery, rather than using combinations of already approved materials.
- As sonopermeation is being established as one of many treatment options, it will be increasingly important to develop tools to stratify tumors and patient groups, to treat only those who are likely to benefit from such treatment.
- Real time feedback-based control appears to be a clear step towards safe and effective sonopermeation, and should be applied whenever possible.
- Understanding the underlying mechanisms and effects of sonopermeation will be crucial to optimize the efficacy and safety to achieve clinical translation.

1. Introduction

Achieving curative treatment of advanced cancer is notoriously difficult and requires that all cancer cells are killed or inactivated. For advanced cancer, chemotherapy is generally required, either alone or in combination with other treatment modalities. However, although the drugs are potent, they are not selective enough and achieving sufficiently high concentrations in tumors without the occurrence of unacceptable toxic effects is often not possible. Off-target accumulation can lead to various side effects and limits the doses that can be administered.

Nanomedicines, which typically rely on the enhanced permeability and retention (EPR) effect for improved tumor accumulation, are designed to improve the biodistribution and thereby therapeutic index of chemotherapeutic drugs [1-3]. Efficiently exploiting the EPR effect in clinical settings, however, has turned out to be relatively challenging [4-6]. Drugs, macromolecules and nanoparticles given intravenously face multiple barriers and restrictions on their way to the target site, complicating efficient delivery. While conventional small molecule drugs suffer from a large volume of distribution and a rapid renal clearance and hence relatively low concentrations in the tumor [7, 8], macromolecules and nanoparticles are in principle restricted to the vasculature, except for areas with inflammation or in tumors, which are both characterized by leaky blood vessels. According to the EPR-effect, nanomedicines may extravasate through the hyperpermeable vasculature in tumors where they are retained as a result of inefficient lymphatic drainage.

Multiple features and facts complicate EPR-based tumor targeting. For instance, the vasculature in tumors is often highly irregular and chaotic, leaving parts of tumors very poorly perfused [9-11]. In addition, the leakiness of the blood vessels tends to be highly heterogeneous [12-14]. After extravasation, the penetration of drug carriers is restricted by the presence of dense stroma [15], the high interstitial fluid pressure observed in many tumors [16-19], and the cell membrane of the tumor cells. Together, these barriers make it very challenging to achieve sufficient degree of targeted drug delivery, especially to the deeper parts of the tumor, precluding curative drug therapy [20, 21]. In the brain, drug delivery is particularly complicated by the blood-brain barrier (BBB), which is formed by endothelial cells and pericytes lining the brain capillaries, connected by tight junctions to protect the brain from potentially harmful blood-borne molecules and materials [22].

Based on these limitations, multiple research groups and pharmaceutical companies are developing methods that can increase the tumor accumulation and cellular penetration of drugs and drug delivery systems [23, 24]. Studies are ongoing to address whether this can be achieved either by administering agents such as vasodilators, blood vessel normalizing agents or molecules that modulate the extracellular matrix, by the use of stimuli-responsive nanocarriers reacting to specific features associated with the target disease (such as enzymes, redox potential or changes in pH), or nanocarriers responsive to locally applied external triggers (such as light, temperature, magnetic fields or ultrasound) [23, 25-27].

2. Ultrasound in drug delivery

Ultrasound in medical diagnostics is a safe and widely applied real-time imaging modality. During the last decades, ultrasound has also been increasingly studied for therapeutic purposes [28-30]. Because it can be focused, it can be used to deliver energy to small volumes deep inside the body without affecting intermediate tissues. Ultrasound is generally non-invasive and localized and can, depending on the desired application, be tuned to create thermal effects such as heating, or mechanical effects such as acoustic radiation force or acoustic cavitation [31, 32]. When ultrasound waves pass through tissue, the waves will be attenuated by scattering and by absorption [33]. The absorption of acoustic energy by tissue causes thermal heating [32-34]. High intensities can be employed to create hyperthermia for applications in physiotherapy [35] and tissue ablation with real time temperature mapping (via magnetic resonance imaging; MRI) [36, 37]. Local mild hyperthermia can also be used to increase drug release from nanocarriers

such as thermosensitive liposomes [38, 39], and to locally increase blood flow [40, 41], vascular permeability [42, 43], diffusion of drugs, and possibly cellular uptake [31, 44], thereby enhancing delivery of therapeutic agents. A radiation force [45] in the direction of wave propagation is caused by a momentum transfer from the ultrasound wave to the transmitting medium [32]. This force can produce a steady flow in fluids (known as acoustic streaming), and may therefore potentially increase convective transport [46]. It could also cause local tissue displacements [33] and disrupt extracellular matrix for increased extravasation and interstitial penetration [32]. In addition, acoustic radiation forces have been reported to modulate the direction and velocity of flow of ultrasound contrast agents, i.e. microbubbles, for instance by pushing them towards the vascular wall while they circulate in tumor blood vessels [45].

The use of ultrasound in the presence of exogeneous gas bubbles can lead to cavitation and local forces strong enough to cause membrane permeabilization. Cavitation refers to the creation and/or oscillation of gas bubbles upon exposure to an acoustic field, in response to the oscillating acoustic pressure [31, 34]. By the use of ultrasound and microbubbles, improved effect of conventional chemotherapeutics has been demonstrated in patients with non-resectable pancreatic tumors (PDAC) [47, 48] and in clinical trials with glioblastoma patients (table 1) [49]. Preclinically, the effect has been evaluated for a myriad of indications. As there are multiple excellent reviews on the topic [30, 50-54], we here focus on how these effects are frequently explained, review some systems created specifically for drug delivery, and suggest future directions to improve tumor-targeted drug delivery and achieve clinical impact.

2.1. Sonopermeation

This review presents studies demonstrating increased drug delivery by ultrasound and microbubbles regardless of underlying mechanisms. The term sonoporation has often used to describe these mechanisms [51, 52]. However, the term sonoporation refers to the formation of 'pores' by the use of sound, which is only a subset of the effects that have been shown for ultrasound and microbubbles. In this review we establish the term "sonopermeation" as a term describing increased therapeutic effect achieved by ultrasound and microbubbles. We suggest that the term "sonoporation" will be used specifically for the formation of pores. Sonopermation describes the non-thermal and mechanical effects achieved with the combination of ultrasound and exogenous microbubbles. It is hypothesized to function both through the formation of transient pores in cell membranes (sonoporation), the opening of intercellular (tight) junctions [51, 55-57], stimulated/altered endocytosis, transcytosis or exocytosis [58, 59], macroscopic changes in perfusion [60] and changes in extravascular, and perivascular space [61]. As pressure waves pass through tissues, microbubbles in the pressure field will expand at low pressures (rarefaction) and contract at high pressures (compression), creating volumetric oscillations in phase with the applied ultrasound [34]. Stable cavitation occurs at relatively low amplitudes, and is characterized by sustained bubble radius oscillation about its equilibrium [32]. These oscillations can be detected as harmonic signals from the microbubbles. Oscillating microbubbles will generate a circulating fluid flow, known as microstreaming, which has velocities and shear rates proportional to the amplitude of oscillation [31, 62, 63] and to the applied pressure. If the microbubbles are close to the endothelium, they can also push and pull on the cell membrane [64], and especially the pulling motion, creating elongation of the cell membrane has been suggested to induce formation of pores [65]. Inertial cavitation occurs when larger amplitude oscillations result from an increased acoustic pressure [31]. The amplitude of oscillation increases until the inrushing fluid has sufficient inertia to overcome the internal pressure of the bubble, and then the bubble will collapse [31, 34]. The extreme compression of the gas by the liquid creates high pressures and high temperatures, and the fragmentation of the microbubble results in smaller bubbles which can again cavitate, grow and collapse [31]. Following the collapse of a bubble, shock waves are created and liquid jets can occur if the bubble collapses near a surface [31, 32, 51]. The oscillation and collapse of microbubbles can also cause formation of free radicals [51], leading to cytotoxicity and potentially cell death [44].

2.2. Ultrasound parameters for sonopermeation

To enable controlled drug delivery without causing tissue damage, careful control of ultrasound parameters is required. For sonopermeation, the ultrasound wave is typically pulsed both to avoid tissue damage from heating and to allow for inflow of microbubbles between the pulses in cases where bubble destruction is expected. The sinusoidal ultrasound wave is often described in terms of its velocity, wavelength, frequency (or period), pressure amplitude, pulse length (or burst duration), pulse repetition frequency (PRF), total exposure time (or duty cycle) and total treatment time [33, 66, 67]. Mechanical index (MI), defined as the peak negative pressure amplitude (MPa) divided by the square root of the center frequency (MHz) of the transmitted ultrasound wave, is often used to classify microbubble behavior, and the probability of inertial cavitation occurring increases with increasing MI [31, 64]. Frequently used parameters for sonopermeation and drug delivery vary greatly between different studies, including frequencies of 0.5-3 MHz with pressures of 0.05-2 MPa, pulse lengths of 2-10 000 cycles with a PRF of 0.25 Hz - 10 kHz, and total exposure times of seconds to hours with duty cycles varying from less than 1% to 50% [52, 66, 68-73]. The response of a microbubble will depend highly on the ultrasound settings [52, 64]. Increasing the pressure, sonication time, burst length or pulse repetition frequency has been shown to give increased permeability of vasculature in the brain [74]. It has also been suggested that higher pressures and thus larger oscillations and a more violent collapse probably induces larger pores, which are required to deliver nanoparticles and gene complexes compared to low molecular weight drugs [51]. By applying real-time feedback of acoustic emission from the microbubbles, the ultrasound parameters can be standardized to the microbubble response in each animal [75, 76]. By doing this, it is possible to eliminate in situ pressure fluctuations caused by variations in tissue absorption of ultrasound, variations in skull thickness when intending to open the blood-brain barrier, or differences in bubble concentration caused by varying vascularization and perfusion between tumors. The harmonic signal may then be used to monitor bubble behavior, with subharmonic and ultraharmonic emissions indicating stable cavitation [77-79], and increased broadband acoustic emission indicating bubble destruction or inertial cavitation [80, 81].

2.3. Biological effects of sonopermeation

Various methods have been reported in the literature to study the mechanisms and effects of bubble-cell interactions [50, 82]. Some examples of how oscillating microbubbles can interact with cells are illustrated in Figure 1. The resulting streaming and shear forces, and/or push-pull-effects on the vessel wall induced by stable cavitation, can cause formation of small pores for increased vascular permeability, and they can also enhance endocytosis which can contribute to transfer of drugs over the membrane [51, 52, 58, 64, 83]. Following the collapse of a bubble, the resulting shock waves and liquid jets can create both temporary and permanent pores in the capillary wall and in cell membranes [31, 32, 51]. Various pore sizes are reported in the literature, from a few nanometers to several hundreds of nanometers, and even larger [84-88]. Membrane integrity is vital for cell survival, hence membrane wound healing processes will quickly start repairing the membrane after sonoporation [89]. Hu et al. investigated the dynamics of pore formation and resealing, and determined which pore sizes are non-resealable [88].

Focused ultrasound has been used to deliver molecules to and into cells in vitro by sonoporation [51, 65, 88], which has also been demonstrated in vivo in endothelial cells [90]. It has been shown that sonopermeation can be employed to increase extravasation across the capillary wall and potentially improve penetration through the interstitium, thereby improving the accumulation and distribution of drugs and drug delivery systems in solid tumors [91-97]. Similar mechanisms have been suggested to be involved in sonopermeation-based BBB-disruption for drug delivery to the brain [66, 98, 99]. Upon sonication, microbubble oscillations will exert mechanical stress on the endothelial cells and their tight junctions, possibly

generating a paracellular transport route [57, 99, 100]. It has also been suggested that transcytosis can be induced by ultrasound [58, 99, 101, 102], and that transient formation of fenestrations in the endothelial cell membrane can contribute to transcellular transport [58, 99]. Additionally, ultrasound combined with microbubbles has been reported to down-regulate the expression of drug efflux pumps (such as P-glycoprotein) in endothelial cells in the brain [103, 104]. By inhibiting drug efflux, the accumulation and retention time of drugs in the brain can be increased. Also, oscillating microbubbles can increase penetration of drugs through the brain parenchyma by the perivascular pump-effect, explained by increased arterial pulsation [105, 106].

Another likely (but less explored) effect of sonopermeation is altered perfusion (Figure 1). Ultrasound and microbubbles have been shown to cause a vasoconstriction or vascular shut down, and reduced perfusion in tumors, brain and other tissues [107-110]. This has also been used in a synergistic manner in combination with radiation therapy [111]. In contrast, locally increased perfusion has also been reported [112]. In a study on repetitive ultrasound exposures, Rix and coworkers found increased peak signal enhancement in tumors after repetitive microbubble injections and speculated that among other reasons, this might be due to the mechanical opening of non-perfused microvessels [60].

2.4.Microbubble platforms and ultrasound transducers

Sonopermeation as a research field is rapidly expanding, and specialized equipment for therapeutic ultrasound procedures is emerging and steadily evolving. The microbubbles which are typically used for this application are ultrasound contrast agents with sizes of 1-10 um, thus restricting them to the vascular compartment [113]. Commercial microbubble formulations have been used for decades in the clinic to enhance echogenicity of blood in diagnostic ultrasound [33]. Various types are commercially available with shells of either protein (Optison®) or lipids (SonoVue®, Sonazoid®, Definity®). They contain heavy gases instead of air for increased stability, which is excreted by exhalation, whereas the shell is excreted by the reticuloendothelial system in liver and spleen (RES) [113]. They can be used with a co-administration of a drug, or the drug may be loaded into or onto the bubbles in various ways [52, 53, 64, 114, 115]. Microbubbles may also be targeted to molecular markers expressed on endothelium of specific diseases [52, 116]. The response of a microbubble to ultrasound depends highly on properties of the microbubble such as size, shell thickness and stiffness [51, 64], and the largest oscillation response of microbubbles is obtained at their resonance frequency, which decreases with increasing size [64]. The majority of studies performed to date (and all clinical trials) are performed with conventional soft-shell microbubbles that are tailored for imaging purposes. These microbubbles are well characterized and approved in the clinic, but it has been shown that the effect of polymeric hard-shell microbubbles can be greater in some situations [117] and that both transfection and nanoparticle delivery by sonoporation is more effective if the nucleic acid or nanoparticle is attached to the microbubble [118, 119]. Sonopermeation has been shown using a multitude of microbubbles such as nanoparticle-loaded [92, 119, 120] or even nanoparticle-stabilized microbubbles [121], hard-shelled microbubbles [122], and clusters of microbubbles and emulsions of liquid perfluorocarbons that change phase and expand upon insonification [91]. Other systems have also been suggested, such as nanodroplets which can be activated in the interstitium [123] and antibubbles where the microbubbles contain a liquid droplet [124]. In general, there is a lack of systematic studies comparing the effect of different microbubbles for drug delivery applications [79]. These studies would also be challenging, as the various microbubble constructs will likely require different ultrasound settings for optimal effect.

For ultrasound platforms, a lot of the early work was done using clinical imaging systems. The advantage is the combination of both imaging and drug delivery simultaneously, however the range of ultrasound parameters available is limited. Gradually, and especially for BBB-applications, there has been a development of more specialized equipment using far lower

frequencies compared to diagnostic ultrasound imaging. In clinical trials on glioma, two very different approaches have been suggested, either implanting the ultrasound device inside the skull (SonoCloud®)[49], or image-guided sonication through the skull from multiple angles to obtain sufficient pressures at the focal spot (Exablate Neuro®)[125]. Other systems have been developed for ultrasound treatment elsewhere than the brain (Sonablate®, Insightec ExAblate® and Sonalleve®). In the clinical trial on pancreatic adenocarcinoma, an unmodified diagnostic ultrasound scanner was used in combination with lipid microbubbles [47].

2.5. In vitro models to study sonopermeation

Several different in vitro models are being employed to investigate the fundamental biological and biophysical processes involved in sonopermeation. Various types of cells, grown as monolayers or cells in suspension, are used to gain insights in microbubble-cell interactions and how the oscillation dynamics affect the cell membrane and transport of model drugs [50, 88, 120, 126-128]. It is unclear how well these assays mimic the in vivo situation and more complex and physiologically relevant models have been designed. 3D models such as cell clusters/spheroids [129], organs-on-chip including vessels [130], ECM components and co-cultures of various cells [131, 132], excised tissues [133, 134], or the chicken embryo model [90] can also be used. Different types of instrumentation have been employed to obtain complementary information on the time- and length-scales of the involved phenomena, as summarized by Lajoinie et al. [50]. Much of the knowledge of microbubble dynamics and the impact on cells upon sonication comes from optical imaging, with fluorescence imaging and high-speed imaging most commonly used [65, 88, 135]. However, also electron microscopy, atomic force microscopy, confocal microscopy and flow cytometry have been used to evaluate perforations in the cell membrane [50, 88, 136-138]. It has been shown that sonoporation can create holes in the cell membrane, both destructively and reversibly [88, 89, 136, 139] and also that tight junctions can be opened [140]. It has been demonstrated that a close contact between the cell and the microbubble is needed [65] and that a certain vibration amplitude of the bubble is necessary for pore formation [127, 141]. Sonoporation has also been used for in vitro transfection of dendritic cells, to achieve a therapeutic effect upon re-injection of the dendritic cells [84] and subsequent studies have indicated that such transfections also can be performed in vivo [142].

2.6. Sonopermeation of tumors

The potential of sonopermeation for delivery of free or encapsulated chemotherapeutics to solid tumors has been demonstrated in several preclinical studies and summarized in reviews [52, 53]. It has been shown that sonopermeation can increase delivery of both drugs and nanoparticles giving reduced tumor growth and in some cases even curative therapy (Table 1.) Perhaps due to less challenging experimental setups, tumor models outside the brain have been used to test novel microbubbles not yet approved for clinical use. There are multiple studies showing that drugs and drug delivery systems loaded onto microbubbles can have improved antitumor effects compared to co-injection regimens (Table 1). This supports the notion that increased effect of sonopermeation can be anticipated as more specialized systems are tested in clinical trials. Another novel concept is the injection of microbubble-microdroplet clusters that will undergo a phase shift upon ultrasound, creating large bubbles that temporarily deposit in and block capillaries. This system was used in combination with Abraxane® to successfully cure the majority of prostate tumor-bearing mice [91]. Interestingly, in the same study, microbubbles alone (as opposed to the clusters) were found to severely reduce the effect of Abraxane®, possibly due to decreased perfusion of the tumor obtained by the selected ultrasound settings and microbubble type.

The only reported clinical trial to date using sonopermeation to treat solid tumors evaluated the safety and potential toxicity of combining gemcitabine with microbubbles under sonication in ten inoperable pancreatic cancer patients [47]. Dimcevski and colleagues reported that the combination of clinically available ultrasound equipment with commercial microbubbles and

chemotherapy resulted in no additional toxicities. Furthermore, the combined treatment enhanced the clinical efficacy of gemcitabine and extended survival in patients with pancreatic adenocarcinoma. Several similar studies have been initiated in patients suffering from breast cancer, liver metastasis resulting from primary colon cancer, and pancreatic cancer (Table 1).

2.7. Sonopermeation of the blood brain barrier

Sonopermeation of the blood brain barrier to access brain tumors is one of the most developed and promising applications of therapeutic ultrasound [66, 143]. The vasculature and biological barriers faced by drugs in the brain and in brain tumors are somewhat different from those in tumors located elsewhere, the BBB with its tight junctions and high density of efflux pumps is a formidable barrier for drug delivery to the brain. Following the first demonstration of reversible BBB-opening by ultrasound in rabbits [68], there have been extensive efforts in further developing the concept in pre-clinical settings [66, 144, 145]. Successful BBB-opening, increased delivery and/or improved therapeutic efficacy have been demonstrated for chemotherapeutic drugs [54, 146], nanoparticles [147-149], antibodies [150-152], interleukins [153] and cells for immunotherapy [154, 155]. Safety has been evaluated in both small animals and in non-human primates, and no adverse effects were observed in awake and behaving primates [156, 157]. It has also been shown by multiple groups that the BBB-opening is temporary and is reversed within minutes to hours and that the window for drug delivery to the brain depends on the size of the drug/nanocarrier [147, 158, 159]. The procedure is generally considered to be relatively safe, but this consensus was recently challenged following the work by Kovacs et al. who showed that BBB-opening could induce a local inflammation [160-162] and suggested that the procedure should be evaluated in more depth before going into clinical practice. Even though small extravasations and mild inflammatory reactions have been observed in the sonicated area by some, ultrasound in conjunction with microbubbles was not reported to result in damage of neurons, neither directly, nor through ischemia or apoptosis, nor by delayed effects up to one month after sonication [163]. One method to increase both the efficacy and the safety of BBB-opening is through real-time feedback of *in situ* sonopermeation, which will reduce the effects of variations in microbubble concentration and ultrasound attenuation. It was recently shown that feedback control through the detection of harmonics from the microbubbles could be used to precisely control the magnitude of the BBB-opening and the amount of drug delivered to the brain [164]. Clinically, the development is being fronted by groups in France and Canada pioneering the development of Sonocloud®, an implantable ultrasound transducer, and ExAblate Neuro®, an image guided transcranial array of transducers, respectively (Table 1). The phase I trial with SonoCloud® reported no adverse effects and it did provide initial indications for therapeutic responses [49].

Besides brain tumors, BBB-opening is also being evaluated for the treatment of other diseases in the brain. Promising results have e.g. been achieved in preclinical models of Alzheimer's disease [75, 165-167] and Huntington's disease [168], as well as in a Parkinson's disease mouse model via the delivery of neurotrophic factors [169]. Furthermore, ultrasound-mediated delivery appears promising for stem cell delivery/treatment [170], for the delivery of viral vectors and gene therapy [171-173], and for the treatment of stroke [174].

3. Conclusion

From pioneering achievements in the last decade using materials and methods intended for imaging, the development is now going in the direction of more specialized systems to achieve maximum, but controlled drug delivery. Targeted drug delivery by sonopermeation is progressing rapidly towards clinical practice; the first clinical trials on BBB opening and treatment of patients with pancreatic cancer have been finalized, and multiple clinical trials with sonopermeation of solid tumors are recruiting. Although our understanding of both mechanisms and adverse effects is still incomplete, the strong pre-clinical evidence and the positive outcome of the performed clinical trials suggest that sonopermeation is a promising approach for treatment of tumors and neurodegenerative disorders.

4. Expert Opinion

Sonopermeation is a technology that is rapidly moving towards clinical practice, based on promising results obtained in proof-of-principle studies in animal models. Multiple clinical trials are currently ongoing, of which the vast majority are exploiting combinations of clinically approved microbubbles and drugs. While it is sensible to break new ground with established methods combining already approved components, the development is now going in the direction of more specialized systems, produced especially for drug delivery. It has been demonstrated pre-clinically that microbubbles developed for therapy can be superior to the clinically approved alternatives, tailored for imaging applications. In addition, many pre-clinical experiments involve ultrasound settings outside the range of diagnostic ultrasound scanners, indicating a need for developing transducers specialized for therapeutic applications. On the other side, there are obviously very appealing advantages associated with the use of systems that are already approved as the road to clinical use is much shorter both financially and regulatory.

Despite the promising results obtained so far, the field is still lacking a complete understanding and explanation of some of the observed effects. The currently most frequent explanation is transient pore formation in the cell membrane or opening of cell junctions, but neither of these are completely described or understood at a microscopic level. These are two distinct mechanisms with different consequences (i.e. intracellular vs. extracellular delivery) and should be evaluated and possibly exploited selectively. However, observations not easily explained by this theory are sometimes encountered. One example is the improved effect of gemcitabine after sonication [47]. Gemcitabine is a small water-soluble molecule that should be able to cross endothelial membranes and diffuse through tissue efficiently. The mechanism is not elaborated in the paper, but it seems plausible that increased perfusion and vessel decompression, in addition to permeabilization of the blood vessel wall, contributed to the enhanced efficacy of gemcitabine. Another example is the detrimental effect of sonopermeation with Sonazoid® on the effect of Abraxane® as seen in a subcutaneous prostate cancer model in mice [91]. Here, the therapeutic effect of Abraxane® was lost if the drug was combined with lipid microbubbles, but greatly improved when combined with the microbubble-microdroplet clusters. The unexpected effect with Sonazoid® could not be further explained based on the study's results. It may be the result of decreased perfusion of the tumor obtained by the selected ultrasound settings.

While a complete understanding is not a prerequisite for clinical success, sonopermeation has almost endless degrees of freedom. Finding the most effective combination of drug, drug delivery vehicle/formulation, microbubbles and ultrasound settings, as well as dosing and treatment schedule through “trial-and-error” seems unrealistic, especially when considering that different diseases require different treatment regimens. Sonopermeation has been proven effective for different types of solid tumors, brain tumors, as well as neurodegenerative disorders, each of which has its own characteristic barriers for drug delivery and hence the potentiating effect from sonoporation differs in these cases. As the toolbox of drug delivery materials and methods expands, it will be increasingly important to develop an understanding of which patients will actually benefit from a specific approach. As sonopermeation is established as one of many treatment options, tools to stratify patient groups, such as magnetic resonance or ultrasound imaging or disease-specific molecular biomarkers, will be needed. However, achieving personalized treatment, tailored treatment regimens and real-time feedback control for sonopermeation requires a better understanding of the (bio) mechanics involved.

In terms of understanding, we are closer to elucidating the mechanism of action for ultrasound-mediated BBB-opening. Increased permeability of the otherwise tightly controlled blood vessel wall has made it possible to deliver drugs to the brain and will likely also increase the drive for development of new drugs for diseases in the brain. The results from clinical trials in France and

Canada will shed light on the possible clinical effects and the strengths/weaknesses of these two different setups. Also the development of feedback-based control - which has been ongoing [76] and which has been recently demonstrated [164, 175] - is a clear step forward in the direction of control and understanding of sonopermeation-based BBB opening.

Specific focus on the limitations in the current application of sonopermeation is needed to produce more effective therapeutic solutions. In Figure 2, we have highlighted four studies that exemplify what should be focus areas in order to advance sonopermeation. Understanding the involved mechanisms and relation to the different biophysical effects will be crucial to optimize the efficacy and safety for ultrasound-mediated drug delivery and achieve translation to clinical benefit (panel 1). Also, indications from pre-clinical research with specialized microbubbles (panel 2) and equipment (panel 3) has shown that therapy-specific setups can be superior compared to combinations of already approved materials. Furthermore, while disease models are invaluable tools in medical technology, the real therapeutic potential of sonopermeation can only be evaluated in clinical trials (panel 4), especially as the ultrasound equipment and relative doses of microbubbles used in pre-clinical research in rodents often is not translatable.

Even though ultrasound can be used for both superficial and deep tumors with imaging guidance, sonopermeation has the limitation of being site-specific, which implies that only tumors with known location can be treated. However, the abscopal effect, which can sometimes be observed after radiation treatment, has shown that localized therapies can have systemic effects [176]. In case of the abscopal effect, local treatment can have systemic consequences as a result of shedding of tumor antigens from the treated region, thereby priming the immune system towards a response (especially when combined with e.g. anti-PD(L)1 and anti-CTLA4 immunotherapies) [177]. As soon as exploitation of the abscopal effect becomes fully understood and a clinical reality, sonopermeation could be an important tool also for the treatment of advanced metastatic cancers.

In our opinion, sonopermeation is developing in a promising manner through collaborative efforts in the field of ultrasound physics, chemistry, pharmacy, biology and medicine. We still have quite a way to go in terms of fundamental understanding, and this may be the limiting step in the development of more disease-specific setups. However, as the results from clinical trials with specialized materials and methods are becoming available, and as more refined systems are being evaluated, we expect the outcomes to be gradually improving. Improved outcomes will generate increased interest and funding, which will eventually lead to specifically developed and properly understood setups that can be applied to a stratified group of patients, resulting in prolonged survival times and improved quality-of-life.

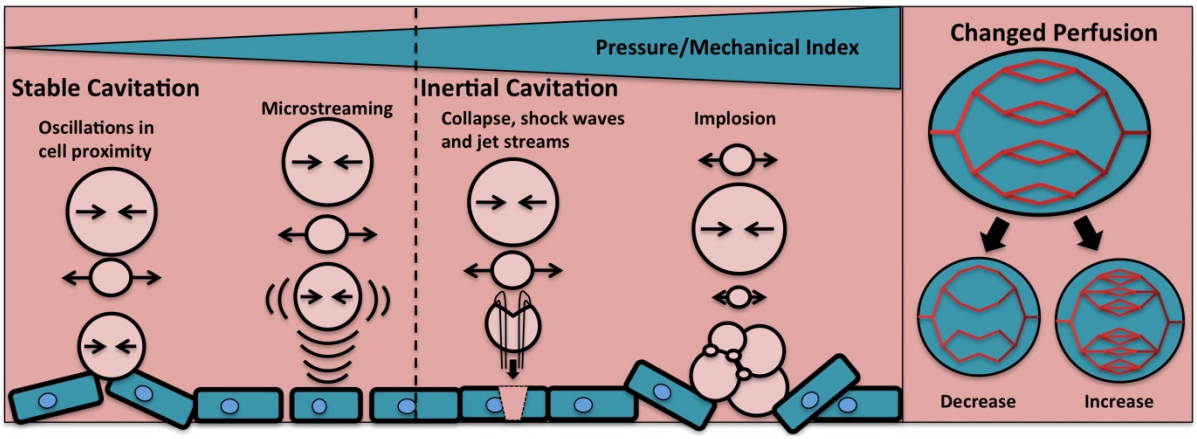

Figure 1: Schematic illustration of possible vascular effects of sonoporation on the capillary wall and on perfusion.

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485 Table 1: Therapeutic studies using sonopermeation.

Selected Preclinical Studies				
Target	**Drug**	**Setup**	**Results**	**Ref.**
Dendritic cells	mRNA	mRNA-lipoplex-loaded microbubbles, 0.8 MPa in Opticells®	Therapeutic effect in two tumor models, no tumor upon rechallenge	[84]
PC3 prostate adenocarcinoma	Paclitaxel / Abraxane®	ACT® 2.25MHz activation, 0.5MHz enhancement	Combined with Abraxane®, complete remission in 6/9 tumors	[91]
Ca9-22 gingival squamous cell carcinoma	Bleomycin	Microbubbles targeted with EGFR-antibodies injected directly into tumor, 1 MHz	Growth inhibition of all 4 tumors only when microbbubles are targeted	[178]
C6 glioma	5FU-loaded nanoparticles	Albumin microbubbles with 5FU loaded nanoparticles attached to the surface, 1MHz, 1.2 MPa	5x increased tumor accumulation compared to without ultrasound, significantly improved therapeutic effect	[92]
MIA PaCa-1, pancreatic adenocarcinoma	Gemcitabine	Lipid microbubbles, 1MHz, MI=0.2	Reduced tumor volume, but not significantly increased survival with ultrasound	[93]
CT-26 colorectal adenocarcinoma	Pegylated liposomal doxurubicin (Doxil®)	Lipid microbubbles, 1MHz	Increased accumulation of doxurubicin in tumors and improved therapeutic effect	[95]
C6 glioma	VEGF-targeted and carmustine-loaded microbubbles	In-house lipid microbubbles, 1 MHz, 0.5MPa	Enhanced local delivery of chemotherapeutic agent, reduced tumor progression and improved median survival time	[179]
9L gliosarcoma	Liposomal doxorubicin	Lipid microbubbles, 1.7 MHz	Reduced tumor growth and improved survival	[148]
4T1 breast carcinoma	Paclitaxel-liposome-microbubble complexes	2.25 MHz	Inhibited tumor growth	[180]
MDA-MB-231 breast carcinoma	Cabazitaxel-loaded nanoparticles	Nanoparticle-stabilized microbubbles, 1MHz	Complete remission in 3 / 3 tumors	[121]
Glioblastoma multiforme	Doxorubicin	PEGylated lipid microbubbles 612.5 kHz	Increased doxorubicin concentration, increased survival and slower disease progression	[181]
MCF-7 breast	Doxorubicin prodrug	Prodrug-microbubble complex, 1 MHz	Higher tumor inhibition rates	[182]
Clinical Trials				
Target	**Deliverable**	**Setup**	**Goal/Results**	**Ref.**
Glioma	Carboplatin	Implantable	Safe BBB-opening above	[49]

		ultrasound transducer, SonoCloud®	0.8MPa	NCT02253212
Pancreatic cancer	Gemcitabine	Diagnostic ultrasound scanner, linear probe MI=0.2	Doubled median survival (from 8.9 to 17.6 months)	[47]
Hepatocellular carcinoma	Yttrium-90 loaded microspheres	Albumin microbubbles and diagnostic ultrasound	Currently recruiting	NCT03199274
Glioblastoma	-	ExAblate® BBB-distruption prior to surgery	Assess safety and feasibility of BBB-opening in patients undergoing surgery	NCT03322813
Breast cancer	Neoadjuvant epirubicin, cyclophosphamide, paclitaxel, carboplatin	Lipid microbubbles, diagnostic ultrasound scanner, linear probe, high MI	Assess increase in tumor perfusion after sonoporation and response to neoadjuvant chemotherapy	NCT03385200
Hepatic metastases from colorectal cancer	FOLFIRI plus bevacizumab	Lipid microbubbles combined with ultrasound	Assess safety and tolerance, decreased tumor size and assessment of vascularity	NCT03458975
Liver metastases from gastrointestinal tumors and pancreatic carcinoma	Oxaliplatin with paclitaxel and gemcitabine.	Lipid microbubbles combined with ultrasound	Assess safety and efficacy	NCT02233205
Brain tumors	Liposomal Doxorubicin or Temozolomide	Transcranial ExAblate®	Demonstrated safety of BBB-disruption using transcranial MRI-guided focused ultrasound	[183] NCT02343991
Liver metastases from breast cancer and colorectal cancer	Paclitaxel or FOLFIRI	Lipid microbubbles with ultrasound	Difference in response between ultrasound-treated and untreated lesions	NCT03477019

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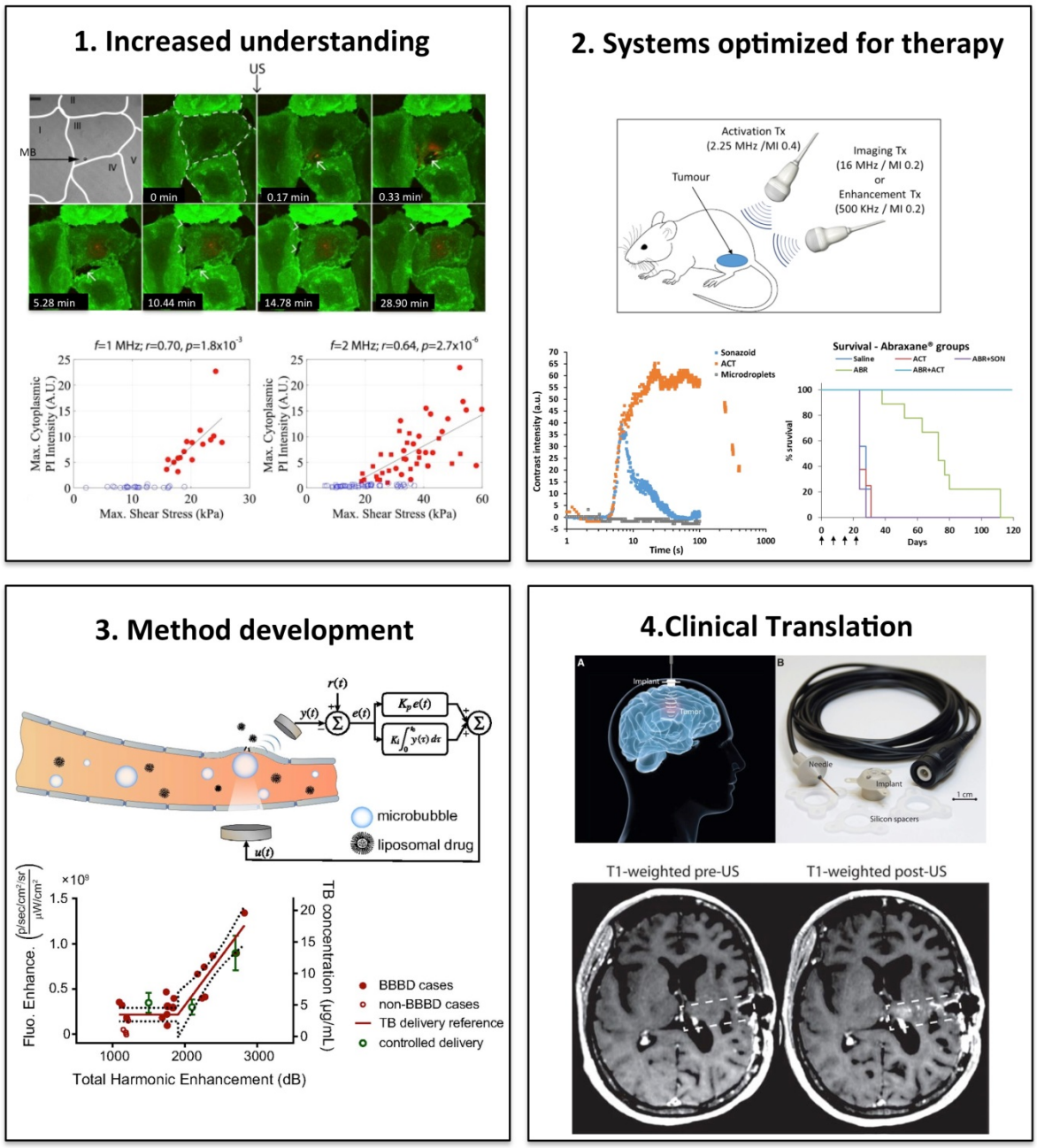

Figure 2: Examples of studies advancing the use of sonopermeation. 1: Helfield et al. demonstrated that sonoporation initially creates a transient hole in the cell membrane allowing for intracellular drug delivery. Subsequently, pores are formed between the endothelial cells possibly creating the basis for BBB-opening and drug extravasation. Figure adapted from [127]. 2: van Wamel et al. demonstrated that acoustic cluster therapy (ACT®) could overcome some of the limitations of standard microbubbles (small size limiting contact with the vessel wall, and short circulation lifetime limiting exposure time), and hence increase the potential for acoustic effects significantly and potentiate Abraxane® for the successful treatment of a prostate cancer model in mice. Figure adapted from [91, 184] with permission from Elsevier. 3: Sun et al. designed a setup for BBB-disruption where feedback from the harmonic signal from stable cavitation was used to control the ultrasound pressure and also the amount of drug delivered to the brain. Figure adapted from [164]. 4: Carpentier et al. demonstrated in a clinical study that the BBB could be safely opened in glioma patients using an implanted ultrasound transducer (SonoCloud®). Figure adapted from [49] with permission from The American Association for the Advancement of Science.

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Conflict of interest

The authors declare no conflict of interest.

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