Ultrasound-Targeted Microbubble Destruction (UTMD) for Localized Drug Delivery into Tumor Tissue

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Abstract

Background: Ultrasound-targeted microbubble destruction (UTMD) is a type of ultrasound therapy, in which low frequency moderate power ultrasound is combined with microbubbles to trigger cavitation. Cavitation is the process of oscillation of gas bubbles causing biophysical effects such as pushing and pulling or shock waves that permeabilize biological barriers. *In vivo*, cavitation results in tissue permeabilization and is used to enable local delivery of nanomedicine. While cavitation can occur in biological liquids when high pressure ultrasound is applied, the use of microbubbles as cavitation nuclei in UTMD largely facilitates the induction of cavitation. UTMD is intensively studied for drug delivery into tumor tissue, but also for the activation of anti-tumor immune responses. The first clinical studies of UTMD-mediated chemotherapy delivery confirmed safety and efficacy of this approach.

Aim: The present review summarizes ultrasound settings, cavitation approaches, biophysical mechanisms of drug delivery, drug carriers, and pre-clinical and clinical applications of UTMD for drug delivery into tumors.

Key points:

- UTMD is used for non-invasive localized drug delivery.
- Microbubbles serve as excellent cavitation nuclei.
- Stable and inertial UTMD cavitation effects contribute to tissue permeabilization.
- Drug carriers can be co-injected or directly coupled to microbubbles.
- A first clinical trial confirmed therapeutic efficacy in pancreatic cancer patients.

Graphical abstract:

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1. Localized drug delivery in cancer

In intermediate and advanced cancer when surgery is not an option, patients are subjected to local radiotherapy, systemic chemotherapy, systemic molecular therapies, or combinations of these. Systemic therapies for cancer entail off-target delivery and toxicity side-effects which require the limitation of doses and decreased therapeutic efficacy [1]. Thus, local drug delivery approaches are required which either limit the drug's activity to the target site, release the drug at the target site, or activate the drug at the target site. Ultrasound-triggered drug delivery is mediated by thermal and/or mechanical effects including cavitation and radiation force. In this review, cavitation-mediated drug delivery is discussed. Ultrasound-targeted microbubble destruction (UTMD) causes cavitation and enhances permeability across natural barriers of tumors including vessel walls and cell membranes, resulting in spatio-temporally controlled enhanced drug delivery into tumors (**Figure 1**). Furthermore, mechanical ultrasound activities destabilize drug carriers and trigger drug release [2]. Ultrasound drug delivery is particularly attractive as it is non-invasive, enables the regulation of tissue penetration depth, and does not rely on ionizing radiation [2]. **In contrast to alternative drug delivery techniques, UTMD allows for targeted drug delivery.**

The present review aims at discussing the approach of UTMD for localized drug delivery into solid tumors. First, ultrasound settings are presented. Then, different approaches to achieve cavitation are elucidated. We then focus on the discussion of the biophysical mechanisms of UTMD. Different drug carrier systems are introduced. Finally, preclinical and clinical applications are summarized.

2. Ultrasound settings

Ultrasound enables the delivery of drugs at different tissue depths by modulation of ultrasound parameters such as frequency, duty cycle, mechanical index, and exposure time [2]. For drug delivery, ultrasound frequencies range from kHz to MHz [16]. Lower frequencies enable deeper tissue penetration as attenuation effects are reduced. Furthermore, the frequency is adjusted to match the resonance frequency of ultrasound contrast agents such as microbubbles (MBs). Ultrasound intensity for drug delivery ranges from 0.3 to 3 W/cm² [16]. Lower intensities of US are used with longer pulse lengths or pulse repetition frequencies to achieve higher duty cycles and similar temporal average intensities than with high intensity ultrasound. The different parameters ensure fine-tuning according to the specific application such as tissue depth and MB type. The mechanical index is dependent on the peak negative pressure and center frequency and proportional to the ultrasound intensity. The FDA predefines the limit of the mechanical index to 1.9 for clinical applications to minimize tissue damage [22]. Duration of ultrasound exposure is fixed to provide sufficient time to induce and maintain cavitation and tissue permeabilization while preventing tissue heating [16].

Focused and non-focused ultrasound transducers have been used for drug delivery in previous studies [23]. Non-focused ultrasound transducers cover bigger tissue volumes at once, which can accelerate the therapy protocol. However, cavitation is not limited to the tumor volume but can occur all along the acoustic ultrasound beam. Focused ultrasound transducers are commonly used for drug delivery as they ensure spatially controlled cavitation which is limited to a few millimeters [23]. While this guarantees specifically localized drug delivery, it brings along the drawback of longer treatment protocols to electronically steer or mechanically displace the transducer in order to insonify the whole tumor volume.

Image guidance ensures correct positioning of the therapy transducer to reach the target tissue. Image guidance further enables assessment of MB cavitation in real-time as MBs appear

as contrast-enhanced regions prior to cavitation and the signal is lost after MB destruction [24]. Thus, image-guidance renders drug delivery more reliable and efficient. However, imaging does not allow tracking drug delivery as drugs diffuse differently from MBs.

3. Cavitation nuclei

Cavitation can be achieved with high pressure ultrasound and without the use of ultrasound contrast agents such as MBs. High pressure ultrasound results in generation and activation of gas nuclei in the tissue or vessels creating a cavitation cloud for pore formation, endocytosis, and vessel permeabilization [4]. Furthermore, ultrasound alone induces acoustic streaming which is weaker than microstreaming but can also trigger biological effects [4]. In these conditions, a high mechanical index is applied, which possibly limits the clinical translation of the approach. The use of MBs allows decreasing the mechanical index to diagnostic ultrasound levels as MBs are highly sensitive to ultrasound exposure and serve as excellent cavitation nuclei [2]. The cavitation response of MBs depends on their size and shell, and the ultrasound settings have to fit the MBs' resonance frequency. Further, the ultrasound pressure influences cavitation activity, and higher pressures have been shown to trigger successive MB implosions. The use of clinical-grade contrast agents, such as BR38 which was previously used for UTMD, further facilitates clinical translation [13–15]. Phase change nanodroplets are another option to provide cavitation nuclei which can be activated by low-intensity ultrasound, and in contrast to intravascular MBs, they can penetrate into the tissue and might further enhance the efficacy of drug delivery [16,17]. The use of molecularly targeted MBs, recognizing tumor endothelial markers, was shown to enhance drug delivery by creating direct contact with cell membranes and vessel walls [6]. Nanometer-sized ultrasound contrast agents, such as perfluorocarbon

nanoemulsions or echogenic liposomes with air pockets, have to be used for targeting of epithelial tumor markers as opposed to endothelial markers that can be reached by MBs [2].

4. Biophysical mechanisms of ultrasound drug delivery with microbubbles

The combination of ultrasound with MBs for drug delivery is most widely studied and presents the focus of the present study. UTMD is based on the interaction of ultrasound and MBs to induce openings in vessels and membranes. Under low-pressure ultrasound, MBs expand and contract inversely proportional to the acoustic pressure waves, a process called stable cavitation. If the pressure is high enough, MBs cavitate non-linearly i.e. they expand more than they contract which leads to an increase in MB size until implosion and collapse (**Figure 2**). Low-intensity ultrasound-mediated stable cavitation and high-intensity ultrasound-induced inertial cavitation trigger different biophysical responses, which are discussed separately.

Stable cavitation

Stably oscillating MBs exert direct mechanical effects on adjacent biological barriers. They push and pull on surfaces and induce fluid jets, microstreaming, and shear stress (**Figure 3**) [3]. The shear stress intensity varies with the ultrasound settings between 100 Pa and 1000 Pa and is much higher than blood flow-associated shear stresses (0.1-4 Pa) [4]. Stable cavitation is mainly associated with formation of small pores and endocytosis [4]. As ultrasound can trigger pore formation, this type of tissue permeabilization is also called sonoporation. Small molecules enter cells through small pores while larger molecules are taken up by endocytosis. Pore formation requires direct contact between cell surfaces and MBs to mechanically disturb the membrane by pushing and pulling, or microstreaming which surrounds the oscillating MB [5]. Interestingly, the use of targeted MBs that bind to cell surfaces requires lower ultrasound intensities to achieve the same membrane permeabilizing effects [6]. MBs are further modulated

by ultrasound waves through radiation forces which push MBs into the direction of the ultrasound beam. This phenomenon can potentially contribute to ultrasound-mediated drug delivery bringing MBs into contact with cell membranes and facilitating permeabilization [4]. Radiation forces might also cause MB compression and pushing into cellular membranes thereby directly permeabilizing the cells [7]. MBs were also observed to be internalized into cells possibly through fusion of cell membrane and MB shell [8]. Sonoporation-mediated membrane pore formation is reversed when ultrasound is switched off [4]. Thus, membrane permeabilization duration is dependent on the duration of the ultrasound treatment, which has to be taken into consideration for effective delivery of drugs. Besides these mechanical effects, stably cavitating MBs in close vicinity of cells produce chemical stress leading to formation of free radicals and reactive oxygen species, which contribute to the permeabilization of cell membranes [4]. Further, stable cavitation was shown to mediate membrane hyperpolarization through activation of ion channels mediating calcium influx and potassium efflux [4].

Inertial cavitation

Under high-intensity ultrasound exposure, the oscillation amplitude of MBs increases with every cycle and ultimately results in MB collapse; a process called inertial cavitation. Upon collapse, MBs fragmentize into several smaller MBs which in turn undergo inertial cavitation. Collapsing MBs exert high shear stresses, and shock waves, which rupture surfaces and induce pores (**Figure 3**) [9]. MBs in contact with cell membranes undergo asymmetrical collapse which results in liquid jet formation [10]. Shock waves and liquid jets not only perforate adjacent cell membranes but have the energy to permeabilize blood vessels [4]. Ultrasound settings such as pressure, exposure time, and pulse repetition frequency determine the size of induced pores which affects the delivery of larger drugs or drug carriers [11]. Indeed, UTMD-mediated membrane pore formation allows cytosolic delivery which is important for gene therapy as DNA

can be degraded in the endocytic uptake pathway [2]. In addition to the biophysical effects described for stable cavitation, inertial cavitation triggers harsh mechanical and chemical insults causing membrane perforation and vessel permeabilization. Inertial cavitation enhances drug delivery even when MBs are not directly interacting with cell surfaces as its effects act over longer distances [4]. In comparison to stable cavitation, inertial cavitation induces membrane pores of larger sizes ranging from hundreds of nanometers to a few micrometers [4].

Both stable and inertial cavitation were shown to trigger endocytosis-mediated uptake of larger drugs (150 – 500 kDa) while smaller drugs (4 – 70 kDa) entered cells through pores [12]. Although, the exact mechanism triggering endocytosis after cavitation is not understood, it is speculated that microstreaming and acoustic streaming deform the plasma membrane, re-arrange the cytoskeleton, and activate mechano-sensors, and all of these processes contribute to endocytosis signaling (**Figure 3**) [4].

5. Drug carriers

In addition to the ultrasound settings and the type of cavitation nuclei, UTMD-mediated drug delivery is affected by drug administration, which can be performed by intra-tumoral, intraperitoneal, or intravenous injection [16]. Due to the invasiveness of intra-tumoral and intraperitoneal injections, and the heterogeneous delivery upon intra-tumoral administration, the intravenous administration route is usually preferred [16]. Drawbacks of the intravenous delivery are systemic toxicity and degradation, which have to be addressed with the preparation of appropriate drug carriers. There are generally two options for UTMD drug delivery: drugs can be encapsulated into drug carriers which will be co-injected with MBs, or drugs are loaded into MBs (**Figure 4**).

During MB synthesis, drug-loaded liposomes can be attached to the MB shell, negatively charged nucleic acids can be absorbed on a cationic MB surface, a second hydrophobic shell can be added to the MB membrane in order to provide a drug storage compartment, or hydrophobic drugs are directly inserted into the MB membrane [18]. These different loading strategies enable loading of different types and quantities of drugs, and sufficient drug delivery requires adjustment of injected MB concentrations [16]. Direct coupling of drugs to MBs is considered a more effective drug delivery strategy because drugs are more proximal to vessels and membranes that are permeabilized by cavitating MBs.

Alternatively, different types of drug carriers can be co-injected with MBs and used in combination with UTMD. Gold nanoparticles (NPs), silica NPs, polymer NPs, nanoemulsions, liposomes, and micelles were studied for UTMD [16,19]. PEGylated poly (lactic-*co*-glycolic acid) nanoparticles (PLGA-NPs), are larger therapeutic carriers (~110 nm), which are biocompatible, biodegradable, and FDA-approved [16]. They protect encapsulated drugs from clearance by the reticuloendothelial system, and allow for slow and prolonged release of drugs in the range of days to weeks so that they have more time to reach the target site [14,16]. The use of encapsulated chemotherapeutics further showed to reduce systemic toxicity, as observed with free doxorubicin while liposomal doxorubicin (Doxil) is better tolerable [20]. To ensure sufficient loading capacity, drug carriers have a size range of 90 to 300 nm and can be efficiently delivered by UTMD applying acoustic pressures below 1 MPa for small molecules (<1 nm) and high pressures at 5-6 MPa for larger drug carriers (>100 nm) [14]. Using PLGA-NPs, Willmann and colleagues were able to achieve the delivery of microRNAs for colon cancer and hepatocellular carcinoma (HCC) therapy in pre-clinical studies [14,15,21].

6. Applications

UTMD is applied for delivery of drugs ranging from nucleic acids, to proteins, and chemotherapies. It is currently intensively studied for blood brain barrier opening and targeted drug delivery in the central nervous system which is naturally well protected against external factors hindering therapy by systemic administration [25]. UTMD-triggered drug delivery was further studied in pre-clinical models of different types of cancer including brain, liver, pancreatic, breast, and ovarian cancers [26–30]. Sonochemotherapy describes ultrasound-mediated delivery of chemotherapy and pre-clinical studies showed positive therapy outcomes when UTMD resulted in increased intra-tumoral drug concentrations [31]. UTMD-mediated drug delivery further proved to decrease accumulation of drugs in healthy tissues such as heart, spleen, liver, lung, and kidney [29]. Though, UTMD can affect tumor perfusion as strong cavitation causes not only blood vessel permeabilization but also damage, and this can cause vascular shutdown which counteracts UTMD drug delivery [31,32]. Thus, control of cavitation localization and dose is required to obtain optimal drug delivery efficacy.

Ultrasound is further used for *in vitro* delivery of immunomodulatory agents such as tumor antigen-encoding and dendritic cell-stimulating mRNA to enable cancer immunotherapy [33]. Furthermore, ultrasound-mediated cavitation was shown to trigger tissue damage at the subcellular level thereby releasing different danger signals that elicit immune responses [34]. Different studies showed that cavitation enabled penetration of dendritic cells, activated cytotoxic T cells, and Natural Killer cells into the tumor tissue [35–37]. Moreover, UTMD was applied to facilitate delivery of immuno-modulatory molecules or genes that encode tumor antigens and co-stimulatory factors [38,39]. Mechanical ultrasound without drugs decreased tumor growth and enhanced immune cell infiltration into subcutaneous solid tumors and across the blood brain barrier [40,41]. The degree of tissue destruction, which is dependent on ultrasound settings and tissue characteristics, is expected to contribute to the resulting biologic responses [42].

236 Researchers from Stanford University developed a novel approach based on ultrasound-
237 guided delivery of molecular drugs encapsulated in NPs for HCC therapy. Their results showed
238 to increase the concentration of delivered microRNA-loaded NPs up to 14-fold, the penetration
239 depth of NPs up to 3-fold, and the concentration of delivered miRNAs up to 7.9-fold in tumor
240 cells compared to treatment without ultrasound [14].

241 Recently a first clinical case study was performed based on sonochemotherapy. Five
242 patients with pancreatic cancer were treated with ultrasound, MBs, and gemcitabine resulting in
243 improved physical state and prolonged survival as the tumor size was transiently or even
244 permanently decreased, or the tumor growth slowed down [43]. A human clinical trial using the
245 same sonochemotherapy approach in inoperable pancreatic cancer patients confirmed safety and
246 therapeutic efficacy as the median survival was doubled, and in five out of ten patients the tumor
247 size decreased [44].

249 7. Conclusions

250 Overall, UTMD-mediated drug delivery proved feasible in multiple pre-clinical studies
251 and in the first studies with human cancer patients. It is a versatile technique for targeted drug
252 delivery into cancer enhancing local drug concentration and reducing off-side delivery and
253 toxicity. UTMD is further studied for cancer immunotherapy enhancing anti-cancer immune
254 responses by antigen release and immune cell infiltration. Developments in ultrasound
255 technology, MB design such as molecular targeting with ligands, drug carrier preparation, and
256 treatment protocols are expected to leverage this technique for improved cancer management.

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Figure Legends

Figure 1: Ultrasound drug delivery via ultrasound-targeted microbubble destruction (UTMD). Microbubbles (MBs) are intravenously injected and circulate freely in the blood vasculature. Upon ultrasound exposure using a focused transducer, MBs in the tumor vasculature undergo cavitation, which leads to breakdown of cell junctions, cell membrane perforation, and tissue permeabilization. Co-injected drugs, such as drug-loaded nanoparticles (NPs), therefore penetrate more easily into the tumor tissue increasing the local drug concentration.

Figure 2: Physical effects of ultrasound on microbubbles (MBs) and biophysical effects of MBs on the endothelium. At low acoustic pressures, MBs oscillate linearly and undergo compression and expansion proportional to the pressure wave. At high acoustic pressures, MBs oscillate non-linearly: they expand faster than they compress and continue to grow until they collapse. Stably cavitating MBs exert forces on the adjacent endothelium: MBs can get pushed to the cell barrier by acoustic radiation force, microstreaming effects around the MBs hit cell membranes, and MB oscillation pushes and pulls on cells. Inertially cavitating MBs have even stronger effects on cells: shock waves disrupt cell junctions and liquid jets perforate cells.

Figure 3: Biological effects of stable and inertial cavitation. Stably and inertially cavitating microbubbles trigger mechanical effects such as pore formation which allows drug diffusion into the cell. Cavitation further produces reactive oxygen species interfering with ion channels or opening the membrane by lipid peroxidation. Stable cavitation induces microstreaming and shear stresses, which deform membranes and activate mechano-sensors. These cavitation-triggered processes contribute to endocytosis and exocytosis.

428

429 **Figure 4: Drug loading strategies for gas-filled microbubbles (MBs).** Lipid-, protein- or
430 polymer-shelled MBs can be used for drug delivery. Drugs can be loaded directly into the shell,
431 attached to the outside or included in an oil layer inside the MB.

432

433

Figure 1

Figure 2

Figure 3

Lipid-shelled MB with
drug in lipid
membrane

Lipid-shelled MB with
drug in oil layer

Positively charged lipid-
shelled MB with
negatively charged drug

Avidinylated lipid-
shelled MB with
biotinylated drug

Protein-shelled MB with
drug in protein layer

Polymer-shelled MB with
drug attached to polymer

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Figure 4: Drug loading strategies for gas-filled microbubbles (MBs). Lipid-, protein- or polymer-shelled MBs can be used for drug delivery. Drugs can be loaded directly into the shell, attached to the outside or included in an oil layer inside the MB.