Ultrasound mediated local drug delivery

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Abstract – *In the field of drug delivery, it has great importance to have sufficient drug accumulation at the diseased site. To obtain this goal it is important that at first, a high concentration of the administered dose at the diseased site has to be present and second, the drug has to be able to extravasate homogeneously at the region to be treated. Ultrasound can be used in different ways to improve these problems. To be able to develop new or better strategies for delivering drugs with the use of ultrasound it is extremely important to understand the underlying mechanisms. There are two interrelated aspects in this research. First, in order to be able to control the process, there has to be understanding about the physical interactions induced with ultrasound. And second, it is important to determine which bioeffects occur as a response on these physical mechanisms. This thesis will give an overview of the used techniques to improve local drug delivery problems with the use of ultrasound.*

Key words: Drug delivery, Ultrasound, Drug carriers, Hyperthermia, Cavitation, Acoustic Radiation force.

INTRODUCTION

In the field of drug delivery, the used drug must satisfy two requirement in order to be successful. First, the drug must be effective in the environment it is used for. And second, it must reach the target site in optimal quantities. In this paper, the last problem is discussed, the delivery of drugs to target site. In the literature it is seen that ultrasound can help improve the concentration of drug at target site in various ways.

Transport

A big problem with drug molecules and particles is their inability to distribute homogeneously in a adequate concentration in the diseased cells. This is due to the fact that it has to pass several barriers, before reaching the diseased cells. The transport of the drug will here be explained for tumors, as they are widely investigated and play a big role in drug delivery. When a drug is administered intra venous, it will enter blood circulation of the tumor after a certain period. Then it first distributes through the vascular space of the tumor. Second, it has to cross the vascular wall. And third, it has to transport through interstitial space. (Jain et al. 1999). These three barriers cause limitations in the homogeneous distribution of the drug in the diseased cells.

Ultrasound

Ultrasound is the transmission of pressure waves through a medium, just like normal sound only with a frequency greater than the upper limit of human hearing, approximately 20 kilohertz and up. These waves can be reflected, refracted, focused and absorbed. Ultrasound is able to actually move molecules, the material gets compressed or expanded at the changing pressure of the wave. The absorption of ultrasound energy in the body is relatively low compared to other forms of electromagnetic radiation. Therefore it can penetrate deep inside the body, which is necessary for medical treatments. Also x-rays and radiofrequency pulses can penetrate enough into the body, but have the disadvantage they cannot be focused, where ultrasound can (Pitt et al 2004).

The interaction of ultrasound with tissue comes to expression in different forms, heat generation, cavitation and acoustic radiation force. These mechanisms cause different bio-effects on the tissue which can be used to enhance the drug uptake and concentration at target site.
Several advantages are accompanied with the use of ultrasound as a drug delivering method. First of all, ultrasound is a non-invasive technique with an external source. Second, it can be applied locally at a very small region of interest inside of the body. Third, it is able to reach deep inside of the human body, in absence of harming the tissue in the beam path. And it does not necessarily require the development of new drugs (Frenkel et al. 2006).

Drug delivery
The first challenge is to overcome dose limiting factors that are caused by the systemic toxicity of the used drug. This is mostly due to the non-specific nature and spread of the drug throughout the blood circulation of the body. This causes the drug to also accumulate in healthy tissue, what is clearly not wanted. As a result, the dose in the target tissue has not the desired concentration and it is not possible to increase the overall dose (Moonen 2007). This limits the effectiveness of the drug in the diseased tissue and treatment is far from optimal.
It is well known that local administration of drugs is a promising strategy, so several solutions have been proposed to increase the target concentration. These methods can be divided into three groups; 'active targeting drug delivery', 'passive targeting drug delivery' and 'physical targeting drug delivery'. Active targeting is usually achieved by combining the drug particle with a targeting moiety, like antigen–antibody and ligand–receptor binding This results in preferred accumulation of the drug in the targeted region. A schematic representation is shown in figure 5(n). Passive targeting takes advantage of the differences in permeability between tissues, allowing the drug to accumulate at regions with higher permeability. Passive targeting also includes the administration of drugs exactly at the desired place, for example invasively into an organ artery. Physical targeting makes use of an external trigger, such as ultrasound or magnetic fields to release the drug at a desired region. In the past most research was performed on the active targeting of drugs to the target region, but in de past few decades increasing numbers of studies are dedicated to passive and physical targeting of drugs, because of the huge improvement of concentrating drug in a very small region (Vasir et al. 2005).
Ultrasound mediated local drug delivery utilizes a form of passive targeting and/or physical targeting for drug delivery. What means that it makes use of the (altered)permeability of the tissue and/or trigger the release of drugs locally. Passive and physical targeting with ultrasound often includes the use of drug carriers. These will be discussed under the section "Drug Delivery Carriers".

The second challenge is to make sure the drug can enter the diseased tissue efficiently, in other words, specific barriers that inhibit the drug to pass, have to be opened or lifted. Several different barriers have been discussed and been topic of multiple studies. In most cases the particles are too large to cross barriers, such as vascular tissue (Lum et al. 2006) and the blood-brain-barrier (McDannold et al. 2008). To overcome these barriers a modification of the target environment is required. The interaction of ultrasound with tissue causes increased permeability in several ways. The vascular wall can be ruptured and as a result drug particles can pass through. Next to that the vascular wall can have increased permeability without being ruptured. In order to create increased permeability, different methods with the use of ultrasound are discussed in this paper under the section "Physical Interactions".
In a lot of studies, triggered drug release and altering the permeability of the tissue is used at the same time. This is possible with the use of certain drug carriers.

APPLICATIONS

As mentioned before, there are a lot of different barriers that have to be crossed for efficiently delivering drugs. The most important ones will be elucidated below.

Cancer

A lot of studies in this area focus on tumors that exist through cancer. Not only because this disease occurs so often, but also because tumor tissue is completely different from healthy tissue. The cellular, genetic and molecular mechanisms of a tumor provide extra barriers for a homogeneous drug distribution and thus effectiveness of the drug. Characteristic for cancer cells is that it has very fast proliferation. Next to that angiogenesis cannot keep up, what results in a lack of blood vessels in the tumor. Also the high density of cells can push blood vessels away or obstruct them (Sinha et al. 2006). Because of this the blood vessel distribution is very irregular, figure 1. The blood flow is disrupted or distorted in many places of the tumor. This is a real problem in the homogeneous distribution of drugs via blood flow. Next to that, due to the high cellular density and low blood vessel proportions.

Second, the drug has to cross the vascular wall of the tumor vessels. The vessels in tumors are often poorly build and have bigger pores than healthy tissue, see figure 2. This brings a great advantage in delivering drug through the vascular wall (Jain et al. 1999).

After that the drug has to be transported through the interstitial space. Here the tumors characteristics may give another disadvantage. Because even if a good functional blood vessel is present, limited distribution of oxygen, nutrition and therefore also drugs occurs, for cells that are distant from a blood vessel (Minchinton et al. 2006).

In cancer therapy it is important to eliminate all tumor cells present to have a curative effect, because one single cell is capable of regrowing the tumor.

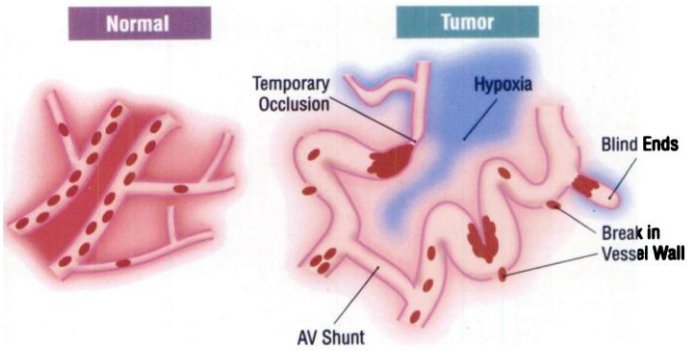

Fig. 1. Diagram showing the principal differences between the vasculature of normal and malignant tissues. Whereas normal tissues have relatively uniform and well-ordered blood vessels that are sufficiently close together to oxygenate all of the tissue, blood vessels in tumors are tortuous, have incomplete vessel walls, have sluggish and irregular blood flow, and have regions of hypoxia between the vessels. (Brown et al. 1998)

Blood-Brain-Barrier

The blood-brain-barrier(BBB) is a selective and structural barrier in the vasculature of the central nervous system, that separated the circulating blood from the cerebrospinal fluid(CSF). The barrier consists of tight junctions between endothelial cells in the vessels of the central nervous system and restricts passage of substances from the circulating blood much more endothelial cells in vessels elsewhere in the body. The main function of the BBB is regulating ion and volume, to create an optimal environment for synaptic and axonal signaling (Choi et al. 2007). However, this property is maintaining a healthy brain, becomes a disadvantage when pharmacological treatment in the brain is needed. It prevents the use of promising drugs. Several strategies have been proposed to circumvent the BBB, a lot of them invasively opening the BBB (McDannold et al. 2009).

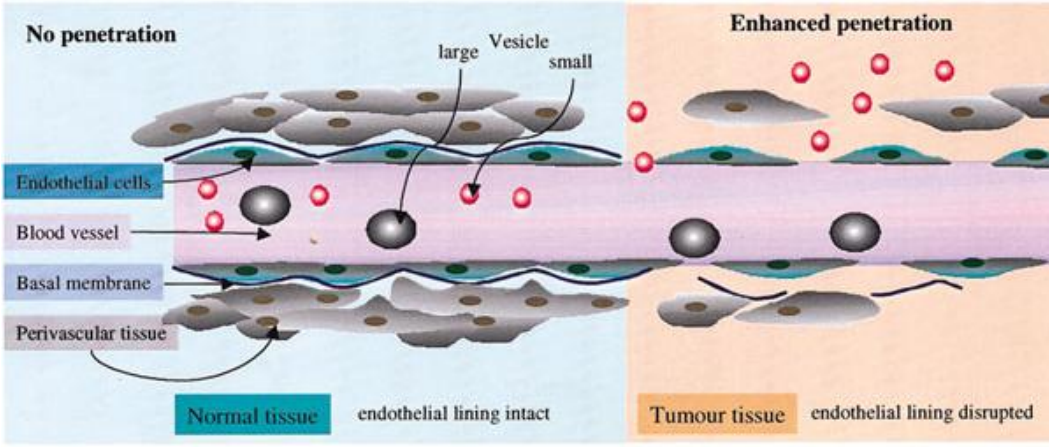

Fig. 2. Schematic representation of particle extravastation from a normal vessel (left) and a tumor vessel (right). (Uchegbu 1999)

There are many other barriers that prevent effective treatment, for example the skin (Park et al. 2007), this however requires a different strategy because of the superficial location of the barrier and will not be discussed in this paper. Or more efficient drug release induced by ultrasound like, antibiotic loaded bone cement (Yan et al. 2006) and induced thrombolysis (Tiukinhoy-Laing et al. 2007) are examples of increased drug delivery with the use of ultrasound.

DRUG DELIVERY CARRIERS

When targeting drugs with the use of ultrasound it is very effective to use drug delivery vehicles. The delivery of free drug, without carrier, has the disadvantage that it extravasates throughout the complete vascular system, and therefore has a high toxicity and lower concentration in the target region can be achieved. The advantage of using a drug carrier is that the carrier can be triggered by ultrasound and release the drug or induce bio-effects at target site. Different types of drug carriers for the use in combination with ultrasound are available, liposomes, micelles and microbubbles. All with other characteristics and therefore interact in different ways with ultrasound and tissue. This results in an adapted distribution, clearance and toxicity of the drug.

Micelles

A micelle [fig. 3b.] has an amphiphilic nature, meaning that it consists of hydrophilic and hydrophobic parts. The core is of the micelle consists of hydrophobic parts due to unfavorable interactions with the aqueous environment. A hydrophilic outer layer is present due to favorable interaction with water, that at the same time protects the core of the micelle. A micelle can be loaded with hydrophobic drugs, stored in the hydrophobic core. The size of micelles in drug delivery ranges from 5 – 30 nm, and therefore is the smallest used carrier for ultrasound. This allows the particles to extravasate at tumor site, but are still large enough to escape renal excretion (Gao et al. 2005). Next to that micelles are not recognized by the reticuloendothelial system and therefore have a longer circulation time compared to the free drug. Further, it is shown that when ultrasound is turned off, the drug is encapsulated by micelles again and therefore possibly preventing further systemic dissemination (Husseini et al. 2005).

Liposomes

Liposomes [fig. 3c.] have, like micelles, an amphiphilic nature, but a different structure. The outer layer consists of hydrophilic parts and attached to that are hydrophobic tails, exactly the same as in micelles. However, liposomes have a same layer in opposite position next to that. Therefore a liposome can be loaded with hydrophilic(in the core of the liposome) and hydrophobic(in between the bilayers) drugs (Needham et al. 2001). Liposomes have a particularly high drug payload. Because of this bilayer, a liposome is much bigger in size, 100 – 200 nm, and does get recognized by the reticuloendothelial system. To prevent liposomes to be cleared from the body rapidly, polethylene glycol (PEG) is grafted onto the outer layer of the liposome (Gabizon et al. 1997). Like micelles, liposomes are in general not gas filled and therefore not susceptible for cavitation.

In general, for liposomes because of their size, it is not possible to extravasete from vessels. It is however possible to create temperature sensitive liposomes(TSL), what is very useful because ultrasound is able to create heat inside of the body. TSL's rely on the fact that lysolipids and/or phospholipids are attached to it. These lipids are solid below a temperature of 39 – 41 ˚C, but become liquid at higher temperatures. When the lipids melt, many pores arise in the shell of the liposome and their contents is released (Ponce et al. 2007).

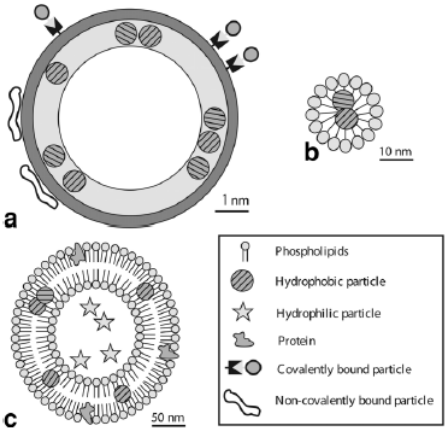

Fig. 3. Overview of different delivery systems used for gene and drug delivery. Particles can be bound covalently and noncovalently to the membrane of the microbubble or the particle can be loaded to the interior of the microbubble **(a)**. Hydrophobic particles can be loaded inside the hydrophobic center of micelles **(b)**. Liposomes can be loaded with both hydrophobic and hydrophilic particles because of their bilayer membrane **(c)**. (Deckers et al. 2008)

Microbubbles

The microbubble [fig. 3a.] is very different from micelles and liposomes and overall mostly used. In most cases the shell is composed of albumin or lipids. The material of the shell determines how easily the microbubble is recognized by the reticuloendothelial system. The core of the microbubble is gas filled, mostly with air or perfluorcocarbon. With sizes ranging from 1 – 12 μm, it can still easily flow through blood circulation, but is not appropriate for extravascular transport. Microbubbles get recognized by the reticuoendothelial because of their size. Circulation an stability in bloodstream is about 10 minutes. To increase the circulation time, it is possible to graft polyethylene glycol (PEG) to the shell of the microbubble, preventing rapid clearance from the system (Lum et al. 2006). Drugs can be loaded onto microbubbles in several ways. The drug can be loaded in the interior of the bubble or it can be embedded or attached to the membrane (Pitt et al. 2004). The microbubble's large size relative to other drug delivery vehicles may allow greater amounts of drugs to be carried. Although the size of the bubbles could definitely be decreased, this is not wanted. This range of micrometers is particularly chosen, because of the good acoustic response of the bubbles (Coussios et al. 2007).

The gas core of the microbubble is very important, because it is sensitive for acoustic activity. Under the influence of ultrasound exposure microbubbles start to oscillate, what causes several important effects that will be explained further on.

PHYSICAL INTERACTIONS

Ultrasound can be used to release drugs from carriers, but it can also be used to induce bioeffects inside the body. The tree physical mechanisms, heat generation, cavitation and radiation force, are used with ultrasound, these are elucidated below. In figure 3 a schematic illustration of the different manners of enhanced extravasation from a vessel is shown.

Heat Generation

The use of thermal elevation in local drug delivery goes back a long time and can be induced in multiple ways. For example, catheters (Ponce et al. 2007), microwaves, radiofrequency infrared, or lasers (Diederich et al. 1999). However these methods are either invasive, only have superficial penetration depth or inhomogeneous distribution of heat. The biggest advantage of using ultrasound to induce local heating is its non-invasive nature and deep penetration into the body. Another very important capability of ultrasound is that it can focus the heat very precisely, into a very small region in the body.

Elevations of temperature results from the absorption of ultrasound energy. This is directly proportional to the absorption coefficient to the tissue. It has a linear relation with the intensity of the ultrasound waves (Frenkel 2008). Other properties of the treated tissue, like diffusion and convection inside of a vessel due to bloodstream, also influence the amount of heat generated (O'Neill et al. 2009).

Heating of tissue has different applications, depending on the temperature. With a great increase in temperature, up to 60˚C, thermal ablation therapy is possible. In lower ranges of temperature elevations, the use of drug carriers is possible, for example the low temperature sensitive liposomes(LTSL) that become sensitive to heat around 41˚C (Ponce et al. 2007). This induces a locally enhanced concentration of the drug, because they only release the drug at target site. Heating inside the body also creates physiological changes in the tissue itself, like increase in blood flow and oxygenation. this process is very complex, not fully understood and therefore hard to predict(Kong et al. 2000).

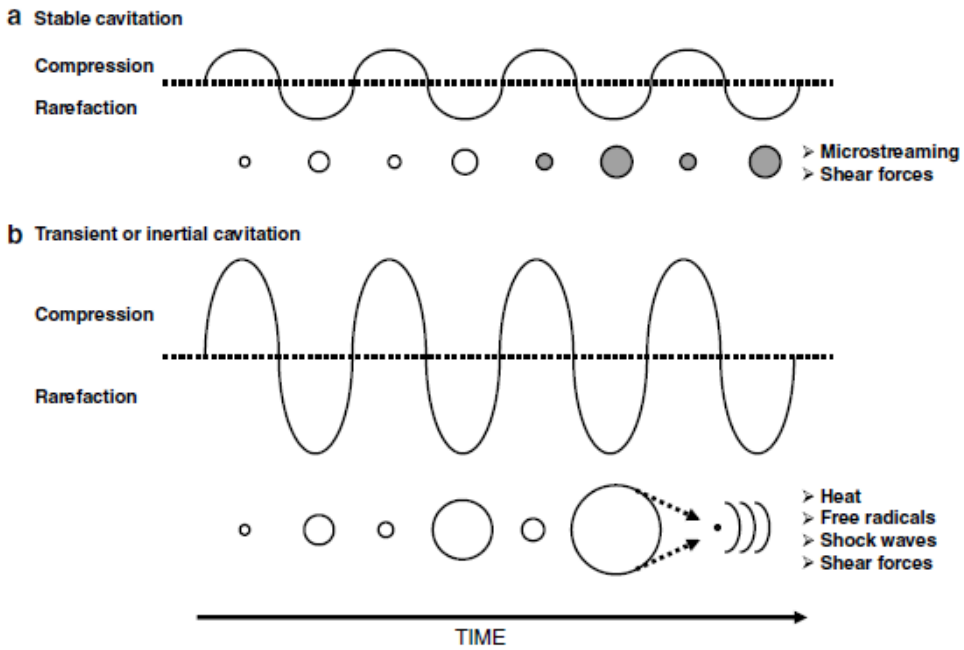

Fig. 4. Schematic diagram illustrating the effects of acoustic fields of identical frequency but differing intensity on microbubble behavior. **(a)** Low-intensity ultrasound induces oscillation, with a gradual increase in microbubble diameter until a resonant diameter is reached, when stable oscillation occurs (filled circles). This process is termed stable cavitation. **(b)** At higher intensities, the microbubbles grow rapidly for a few cycles. However, the inertial energy of the fluid surrounding the microbubble becomes so great that it cannot reverse direction and collapses violently. This in turn generates shock waves, free radical production and local heat. This process is termed inertial or transient cavitation. (Newman et al. 2007)

Acoustic Cavitation

In the field of drug delivery, cavitation is the most important non-thermal ultrasound mechanism. It is defined as the growth, oscillation and collapse of small gas bubbles in a fluid under the influence of ultrasound waves. There are two basic types of acoustic cavitation, stable/non-inertial cavitation and transient/inertial cavitation. Stable cavitation occurs at low intensity ultrasound energy and bubbles oscillate stable around resonant diameter. Initially the bubbles slowly grow in size each cycle, until it reaches the resonant size. (Fig. 4a.) Stable cavitation can last for a long time and induces shear forces in nearby tissues and microstreaming. (Fig. 5c.) Inertial cavitation occurs at higher intensities causing the bubble to violently collapse. The bubble rapidly grows in size in only a single or few cycles. Due to the great forces of inertia from the surrounding medium, the bubble is not able to reverse direction and collapses. (Fig. 4b.) Inertial cavitation creates local increase of temperature and pressure (Newman et al. 2007).

To induce increased drug delivery with cavitation forces, an air or gas filled nuclei is necessary. Microbubbles are widely used for this purpose and can also be loaded with drugs at the same time (Lum et al. 2006). In that case the microbubble has to collapse in order to release the drug it is containing. Beneficial bioeffects, induced by the collapsing bubble, may enhance the uptake of the locally released drug from the microbubble. It is also possible to administer free drugs into the body and induce bioeffects with the use of microbubbles in the target area (Guzman et al. 2003). In this way cavitation does not trigger a local release of drug, but an enhanced uptake at target site, by enhancing permeability. To prevent a high toxicity for the body, the free drug can be encapsulated in micelles (Husseini et al. 2005) or liposomes (Schroeder et al. 2007). Here the micelles or liposomes use the cavitation forces to shear and locally release the containing drugs.

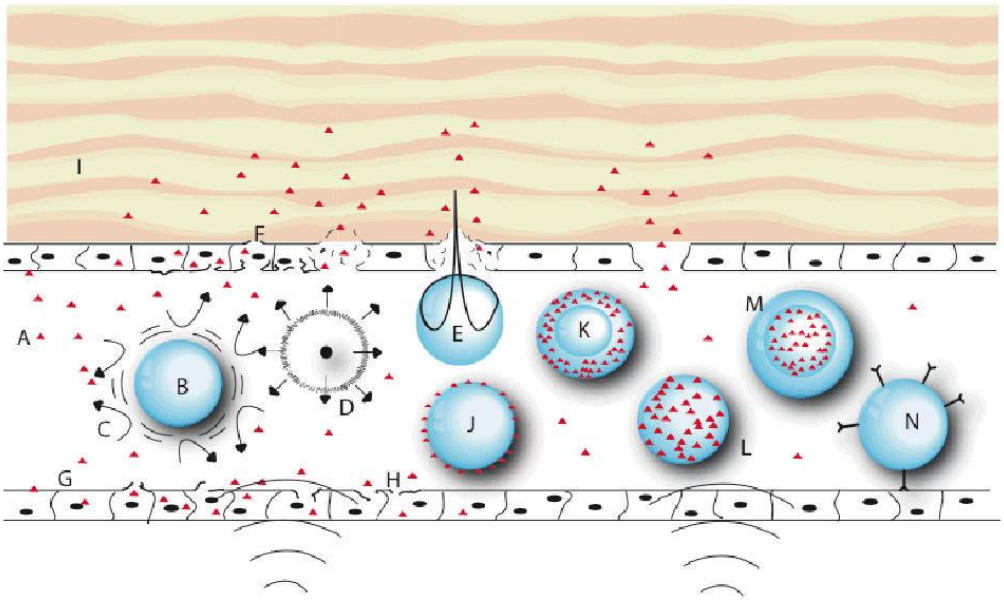

Fig 5. Schematic representation of various modes by which drug delivery can be enhanced by ultrasound. (**a)** therapeutic agent (triangles) **(b)** gas bubble undergoing stable cavitation **(c)** microstreaming around cavitating bubble **(d)** collapse cavitation emitting a shock wave **(e)** acoustic radiation force **(f)** completely pierced and ruptured cell **(g)** non-ruptured cells with increased membrane permeability due to insonation **(h)** cell with damaged membrane from microstreaming or shock wave **(i)** extravascular tissue **(j)** microbubble decorated with agent on surface **(k)** microbubble with agent in lipophilic phase **(l)** micelle with agent in lipophilic phase **(m)** liposome with agent in aqueous interior **(n)** vesicle decorated with targeting moieties attached to a specific target. (Pitt et al. 2004)

Acoustic Radiation Force

Acoustic radiation forces occur at all pressures with the use of High Intensity Focused Utrasound(HIFU), causing the interacting material to displace in the direction of the ultrasound wave. This phenomena occurs when a traveling ultrasound wave is absorbed or reflected by a particle, the momentum associated with the wave produces "primary ultrasound radiation force (USRF)," causing the radiating sound wave to transfer to the particle. Radiation forces are proportional the absorption coefficient of the tissue and the applied energy (Dayton et al. 2004).

In fluid mediums the radiation force can produce a steady flow, also known as acoustic streaming, making it possible to direct fluids in some direction. Compressible objects such as gas filled microbubbles experience much larger forces and are easier to displace (Frenkel 2008). Therefore acoustic radiation forces are commonly used to displace microbubbles. It is possible to push microbubbles that are circulating in the blood stream towards the vessel wall to increase uptake in that area. (fig. 5e.) The movement of microbubbles to the vessel wall induces shear forces and can cause gaps in the endothelium of the vessel wall (Lum et al. 2006).

In addition to primary USRF, which acts in the direction of acoustic wave propagation, a "secondary USRF" also acts between individual bubbles. The second USRF acts on the individual bubbles to attract each other, causing a greater concentration at the target site (Dayton et al. 2004).

RESEARCH

To be able to develop new or better strategies for delivering drugs with the use of ultrasound it is extremely important to understand the underlying mechanisms. There are two interrelated aspects in this research. First, in order to be able to control the process, there has to be understanding about the physical interactions induced with ultrasound. And second, it is important to determine which bioeffects occur as a response on these physical mechanisms.

Heat Generation

As previously said, it is possible to enhance delivery by ultrasound induced heat generation. This can be done with the use of thermo sensitive liposomes (TSL), non thermo sensitive liposomes (NTSL) or free particles. It is important to investigate their influence on drug delivery and their sensitivity to hyperthermia (HT). The carriers have great importance, because they can provide local release of drugs. Furthermore, physiological changes in tissue can induce an increase extravasation of drugs from the vessel. Important aspects of heat generation in the body are a homogeneous distribution of heat in the tissue, the time of administering the drug in relation to the thermal state, the size of the carriers and drugs, good scheduling of heat exposures and a homogeneous extravasation of the drug.

The group of Ponce et al. uses thermo sensitive liposomes loaded with doxorubicin in combination with hyperthermia. Next to doxorubicin, also manganese was loaded into the liposome. Manganese was used to monitor the drug uptake with MRI. The temperature elevation was not induced by ultrasound and located in the center of the tumor, causing an inhomogeneous distribution of heat. In the centre of the tumor, a temperature of 45 – 46 ˚C was reached, whereas the border of the tumor (5-6mm from centre) was elevated to 38.5 – 39 ˚C. They investigated the effect of applying hyperthermia and TSL's in different time schemes by comparing the drug delivery patterns. They also measured antitumor effects, by comparing growth rates. They used three different time schemes; (1) first reach stable heating in the tumor and then administer TSL's, (2) first administer TSL's and then start with heating, (3) administer ½ TSL's dose first, then start heating and when a stable state of heating is reached administer the other ½ TSL's dose. The results show that protocol 3 delivers the drug most homogeneously, see figure 6a. In figure 6b. however, it is shown that TSL's release their content very rapidly when they come in contact with heat, therefore the drawback in administering TSL's before steady HT state is, that the liposomes not completely release their content. When comparing antitumor effects it shows that protocol 1 is most effective. The use of manganese to image the extravasation of drug, may not be very accurate. There is no evidence that manganese and doxorubicin remain co-localized after liposome release.

The group of Kong et al. uses NTSL's in combination with HT. Their source of heat was like Ponce et al. also not induced by ultrasound. Here they did create a homogeneous thermal field for the target tissue, with the use of a heat chamber. They used a temperature range from 39 to 42 ˚C in steady state before administering the drug. This is the same protocol as evaluated the best in Ponce et al. The difference is that here they use NTSL's. The results show a threshold between 39 and 40 ˚C for extravasating liposomes through tumor vascular and increased extravasation at higher temperatures. Because of the use of NTSL's, at this threshold the vascular walls permeabiblity increases, that causes more liposomes to extravasate. They also showed that the effects of HT are transient, but still show some extravasation up to 6 hours after HT. Furthermore, they proved that tissue develops, thermo tolerance. When treated tissue was given HT after 8 hours again, no extravasation of liposomes was observed. Therefore it is important to schedule a series of HT exposures.

In the studies of Ponce et al. and Kong et al. they both use liposomes with a size of 100nm and both use the same protocol of administering heat and liposomes. Their results also agree that heat is capable of increasing extravasation from tumor vasculature. However these results do not rely on extravasation of the same particles. Ponce et al. use TSL's, and therefore the drug is released from the liposomes at target site. This induces the extravasation of only the drug from the vasculature. Kong et al. use NTSL's and therefore the drug remains encapsulated inside of the liposome. They describe the extravasation of the complete liposome from the vasculature. The approach of Kong et al. brings a disadvantage. Because the complete liposome has to be extravasated, they are restricted in the size of the liposome. Ponce et al. could use much bigger liposomes and therefore increase load capabilities, what possibly increases concentration at target site.

So, it is shown that hyperthermia can increase extravasation, however the bioeffects allowing it are not clear. Ponce et al. states that the endothelial wall of the blood vessels might be damaged, and therefore the blood supply to the tumor is decreased and the growth of the tumor slows down. Kong et al. state that the increased extravasation relies on the changed tumor vessel wall environment.
In these two studies, heat is not induced by ultrasound. It is however possible to create heating within tissue with an ultrasound source. There are several advantages of using ultrasound to induce heat with respect to the heat sources used in these papers. Ultrasound is non-invasive and can be focused very locally. Therefore, in future research the use of ultrasound to induce heat should be investigated.

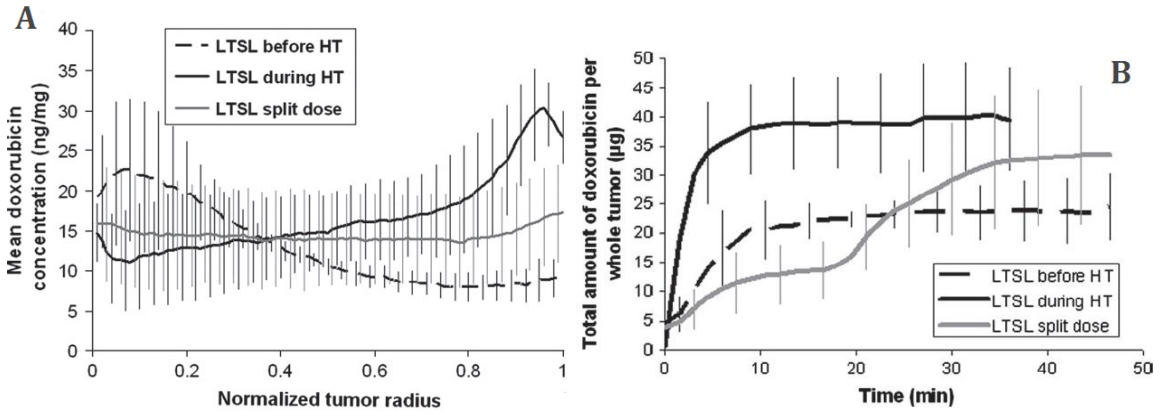

Fig 6. (a) Mean doxorubicin concentration (nanograms per milligram of tissue) profiles for each of the tree therapeutic groups as a function of the normalized tumor radius (n = 6 – 7 rats per group) **(b)** Total amount of doxorubicin delivered to whole tumor as a function of time for three different protocols of lysolipid-based temperaturesensitive liposomes (LTSLs) plus hyperthermia (HT), calculated using magnetic resonance images. Mean amounts of doxorubicin are shown for each group (n = 6 – 7 animals per group). (Ponce et al. 2007)

Summarized, if the generation of heat is used to deliver drug locally, the use of TSL's would be recommended, because of the higher uptake compared to NTSL's (Ponce et al. 2007). This is due to the higher intravascular concentration, by the release of drug from the liposomes in the lumen. Another benefit of TSL's is that they are not limited to sizes that can still be extravasated by the tissue. The size of drug particles is much smaller than that of liposomes, the extravasation of drug particles is therefore much easier. The best results are found when TSL's are administered when a steady temperature state is reached. Further it is very important to schedule a hyperthermia treatment carefully when treatment with hyperthermia is used repeatedly, as thermo tolerance plays a role in the physiologic response of the tissue. The temperatures used are relevant for clinical studies, because the threshold temperature is around 39 ˚C.

Acoustic Cavitation

Cavitation is probably the most investigated physical interaction of ultrasound and is most commonly used in combination with microbubbles. In order for cavitation to happen, the acoustic parameters, like pressure, centre frequency and insonication time, are very important. For the studies that will be discussed below, the used parameters are listed in table 1. Many studies have shown to trigger cavitation at a certain parameter setting. Next to that, air or gas filled nuclei are obligatory for cavitation and therefore have great influence. The concentration of microbubbles and their position towards surrounding cells is very important.

Tran et al. investigated the effects of cavitation on a single cell of a mammary tumor. The effects on the cell was measured using the patch-clamp technique. This technique makes recordings of the cell membrane potential. They used microbubbles with a diameter of 1 – 12 µm at a concentration of $2*10^8$ bubbles/ml. The microbubbles were infused at a rate of 1 ml/min. The ultrasound parameters were as follows; 1 MHz, up to 500 kPa, 5 – 50 cycles, 100 µs repetition time and 2 – 20 s exposure time. The results show that microbubbles or ultrasound alone do not affect the cells. When using both at the same time, a threshold of 150 kPa is found. This means that with these settings, below 150 kPa cells are not affected by ultrasound and microbubbles. At all pressures the cells remain viable. In this investigation it is shown that in order to induce any effects by applying microbubbles and ultrasound, the microbubbles have to be in direct contact with the cell. Furthermore, they investigated whether the effects induced by oscillating microbubbles are caused by mechanical stresses on the cell membrane. The oscillatory movements of the microbubbles were imitated using a glass rode poking the cell. They observed similar results compared to the microbubbles, see figure 7. These results show that the oscillations of the bubble cause mechanical stress on the cell membrane, which induces hyper polarization. Hyper polarization is a change in membrane voltage that makes it more negative, it has been shown to be reversible and reproducible. A drawback in this study is that they do not show if the induced hyper polarization enhances permeabilization of the cell.

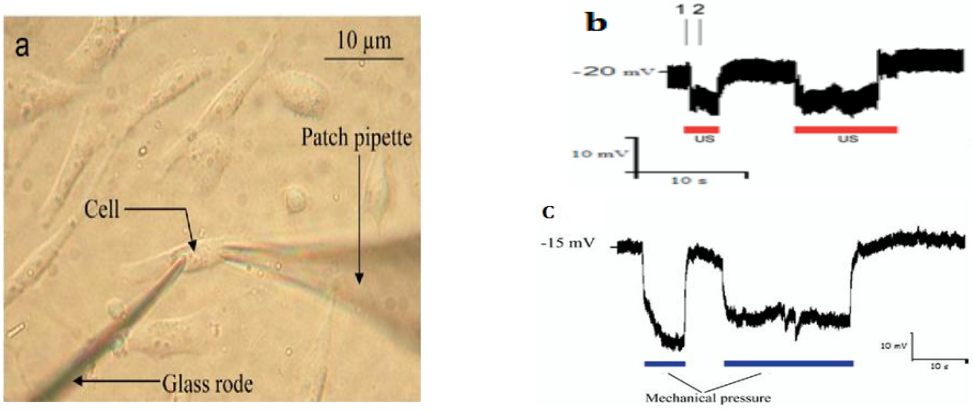

Fig 7. (a) Optical image of a glass rod pressing on a patched cell. **(b)** Cell membrane potential of a patched cell with ultrasound (1 MHz, 200 kPa, pulse length 10 µs, repetition time 100 µs) and microbubbles. **(c)** Membrane potential of the patched cell under the rod's mechanical pressure. (Tran et al. 2007)

Also the group of van Wamel et al. investigated the effect of oscillating microbubbles on a single cell. They used microbubbles with at diameter of 1 – 12 µm at a concentration of 1 – $2*10^5$ bubbles/ml. The model drug was a fluorescent nuclei (0.8 nm), that normally does not cross the cell membrane of the endothelial cell cultures used in this experiment. The model drug was not attached or loaded onto the microbubbles. The ultrasound parameters were 1 MHz, 0.4 MPa, 10 cycles, 50 Hz repetition rate and 5 s of duration. They investigated sonoporation and recovery time of the cells, with timetables as shown in figure 8. The results show that all cells that experienced membrane deformation had uptake of the model drug. And all cells that not experienced membrane deformation showed no uptake of the model drug. This observation is also seen in the study of Tran et al, who say that direct contact of the microbubble and cell membrane is obligatory. There was no correlation between the magnitude of the oscillation and uptake of model drug. The enhanced permeability of the cell membrane is reversible, because the concentration of model drug decreased over time after insonication. Also the experiment about recovery time show that enhanced permebilization is transient. The recovery times varied from 0 – 60 s. The results show that direct administering of drugs is preferred, because of the highest uptake of the model drug.

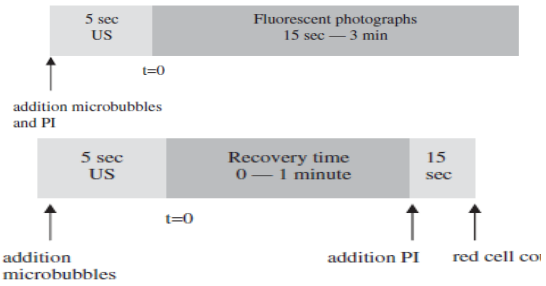

Fig 8. (top) Timetable of the sequence of events of *sonoporation* of fluorescent nuclei (PI). **(bottom)** Timetable of the sequence of events of *recovery time* depended fluorescent nuclei (PI) uptake. (van Wamel et al. 2006)

This study investigated the effect of microbubbles on a single cell, like Tran et al. Their results are similar as they both claim that direct contact of the microbubbles and cell membrane is necessary, to induce hyperpolarization (Tran et al.) or enhanced permebilization (van Wamel et al.). It is however not clear if they are working with the same principles. Tran does not prove enhanced permebilization and van Wamel has no evidence for hyperpolarization. More investigations are needed.

Rahim et al. used microbubbles with at diameter from 1 – 10 µm at a concentration of 2 – $5*10^8$ bubble/ml. They used a plasmid that was not bound to the microbubbles, but present in the same solution at a concentration of 0.5 mg/ml. They used a 1 MHz transducer, the pulse length was varied up to 80 cycles, the repetition up to 2.5 kHz and the exposure time up to 60 s. They investigated the effect of plasmid concentration and microbubble concentration. The results show that microbubbles or ultrasound alone do not have any effect. Increasing concentration of either the plasmid or microbubbles did not enhance sonoporation. Also the repetition rate and cycles did not affect sonoporation significantly, what suggests that cavitation occurs within the first 10 cycles. However, the pressure and duration of the exposure are of great importance. By increasing the duration of the exposure the viability rapidly decreases. The same thing happens with increasing pressure. Here a threshold for cavitation to occur lies around 0.13 MPa. This group suggests optimal parameter settings of 0.50 MPa, 40 cycles, 1 kHz repetion rate, 10 s exposure at 1 MHz for maximal transfection and viable cells.

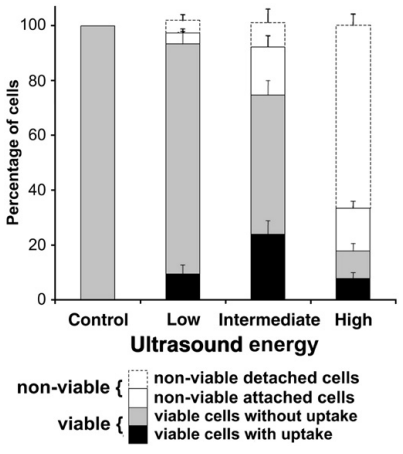

Fig 9. Quantification of endothelial bioeffects following ultrasound exposure. (Hallow et al. 2007)

The group of Hallow et al. uses microbubbles with a diameter of 3 – 5 µm and a fluorescent nucleic that is impermeable to the intact arteries that were used in this experiment. The model drug was not loaded on/in the microbubbles, but administered separately. The artery was placed in a chamber filled with a solution of 4µM model drug and

$1.1*10^7$ bubbles/ml, at physiological pressure (100 mmHg). To test the influence of parameters, three different protocols were used with varying pressure and exposure time: (1) 5.0 J/cm^2 = 0.7MPa, 300ms, (2) 66 J/cm^2 = 1.4 MPa, 1000ms, (3) 630 J/cm^2 = 2.5MPa, 3000ms. All with an frequency of 1.1 MHz and duty cycle of 1% to prevent heating. The results show that increasing energy results in increased uptake of drug in the endothelial cells of the artery, but also increased cell death, shown in figure 9. At the highest energy only 25% of the cells remained viable after treatment. These observations are also seen in the study of Rahim et al. and both advise pressure intensities at a lower range. They also tested if the smooth muscle cells behind the endothelial cells in the artery showed uptake of the model drug. Unfortunately this was only the case at the highest energy protocol (3), when the endothelial cells are almost all non viable. This suggests that the internal elastic lamina, located between the endothelial cells and smooth muscle cells, serves as main barrier. It seems that the penetration depth of cavitation events is very limited due to this barrier in arteries.

The studies of Tran et al. and van Wamel et al. observed that in order for cavitation events to occur, direct contact of microbubbles and cell membrane is necessary. In the this study, Guzmán et al. go one step further by trying to understand the cell to bubble interactions that are related to acoustic cavitation and looking at the range a bubble is capable of influencing multiple cells and neighboring bubbles. They used gas filled microbubbles with a diameter in a range of 2 – 4,5 μm at varying concentrations from 4*10^4 bubbles/mL to 9*10^7 bubbles/mL. . Human prostate cancer cells were grown and also used at varying concentrations from 2.5*10^5 cells/mL to 4.0*10^7 cells/mL. To monitor the transport of molecules across the membranes of viable cells a green fluorescent molecule was used, with a size of 0.6 nm. These molecules are not able to cross the membrane of intact cells. They used a 500 kHz ultrasound transducer and 60 ms pulses at a 6% duty cycle. Any effects of heating were excluded, because temperature measurements did not rise enough, mean 1˚C and a maximum of 5 ˚C . For all varying cell and bubble concentrations, different energy exposures were applied. They ranged from 2 to 817 J/cm^2 for the bubbles and from 12 to 332 J/cm^2 for the cells. This was achieved by varying the pressure (0.64 – 2.96 MPa) and exposure time (120 – 2000 ms). The results show that the cellular uptake is homogeneous and a lower energy exposure is preferred, when using as a drug delivery mechanism. A high cell density results in a higher viability overall. However, using greater bubble concentrations does not benefit the efficiency of permeabilizing the cells. It seems that neighboring bubbles influence each other so that the number of cells affected by each bubble decreased with increasing bubble concentration. From these findings Guzmán created a unifying parameter that captures the inverse dependency of the cell density and bubble concentration, the cell-to-bubble ratio. However, this parameter is not a unifying parameter, because of contrasting results within the range of a parameter.

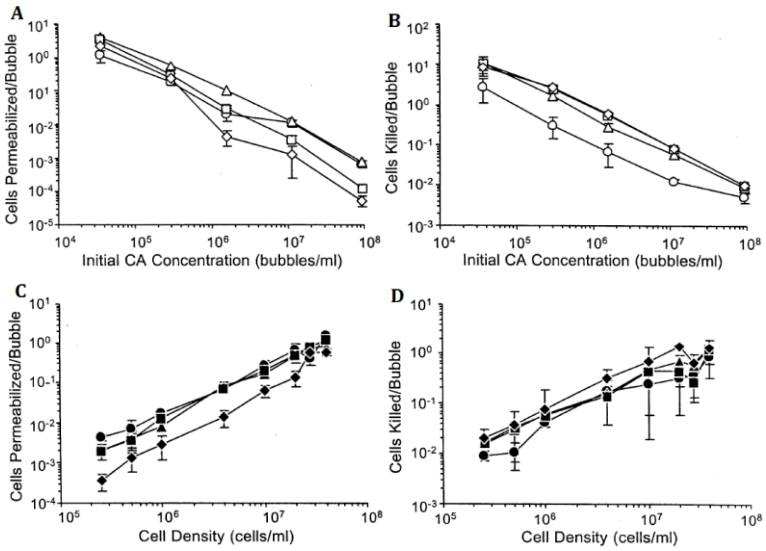

Fig 10. (a,b) Effect of initial contrast agent concentration on molecular uptake and cell viability. **(c,d)** Effect of cell density on amount of permeabilized and killed cells. (Guzmán et al. 2003)

The group of Karashafian et al. investigated the effect of acoustic parameters on cavitation effects. They used gas filled microbubbles with a diameter in a range of 1 – 18 µm, at a concentration of $33*10^6$ bubbles/ml. They used fibrosarcoma cell cultures at a concentration of $1.2*10^6$ cells/ml. To measure permeability a fluorescent molecule was added, but not bound to the microbubble. After sonication, the cells were investigated on their viability with another fluorescent molecule. They used three different transducers, 500 kHz, 2.25 MHz and 5 MHz. In total they used 87 different exposure conditions with varying pressure (0 – 3500 kPa), pulse durations (4 – 32 µs), pulse repetition frequency (10 – 3000 Hz), duty cycle (0.032 – 9.6 %) and insonication time (0 – 120 s). The results show that there is an inverse correlation between cell permeability and viability for all conditions, this is also shown in figure 7. They say that bubble disruptions is necessary, but there is no correlation with permeability. To evaluate the results, Karashafian makes use of the therapeutic ratio (TR), the proportion of cells in which reversible permeability increases are induced compared with those that are killed by the same exposure. They state that maximum permeability occurs at 500 kHz, 570 kPa, 32 µs (16 cycles), 3 kHz and 120 s insonication time. The highest TR is seen at 500 kHz, 570 kPa, 8 µs (4 cycles), 3 kHz and 12 s insonication time. The studies of Rahim and Hallow both agree with using lower intensities for inducing cavitation events. This because of the high rate of cell death at higher intensities, like the therapeutic ratio (TR). Karshafian uses a lower centre frequency, but the other parameters are around the same range.

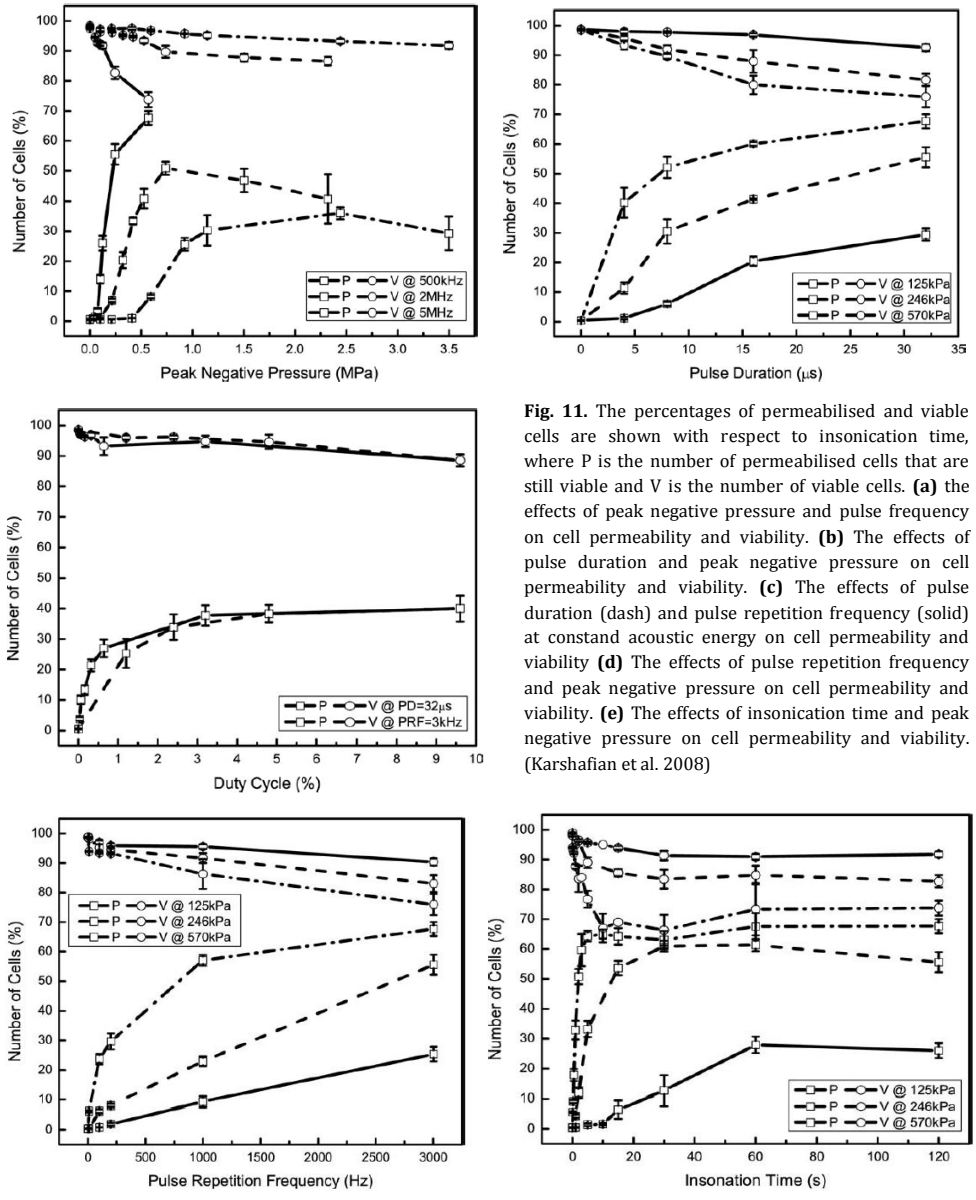

Fig. 11. The percentages of permeabilised and viable cells are shown with respect to insonication time, where P is the number of permeabilised cells that are still viable and V is the number of viable cells. **(a)** the effects of peak negative pressure and pulse frequency on cell permeability and viability. **(b)** The effects of pulse duration and peak negative pressure on cell permeability and viability. **(c)** The effects of pulse duration (dash) and pulse repetition frequency (solid) at constand acoustic energy on cell permeability and viability **(d)** The effects of pulse repetition frequency and peak negative pressure on cell permeability and viability. **(e)** The effects of insonication time and peak negative pressure on cell permeability and viability. (Karshafian et al. 2008)

McDannold et al. used gas filled microbubbles with a diameter of 2 – 4.5 µm at a concentration of 5 – 8*10^8 bubbles/ml. Their goal was to disrupt the Blood-Brain-Barrier (BBB) in vivo. The disruption was measured by injecting a magnetic contrast agent intravenously and immediately after sonication capture images with the use of MRI. They used an ultrasound transducer with a frequency of 690 kHz and changed the following parameters: pressure (0.4 – 1.5 MPa), pulse length (0.1 – 10 ms), pulse repetition frequency (0.5 – 5 Hz) and bubble concentration up to five times the standard concentration. The results show that with these setting only small and limited damage to the vasculature was present. From the parameters tested, only pulse length made a significant difference. Therefore they conclude that the pulses repetition frequency and the concentration of microbubbles does not influence disruption of the BBB. The pulse repetition frequency are also considered less important by Rahim et al. and karshafian et al. The observation that microbubble concentration does not influence disruption is in contradiction to the study of Guzmán, who claims that bubble concentration does influence efficacy of cavitation. This difference could be explained by the used range in bubble concentration. McDannold used only a small range, where Guzmán used a much bigger difference.

	Centre Frequency	Pressure (MPa)	Pulse Length	Pulse Repetition	# Cycles	Duty Cycle	Exposure Time	Carrier Size	Carrier Concentration
Tran et al. 2006	1 MHz	up to 0.5	10 / 100 µs	-	5 - 50	-	2 – 20 s	Microbubbles 1 – 12 µm	2*10^8 mb/ml
Van Wamel et al. 2006	1 MHz	0.4	-	50 Hz	10	-	5 s	Microbubbles 1 – 12 µm	1 – 2*10^5 mb/ml
Rahim et al. 2006	1 MHz	0.13 - 0.5	-	0.5 - 2.5 kHz	10 - 80	-	10 - 60 s	Microbubbles 1 – 10 µm	2 - 5*10^8 mb/ml
Hallow et al. 2007	1.1 MHz	0.7 – 2.5	1 / 100 ms	-	-	1 %	300 – 3000 ms	Microbubbles 3 – 5 µm	1.1*10^7 mb/ml
Guzmán et al. 2003	0.5 MHz	0.64 –2.96	60 ms	-	-	6 %	120 – 2000 ms	Microbubbles 2 -4.5 µm	4*10^4 - 9*10^7 mb/ml
Karashafian et al. 2008	0.5 – 5 MHz	0 – 3.5	4 – 32 µs	10 – 3000 Hz	-	0.032 – 9.6 %	0 – 120 s	Microbubbles 1- 18 µm	33*10^6 mb/ml
Mcdannold et al. 2008	0.69 MHz	0.1 – 1.5	0.1 – 10 ms	0.5 – 5 Hz	-	-	20 s	Microbubbles 2 – 4.5 µm	5 – 8*10^8 mb/ml
Husseini et al. 2005	0.07 MHz	*0.25 - 0.52 W/cm2*	-	-	-	-	-	Micelles 10 – 30 nm	10 ug/mL
Gao et al. 2004	1 Mhz	*3.4 W/cm2*	-	-	-	33 - 50%	30 s	Micelles 8.9 – 12.9 nm	1.5 – 3 mg/kg

Table 1. Overview of used parameters in articles.

The group of Husseini et al. works with micelles instead of microbubbles. Where most others papers use a free (model) drug to circulate, they introduced micelles, 10 – 30 nm, to encapsulate the drug. These micelles are inserted in a water bath and compared with the free drug, without the use of cells or tissue. They measure the fluorescence intensity from the drug and compare that to the free drug inserted in the water bath. When this drug comes in contact to aqueous solutions it exhibits a decrease of fluorescence. This will happen when a micelle releases its content. Also the ultrasound parameters differ from other studies, with a low frequency of 70 kHz. The results show a local release of the drug from the micelles, with a threshold at 0.3 W/cm^2. They suggest that the release depends upon collapse cavitation of bubbles near the micelles. This in accordance with the findings of Tran, van Wamel and Guzmán, they also claim that bubbles have to be in direct contact with the cell membrane to induce effects on the cell. In this case the micelle has to be in direct contact with a bubble to experience effects from the cavitation events from the bubble. A micelle immediately adjacent to a collapsing bubble would be subject to very high shear stresses. This perhaps is sufficiently enough to shear apart the micelle and release the drug for a time until the micelle reforms and re-encapsulates the drug. From acoustic measurements they are very confident of the presence of cavitation events, but the distinction between inertial and non-inertial is not certain.

Also the article of Gao et al. encapsulates the drug into micelles, here the micelles have a diameter of 8.9 to 12.9 nm. They used ovarian carcinoma cells inserted into mice. An ultrasound transducer of 1 MHz was used, exposure time of 30 s with duty cycles of 33 or 50 %, with an intensity of 3.4 W/cm^2. The goal of this study was to investigate the survival and growth rates after insonication compared with the free drug and the influence of ultrasound. They measured the intensity of the fluorescent drug in several parts of the mouse organs. The results show that the tumor growth rates and mice survival rates were slower/better with the use of micelles and ultrasound. Ultrasound is effective in targeting drugs into tumors, but the effect depended on the time between drug injection and ultrasound application. The drug needed time to accumulate in the tumor, but ultrasound did not enhance micelle extravasation of the drug. With ultrasound the intracellular uptake was much higher and accumulation in other organs much lower. Gao et al. suggest that these differences were presumably cell membrane susceptibility to ultrasonic irradiation. With increased duty cycle they investigated the influence of heat, but the effects were not significant. What does cause the enhanced intercellular uptake under ultrasound is not clear. They should have used a method that includes acoustic measurements to help understand the mechanisms that play a role in the enhanced delivery.

Although Husseini and Gao both use micelles to encapsulate drug, their studies are completely different. First of all, Husseini experiments purely in vitro, where Gao uses mice to test the efficiency of micelles. The used ultrasound parameters are also very different. In the results, Husseini claims that ultrasound is capable of releasing the drug from the micelle. In the results of Gao it is shown that this is not induced by ultrasound. Gao claims that the micelles completely extravasate through the vasculature and do not release their content under the exposure of ultrasound. A very promising result of Gao et al. shows that micelles lower the toxicity for the rest of the body while increasing the concentration in the tumor. Much more investigations have to be done in order to understand the principles of using ultrasound and micelles.

Several studies that were treated in this paper are not sure whether they are dealing with a form of cavitation (either inertial or non-inertial), like McDannold et al, Karshafian et al. and Gao et al. Other studies do have the proof that they are dealing with cavitation, but cannot make the distinction between inertial and non-inertial cavitation by evaluating the observed bioeffects, like Husseini et al. This however is of great importance in the understanding, improvement and development of new strategies. For this reason Farny et al. investigated the improve of spatial sensitivity for inertial and non-inertial cavitation during sonication. They used a commercial diagnostic ultrasound system equipped with an array transducer. The results demonstrate that a commercial diagnostic imaging system can be used to apply standard cavitation detection techniques to expand the detection region for both inertial and stable cavitation.

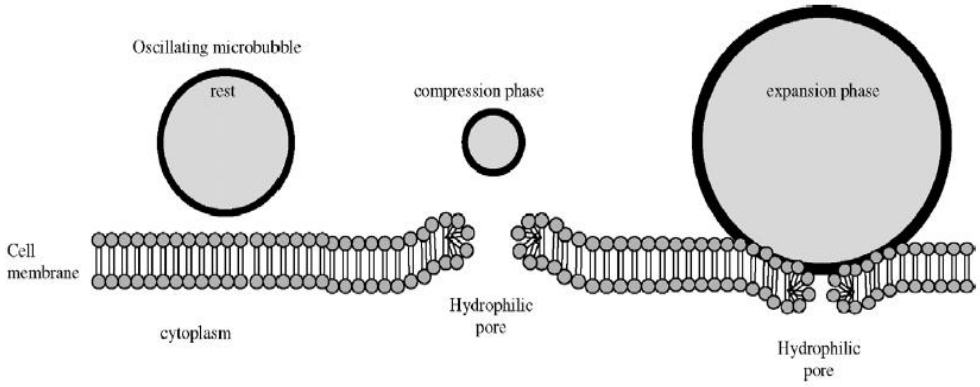

Fig 12. The pushing and pulling behavior of the microbubble causes rupture of the cell membrane creating a hydrophilic pore allowing trans-membrane flux of fluid and macromolecules. (van Wamel et al. 2006)

A big contradiction is found in the articles of Rahim et al. and Guzmán et al. Where Rahim claims that increasing bubble concentration does not affect sonoporation, Guzmán claims the opposite. Both used a similar range in bubble concentration, but Guzmán used a much greater range of pressure values. This seems to make the difference, because this parameter is especially important to the oscillation behavior of the bubbles. The bigger the pressure, the bigger the oscillation movements of the bubbles, and therefore will neighboring bubbles have much more effect on each other. Guzmán explains that neighboring bubbles influence each other negatively, because a neighboring bubble might absorb the energy of the other bubble, what decreases permeability efficiency. Also McDannold et al. claims that the microbubble concentration does not affect sonoporation, although using quite a big range in pressure intensities. This difference might be caused by the used range in bubble concentration. McDannold used only a small range, where Guzmán used a much bigger difference. Also the location of insonication, the blood brain barrier, may contribute to the different results.

It is known that acoustic cavitation is capable of inducing several bioeffecs in tissue. It is however not clear how these bioeffects actually induce enhanced permeabilization. Therefore the opinions and suggestions by different papers differ extensively. Studies that focus on the interaction between a single cell and microbubbles found that direct contact is needed and therefore the mechanical forces induce the effects. Tran et al. observed hyper polarization of the cell membrane after mechanical oscillation. What suggests that permebilization of the cell would not be induced by pore formation, this is based on the results in the paper of Deng et al. 2004. Deng showed that induced permeability, due to formation of pores in the membrane, results in depolarization of the cell membrane, opposite of hyper polarization. However, Tran did not prove there was enhanced permebilization, because they only measured the electrophysical properties of the cell membrane. Therefore more research, including drugs is needed.
Other studies make use of cell cultures or test in vivo, this makes it possible to investigate the effects of microbubbles on multiple cells. Different models have been proposed to explain the enhanced extravasation. Van Wamel et al. proposes a model of oscillating microbubbles enforcing pore formation in the cell membrane as shown in fig 12. The rupture of the cell membrane is most likely caused by microstreaming, induced by rapidly oscillating microbubbles (non-inertial cavitation). The cell membrane can withstand compression better than elongation and therefore it ruptures at compression phase. This theory is supported by Rahim et al. and Karshafian et al.
Guzmán proposes a model that describes the interactions of a bubble between multiple cells, this model is illustrated in figure 13. It gives a schematic illustration of the killed/permeabilized/unaffected cells from a bubble collapse. They introduce the terms blast radius R_b and killing radius R_k. According to the model shown in Fig. 13, cells within the blast radius (R_B) of a bubble are affected by that bubble and those within the killing radius (R_K) are rendered nonviable. Cells that are outside the blast radius are not affected. These radii are dependent on cell density.

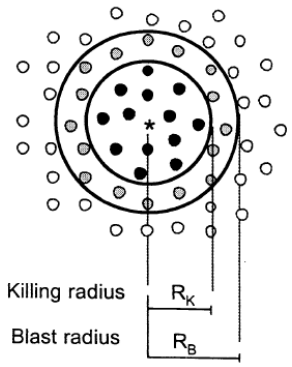

Fig 13. Conceptual illustration of length scales over which cavitation bubble bioeffects occur. Cells within the killing radius R_K are rendered nonviable (*black circles*). Cells located farther than R_K, but within the blast radius R_B, are reversibly permeabilized (*grey circles*). Cells outside the blast radius are considered unaffected (*white circles*). Bubble location is indicated with an asterisk. (Guzmán et al. 2003)

Summarized, it is proven by many groups that permeabilization of cell membranes is reversible and reproducible. However, with the use of higher intensities, cells may irreversibly be killed. In the field of drug delivery this is not wanted and a lower intensity should be chosen. The optimal parameters differ in each study, but most of the results lie around this measure: center frequency from 0,5 to 1 MHz, acoustic pressure from 0.25 to 0.8 MPa, insonication time around 10 s and the duty cycle needs to be low to prevent heating. These are the parameters determined the most important by the previous studies, also the ranges given are indicated by the previous studies only.
Gas or air filled nuclei are needed in order for cavitation to happen, but the effects caused by the bubbles are restricted to the cells in direct contact of them and therefore superficial. These effects rapidly vanish so the drug should be available at the time of insonication. The used drug should be attached/loaded to the microbubbles or encapsulated in micelles. This will lower the toxicity for the body, because the drug is released at target site and is not able to extravasate in high concentrations in healthy tissue. To determine if inertial or non-inertial cavitation causes the occurred bioeffects, a good detection system should be used, as proposed in the paper of Farny et al.

Acoustic Radiation Force

Next to the two most widely investigated ultrasound related mechanisms, hyperthermia and cavitation, a third mechanism occurs and produces biological effects in tissue, this mechanism is called acoustic radiation force.

The group of Lum et al. investigates the use of acoustic radiation forces with the help of drug loaded microbubbles at a concentration of 10^6 or 10^7 bubbles/ml. Fluorescent beads, with a size of 40 – 200 nm, are loaded on the shell of the bubble. A cellulose tube was prepared to function as a test vessel, blood flow was imitated with the microbubbles inside. They use a ultrasound pulse sequence to create radiation forces. An 1.3 s ultrasound pulse of 3 MHz and 150 kPa was used with a 1.5 MHz and 1.1 MPa fragmentation pulse. The results show that fluorescent beads not bound to a bubble are not influenced by ultrasound, what suggests that bigger particles are more susceptible for radiation forces. The effectiveness of radiation forces is dependent on bubble concentration, shear stress, bead size and insonication time. This is shown in fig. 9, the beads of 200 nm had a greater deposition than the 40 diameter beads. Also a higher concentration created a much higher deposit on the vessel wall. The shear stress elevation caused a lower deposit to almost nothing. Radiation force is able to direct bubbles to the wall of a vessel. They also found that the shell of the bubble stays attached to the fluorescent beads after sonication.

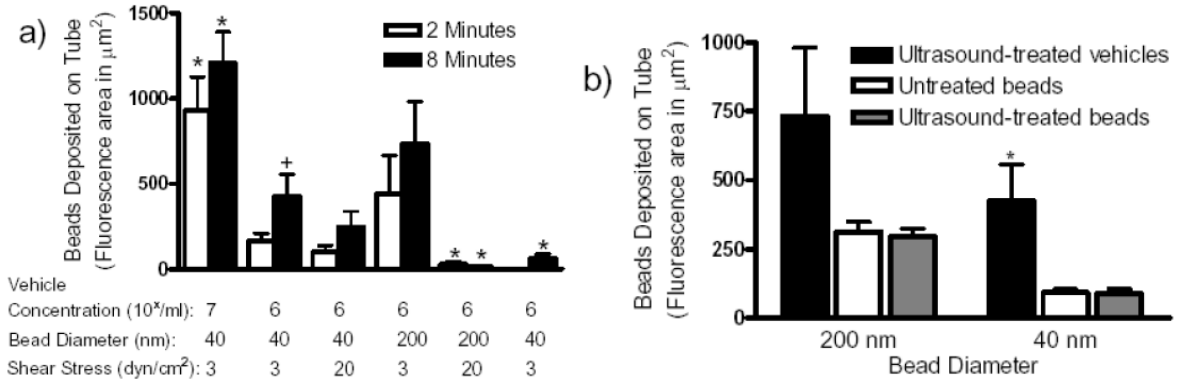

Fig 15. Quantification of the deposition of fluorescent nanobeads on the surface of a cellulose tube. **(a)** Data were collected with varied concentration, wall shear stress, and length of insonation. **(b)** The samples presented were sheared at 3 dyn/cm2 for at least 8 minutes with or without insonation. (Lum et al. 2006)

Frenkel et al. used a drug encapsulated in liposomes with a diameter of 100 nm at varying concentrations of 1, 2, 6, and 10 mg/kg. Fluorescent nanoparticles with the same diameter were used to measure the uptake. Ultrasound parameters of 1 MHz transducer, 1Hz repetition frequency and a duty cycle of 9% were used. The results show that an increase in drug dose administration, slows down the growth of the tumor. A temperature elevation with a mean of 5.1 ˚C was seen. No significant differences in growth rate were found between tumors that were treated with ultrasound and those that were not. Also no significant differences in drug concentration in the tissue were found for ultrasound treated and untreated tissue. However, the fluorescent nanoparticles were found extensively throughout the tissue after HIFU treatment, and not in untreated tissue, see figure 23. This is very strange, because both particles have the same diameter, the difference is not clear and only speculations towards tumor interstitial pressure and radiation forces have been raised.

The study of O'Neill et al. uses no carriers, but fluorescent nanoparticles with a size of 200 nm at a concentration of 10^{11} m/L. Without the use of carriers the free drug circulates through the complete blood stream and affects also healthy tissue. Also the induced interactions with ultrasound are dependent on the tissue properties that might be susceptible for ultrasound. Ultrasound parameters with an intensity of 1 MHz, 8.95 MPa, 1 Hz repetition frequency, 100 pulses and a duty cycle of 5% were used. In this study they investigated the presence of thermal influences with pulsed high intensity focused ultrasound (p-HIFU). To compare the ultrasound induced heating with a non-ultrasound source of heat, a lamp was used and induced heating up to 42 ˚C to exceed or match p-HIFU. Base temperatures of 34 and 37 ˚C were used and elevated to 39 or 42 ˚C respectively. The results show an increased uptake for both p-HIFU treatments compared to the control sample. The heating induced with the lamp did not show an significant increase, implying that the increased extravasation with p-HIFU is not induced by the temperature elevation. They also investigated the presence of cavitation events, by spectral analysis. From those results cavitation cannot be excluded as possible mechanism. They also show that the p-HIFU treated tissue is still able to extravasate particles up to 24 after insonication, which is longer than the effects of hyperthermia or cavitation. This suggests a third mechanism may play a role, acoustic radiation forces.

There is a big difference seen between the article of Lum et al. and the articles of Frenkel et al. and O'Neill et al, considered the goals of applying ultrasound exposures. Where Frenkel and O'Neill try to enhance extravasation from tumor vasculature, Lum wants do deposit microbubbles against vessel walls to obtain a much higher concentration inside of the vessel than can be achieved with regular blood flow.

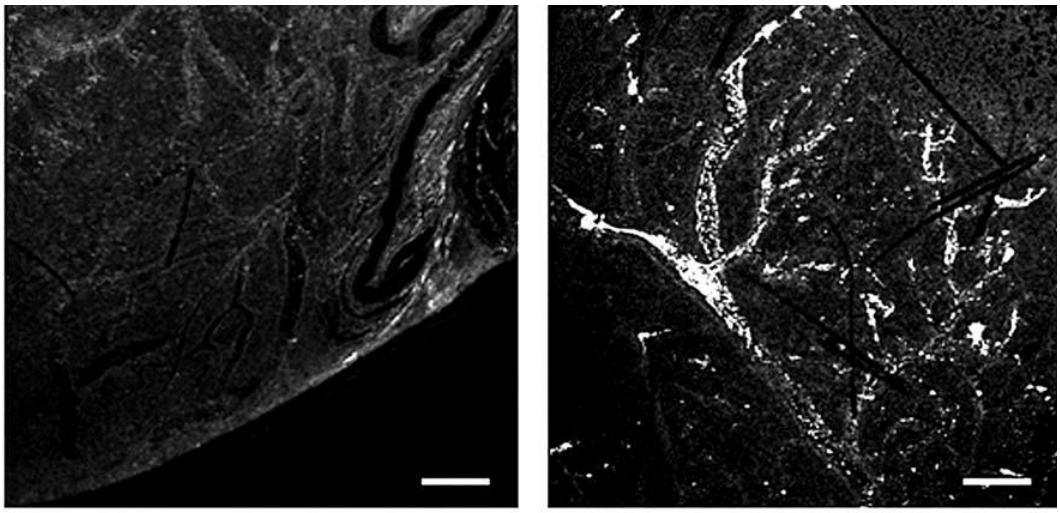

Fig 14. Three-dimensional reconstructions produced from laser scanning confocal micrographs. Whereas few fluorescent polystyrene nanoparticles were observed in control tumors **(a)**, they were found extensively in tumors treated with pulsed high-intensity focused ultrasound **(b)**. Bar = 200 µm. (Frenkel et al. 2006)

Different types of carriers are used and because of that these studies rely on different effects. Lum et al. uses the fact that these forces are able to displace present microbubbles towards a vessel wall, bigger particles are needed to be susceptible for the radiation forces. Frenkel and O'Neill make use of a high wall shear stress that creates ruptures, allowing bigger molecules to cross. They hypothese that radiation forces mechanically manipulate the tissue so that a vasodilating effect occurs. This action might release pressure on the intratumoral vessels and lower the interstitial fluid pressure. It also may have caused an improved transport of nanoparticles. It must be said that these explanations from Frenkel et al. are only hypothetical.

Lum et al. uses radiation forces to deposit microbubbles on vessel walls successfully. However, they do not investigate if this can be used to enhance extravasation of drugs through the vessel wall. In this study there is no evidence that these radiation forces may also be capable of enhancing permeability of the tissue. These factors should be investigated in the future. It might be possible to combine the exposures of Lum et al, to first locate the drug encapsulated in microbubbles very locally at a vessel wall, with a cavitation exposure to release the drug from the microbubbles. The drug is then released in high concentrations at target site, what benefits the extravasation from the vasculature, because of the small sizes of the drug particles. This is only a idea for the future, and more has to be known for it to be considered feasible. For example, there is nothing known about the period the microbubbles stay deposited at the vessel wall or the risks that may be accompanied with the microbubbles presence at the vessel wall.

To be able to use acoustic radiation forces efficiently, a lot of more research has to be done in this area. Little is known about the mechanism and the induced bioeffects. This is very important to be able to develop new strategies or improve the current.

DISCUSSION

Ultrasound uses several mechanisms to target drugs locally, namely by heat generation, acoustic cavitation and acoustic radiation forces. Although the use of microbubbles in combination with cavitation events seems very promising, hyperthermia and radiation forces should not be forgotten. Hyperthermia has been proven to be very effective with heat sources other than ultrasound. The drawback of these sources is that they are invasive or cannot generate a homogeneous distribution of heat (Ponce et al. 2007). Ultrasound might be capable to overcome these problems. However, it has been shown that heat induction with HIFU is probably not feasible, because of radiation forces that will have a big influence (O'Neill et al. 2009). Radiation forces are not only able to enhance permeability by altering the tissue properties (Frenkel et al. 2006), it has been shown that it can displace microbubbles towards the vessel wall, creating higher concentrations than can be achieved with regular blood flow (Lum et al. 2006).
At the moment a lot of research in targeting drugs is investigated with the use of ultrasound and many studies report promising results. However, critical questions need to be asked continuously.

A lot of groups investigate the targeting of drug into tumors. Tumors have their own characteristic barriers and are because of that reason especially complicated. These studies are often tested on cell cultures in vitro or in small tumors grown in animals, in this way however, a lot of distinctive features of the tumor might get lost. In these problems the size of the tumor plays a big role. As described before, in tumors the cell density is very high. Therefore the cells that are further away from a vessel are supplied with less nutrition's, oxygen and also drugs. When a tumor is relatively small (Kong et al. 2001) the cell density is less high and the drug is able to distribute more homogeneous. Also skin flap chambers (Ponce et al. 2007) are not a good imitation of a tumor as the tumor is completely squashed. There are only a small number of groups that investigate the effect of tumor size on the drug distribution and antitumor effects, this should be investigated more frequently in the future.

Next to that the concentrations of drug carriers are relatively high under the investigated conditions. Is it feasible to achieve such a high concentration of drug in the human body. It has been proved more than once that this concentration does influence the bioeffects and delivery of drug at target site, for example by Guzmán et al. 2003.
Furthermore, mainly the first two barriers are investigated in current studies. These two barriers are the homogeneous distribution in the blood pool at target area and the extravasation through the vasculature. It is however also very important to have a big enough penetration depth through interstitial space. In tumors this causes problems due to the high interstitial pressure, but also other types of tissue may cause problems, like the elastic lamina in arteries (Hallow et al. 2007). More research should be focused on the penetration depth of the drug with sonication.
A lot of research is done in the drug delivery into tumors. There are many different tumor types, from slightly leaky to very leaky for example. To test the protocol that is used in a study it might be very useful to test on different types of tumor in the same study. This will give more information about the distribution of the drug and the effects of ultrasound parameters.
In the end it is very important to find a unifying parameter to indicate the performance of the experiment. Such a parameter will support the results even better. A good parameter is the therapeutic ratio (TR) used in the article of Karshafian et al. This parameter gives information about the amount of killed cells, and viable cells.

As been stated before, ultrasound is a passive targeting drug delivery strategy. This offers several advantage, it has been shown to be non-invasive, can be focused locally, is able to reach deep inside the body and it does not need the development of new drugs (Frenkel et al. 2006). In contradiction to many active targeting drug delivery strategies, that make use of specific interactions at target site, including antigen–antibody and ligand–receptor binding (Vasir et al. 2005), these strategies require the development of new drugs for new targets. In the past decades the local targeting of passive strategies have been improved in such a way, they are not as underestimated anymore. These facts make ultrasound a powerful strategy to trigger local drug delivery.

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