Crucial factors and emerging concepts in ultrasound-triggered drug delivery.

Geers Bart[1], Dewitte Heleen[1], De Smedt Stefaan C.*[1], Lentacker Ine[1]

[1]Ghent Research Group on Nanomedicines, Laboratory of General Biochemistry and Physical Pharmacy, Faculty of Pharmaceutical Sciences, Ghent University, Harelbekestraat 72, B9000 Ghent, Belgium

*Corresponding author: Laboratory of General Biochemistry and Physical Pharmacy, Harelbekestraat 72, 9000 Ghent, Belgium, Tel: +32 9 264 80 78, Fax: +32 9 264 81 89

1 ABSTRACT

Time and space controlled drug delivery still remains a huge challenge in medicine. A novel approach that could offer a solution is ultrasound guided drug-delivery. “Ultrasonic drug delivery” is often based on the use of small gas bubbles (so-called microbubbles) that oscillate and cavitate upon exposure to ultrasound waves. Some microbubbles are FDA approved contrast agents for ultrasound imaging and are nowadays widely investigated as promising drug carriers. Indeed, it has been observed that upon exposure to ultrasound waves, microbubbles may (a) release the encapsulated drugs and (b) simultaneously change the structure of the cell membranes in contact with the microbubbles which may facilitate drug entrance into cells. This review aims to highlight (a) major factors known so far which affect ultrasonic drug delivery (like the structure of the microbubbles, acoustic settings, etc.) and (b) summarizes the recent preclinical progress in this field together with a number of promising new concepts and applications.

2 INTRODUCTION

Time and space controlled drug delivery remains a holy grail in medicine. It is the wish of every scientist or physician to design or have access to a device that only delivers therapeutic molecules at a certain target site (e.g. a tumor), leaving healthy tissue unharmed. To design such a "magic bullet", a concept that was first described by Ehrlich in the beginning of the 20th century, one needs to develop a drug carrier that responds to a stimulus applied by an external force or produced by the target tissue itself. Different external stimuli such as electromagnetic waves (IR, UV of visible light) or magnetic and electrochemical forces can be used to achieve such a trigger, as reviewed by *Timko et al* [1]. Another type of local stimulus can be generated through mechanical waves exerted by ultrasound.

Ultrasound waves are used in medical imaging (e.g. echography). An ultrasound image is formed due to the fact that ultrasound waves become reflected by tissues with a density different from the surrounding medium [2]. To enhance the contrast of the vasculature or other blood containing tissues that do not reflect ultrasound due to their compressibility, ultrasound contrast agents (UCAs) can be intravenously injected. UCAs are micron sized (1-10µm) gas bubbles (microbubbles) with a shell composed out of phospholipids, polymers or proteins. These microbubbles are suitable contrast agents because of their interaction with the ultrasound wave [3]. A microbubble will start to oscillate in response to the exerted cycles of negative and positive pressure. These oscillations itself produce ultrasonic signals that can be detected by the ultrasound transducer. Increased pressure will compress the microbubbles while negative pressure will induce rarefaction of the bubbles. This process,

called cavitation, has been investigated in detail by *Bouakaz, Versluis and de Jong [4]* using high speed light microscopy .

Cavitation at higher acoustical pressures will induce more violent microbubble oscillations, eventually resulting in microbubble destruction (so named 'inertial cavitation') [5]. Inertial cavitation can be useful in imaging, this to evaluate blood flow abnormalities by means of destruction replenishment imaging [6], but is particularly useful in drug delivery as it can trigger (a) release of drugs from the microbubbles and (b) uptake of the released drugs into the cells whose membranes become temporarily permeablized due to the localized mechanical effects related to microbubble implosion [7]. Since ultrasound is only applied at a certain location, time- and space-controlled drug delivery may become feasible.

3 ADVANCES IN MICROBUBBLE DESIGN FOR ULTRASOUND GUIDED DRUG DELIVERY

As schematically represented in Figure 1, four types of 'microbubble modficiations' have been reported so far for ultrasonic drug delivery: (a) drug-loaded microbubbles; (b) *in situ* formed microbubbles or nanodroplets; (c) acoustically active liposomes (sometimes called 'nanobubbles') and (d) targeted microbubbles.

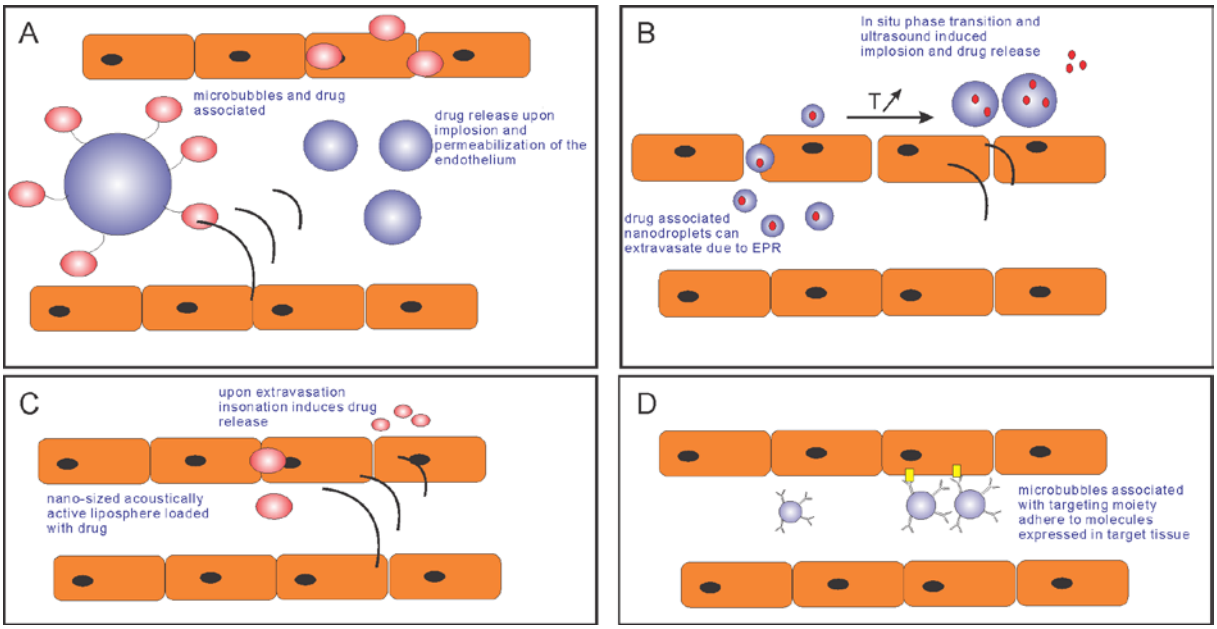

Figure 1: Schematic overview of microbubble modficications reported for ultrasonic drug delivery. **A)** envisions drug-loaded microbubbles releasing their associated payload upon insonation, **B)** shows nanodroplets that are able to extravasate due to Enhanced Permeation and Retention (EPR) and form microbubbles after phase transition in situ, **C)** depicts nano-sized acoustically active lipospheres in tumor tissue and finally **D)** shows microbubbles associated with a targeting moiety that adhere to target molecules in tissue expressing pathophysiologic epitopes.

3.1 Drug-loaded microbubbles

Since the 1990's a number of research groups have attempted to design microbubbles which can carry a therapeutic payload. As Figure 2 summarizes, drug delivery from microbubbles by ultrasound is an attractive concept for various reasons; (a) using low acoustic pressures the drug loaded microbubbles can be visualized, being attractive for 'image guided drug delivery'; (b) many drugs, especially biological drugs like nucleic acids and proteins need to be protected from degradation upon administration, which can be accomplished by formulating them associated with microbubbles; (c) the loading of the drugs into

microbubbles can also prevent their uptake in untreated tissue (i.e. tissue that is not exposed to ultrasound) and thus reduce side-effects; (d) upon applying ultrasound, both local drug release and cell membrane permeabilization (sonoporation) can occur.

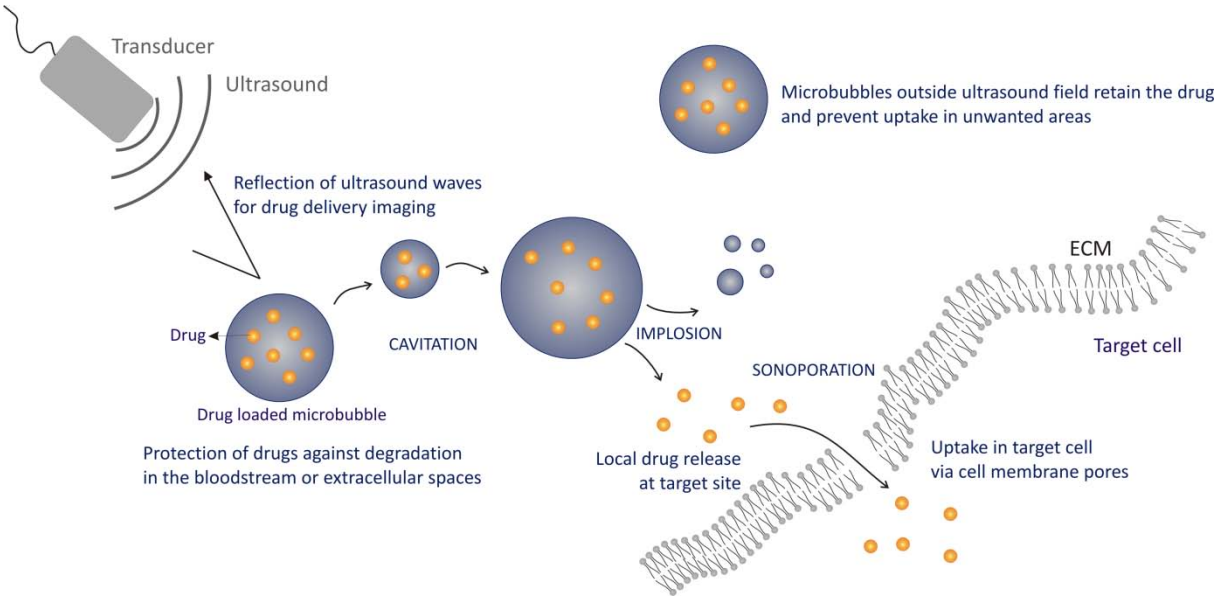

Figure 2: Schematic overview of the wide potential of drug-loaded microbubbles.

A straightforward strategy to load the microbubbles with drugs is associating them with the shell or more particularly with its building blocks. Another way of loading is by encapsulating the drug into an oil reservoir present in the core of the microbubble. Finally, drugs can also be packed into nanoparticles that are subsequently attached to the microbubble's surface. The following section gives an update on the recent progress in the design of drug-loaded microbubbles . For a complete overview on drug-loaded microbubbles we refer to *lentacker et al.* and *Tinkov et al.* [8,9].

3.1.1 Loading through electrostatic binding.

A first strategy to attach therapeutics to the microbubble shell is through electrostatic interactions, initially explored to load microbubbles with pDNA. As an example, *Frenkel et al* [10] used albumin-shelled microbubbles as a template for pDNA attachment. A layer-by-

layer approach can be used to alternately deposit cationic polymers and anionic nucleic acids on the microbubble shell, which clearly improves the electrostatic loading of the microbubbles with nucleic acids [11]. *Sirsi et al* [12] recently reported on lipid-shelled microbubbles that were covalently coated with PolyEthyleneGlycol-PolyEthyleneImine (PEG-PEI) copolymers, followed by the electrostatic deposition of pDNA onto such cationically charged microbubbles. Ultrasound induced gene expression in tumor tissue was clearly observed, being significantly higher than the gene expression in control samples being untreated tumors. Similar results were obtained with plasmid conjugated microbubbles, that showed a significant increase in gene transfection in smooth muscle cells as well [13].

Tinkov et al. [14] described an attractive method to complex Doxorubicin (DOX) onto microbubbles using electrostatic interactions. They prepared an anionic microbubble by incorporating an anionic phospholipid 1,2-dipalmitoyl-sn-glycero-3-phospho-1'-rac-glycerol (DPPG) in the shell which can form a complex with the cationic glycane-group in the DOX molecule. This method showed efficient incorporation of DOX into the microbubbles' shell (up to 40 µg DOX per ml microbubble dispersion); tumor cell destruction was obtained after injection of these bubbles in rats bearing pancreatic tumors and exposure of the tumor to ultrasound. The same strategy was recently used by *Ting et al.* [15] to prepare 1,3-bis(chloroethyl)-1-nitrosurea (BCNU) loaded microbubbles. Focused ultrasound was used to locally implode BCNU carrying microbubbles at the blood brain barrier (BBB) of rats. This resulted in a significantly higher uptake of BCNU in glioma tumors implanted in the rats and a slower tumor progression when compared to BBB disruption with microbubbles and ultrasound co-administered with BCNU.

A serious drawback of electrostatic drug loading of microbubbles could be premature release of the drug in the body. Indeed, once injected in the bloodstream charged blood components like serum albumin can compete or interact with the charged microbubble shell. This can result in a release of the attached drug or in the formation of large aggregates which can block the vasculature [16]. *Sirsi and colleagues* also showed that charged microbubbles can influence circulation times by adhering nonspecifically to the vasculature close to the injection place. This can have a significant impact on the amount of microbubbles reaching the target tissue.

3.1.2 Drug reservoirs.

As an alternative, some research groups have tried to create a drug reservoir inside the microbubble. One example is the use of double emulsion techniques to obtain polymer coated oil–filled microcapsules [17]. Another technique, as described by *Tartis et al* [18], involves the incorporation of a drug-containing oil-phase within lipid-coated microbubbles. These oil filled microbubbles retain their responsiveness to ultrasound and can be destructed at higher ultrasound intensities thereby releasing their content. The fact that only lipid-soluble drugs can be incorporated however, limits the use of such systems.

Another, more versatile method to prepare drug-loaded microbubbles, is the attachment of multiple drug reservoirs (i.e. drug loaded nanoparticles) to the microbubbles' surface. The major benefit of this concept is that it creates a higher drug loading capacity, as plenty of small drug-filled pods are attached to the surface. Another advantage is that different types of therapeutics can become stored in microbubbles. Liposomes, for example, can be loaded with both hydrophilic and hydrophobic drugs and can carry larger molecules like pDNA, siRNA or mRNA [19]. Biotinylated lipid coated bubbles can be easily prepared by introducing

a phospholipid containing a PEG-biotin group into the microbubble shell. Such biotin containing bubbles can be incubated with avidin, enabling the subsequent attachment of biotinylated (drug containing) nanoparticles. Our group showed that this concept can be used to enhance the uptake and therapeutic efficiency of both small (DOX) [20] and high molecular weight drugs (pDNA, siRNA, mRNA) [21-23].

Although avidin-biotin binding is an easy and straightforward method to obtain nanoparticle decorated microbubbles, it limits the *in vivo* application of nanoparticle loaded microbubbles due to the immunogenic nature of the avidin molecule [24]. Furthermore, the loading procedure requires several washing steps that influence microbubble stability and inter-batch reproducibility. This stimulated us to design "self-assembling liposome loaded microbubbles" [25]. In this concept, maleimide functionalized liposomes (containing DOX) are mixed with a lipid solution containing dipalmitoylphosphatidylcholine (DPPC) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[PDP(polyethylene glycol) (DSPE-PEG-SPDP) and covered with perfluorobutane gas. As shown in Figure 3 shaking of these vials in a high-speed mixing device (Capmix^{TM}) spontaneously results in the formation of self-assembled liposome loaded microbubbles. Note that in this way the liposomes become covalently bound to the microbubbles, in opposite to other methods that enable the attachment of nanoparticles like electrostatic loading or avidin-biotin approaches.

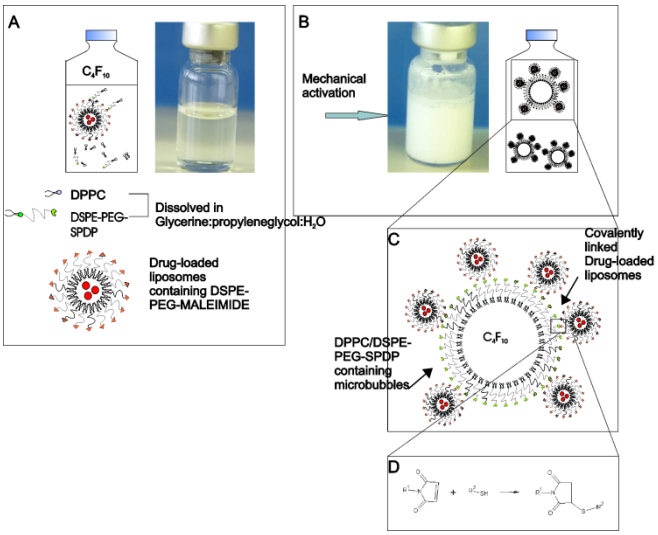

Figure 3: Schematic depiction of the production procedure of self-assembled liposome-loaded microbubbles. Reprinted with permission from Elsevier (*Geers et al. 2011a*) [25].

An important question when using drug-loaded microbubbles remains whether a sufficient amount of drug can become incorporated in the microbubbles. As recently reviewed by *Barenholz* [26], the first clinical trials with liposomal DOX in humans reported a dosing between 25 and 50 mg/m^2 [27], this corresponds with approximately 0.5-1 mg/kg. This implies that a dose of 40-80 mg should be administred to a normal 75 kg patient. One can wonder whether it is feasible to administer this dose to a patient when the drug is formulated as a microbubble dispsersion. According to our calculations (*Geers et al. 2011*a [25], *lentacker et al. 2010 [20]*) and the work performed by *Tinkov et al [14,28]* between 10 and 40 µg of DOX can be loaded in 1 ml of bubble dispersion which allows us to suggest that liters of a bubble dispersion should be infused, which is practically impossible. Note that in ultrasound imaging with diagnostic bubbles, one is allowed to inject maximal 1 ml of bubble dispersion [2,29,30] per treatment. One can expect however, that using the self-assembled microbubbles the amount of bubbles needed for efficient treatment would be reduced. Indeed, we have shown that ultrasound assisted drug delivery can enhance the efficacy of

DOX, at least *in vitro* (Figure 4). We observed a stronger cytotoxicity of DOX, even at doses that did not show toxicity in the free form (which shows more cytotoxicity than liposomal DOX). Given the stronger effect of liposomal DOX loaded on microbubbles upon insonation, one could envisage that lower doses of DOX can be administered to obtain the same effect and thus a lower dispersion volume is needed.

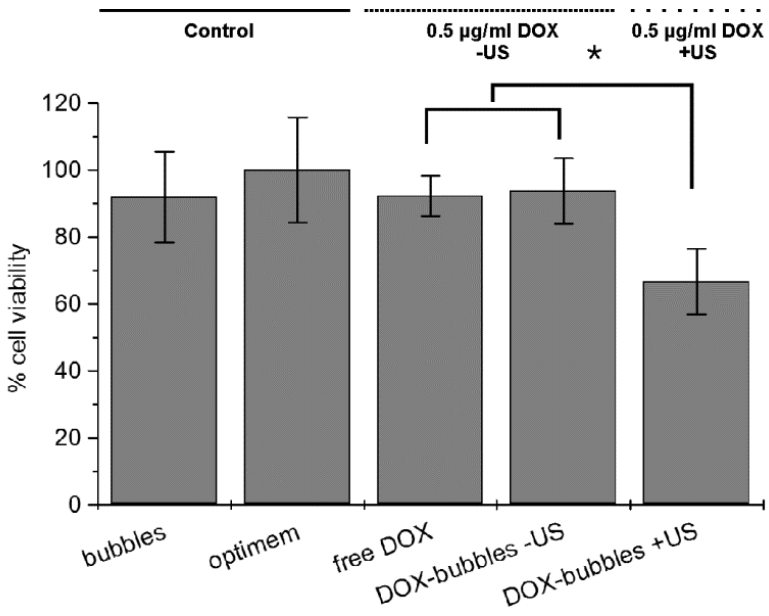

Figure 4: Cell viability experiments showing an increased cell killing efficiency of DOX when the drug was encapsulated in liposomes and bound to microbubbles, followed by ultrasound treatment. Reprinted with permission of Elsevier (*Geers et al 2011a*) [25].

We would like to note however that this issue is relevant only for small molecules. In the case of genetic drugs (like siRNA, pDNA and mRNA) the efficient delivery of a few strands should be enough to obtain sufficient therapeutic response.

3.2 Ultrasound responsive liposomes (nanobubbles).

A major disadvantage of microbubbles as drug carriers is their relatively large size (1-6 µm). Due to this feature, microbubbles have a rather short half-life, i.e. in the order of minutes

[19,31]. Upon injection such microbubbles will circulate a few times, but will inevitably get stuck in the lungs where gas exchange occurs. Consequently, microbubble-ultrasound triggered drug delivery will be mainly restricted to cardiovascular targets and to tumor endothelia.

To solve this problem, several papers report on so-called nanobubbles [29,30], also named 'bubble liposomes' [32], which are smaller than 1 μm, combining the benefits of a liposome (small size, long circulation time) with ultrasound responsiveness. These small bubbles are generally prepared by sonicating liposomes in the presence of fluorinated gases. With these nanobubbles successful delivery of pDNA, siRNA and coumarin [33] has been demonstrated both in *in vitro* and *in vivo* models.

3.3 In situ generation of microbubbles from nanodroplets

A very intelligent suggestion for circumventing the short half-life of drug-loaded microbubbles is the design of nanoscopic droplets based on perfluorocarbons with a relatively low boiling point (e.g. perfluoropentane or perfluorohexane). These so-called 'nanodroplets' can convert into their gaseous form upon ultrasound exposure. The advantage of the use of liquid perfluorocarbons is that they can be emulsified in water when stabilized by an appropriate surfactant (e.g. pluronic, lipids) [34-36]. Such nanodroplets are typically smaller than 200 nm, which allows them to extravasate from the leaky tumor vasculature [37]. When the tumor tissue is subsequently treated with ultrasound, a liquid to gas phase transition occurs due to a local temperature increase in combination with the low pressure phase generated by the ultrasound [38]. As a consequence, ultrasound responsive microbubbles are formed in situ.

Alternatively, perfluorocarbons with a low boiling point can be encapsulated in inorganic mesoporous silica-nanoparticles which can incorporate various types of drugs as well [39][40]. Perfluorocarbon-drug-loaded silica-nanoparticles may well provide a solution for the different challenges we are facing with regard to drug-loaded ultrasound contrast agents, namely sufficient extravasation in tissues and high loading of (multiple) compounds.

3.4. Targeted microbubbles for drug delivery.

Recently there is growing interest in the use of 'targeted microbubbles' for diagnostic molecular imaging. Such microbubbles should be able to interact with molecules that become expressed in specific pathologies. In this case antibodies [41] or even nanobodies [42] are coupled to the surface of the bubbles, typically through avidin-biotin coupling [43]. Aptamers, nucleic acids that show affinity for specific molecules, can be used as targeting moieties as well [44]. Aptamer-loaded nanobubbles [45] have been described and show potential for targeting specific cell types. Clearly, targeted microbubbles can be of interest for drug-delivery as well as the amount of drug closely located near the target tissue may become enhanced [46].

4 THERAPEUTIC MOIETIES BENEFITING FROM ULTRASOUND TRIGGERED DELIVERY

The therapeutic moieties reported in the context of ultrasonic delivery can be divided in three different groups (Figure 5): (a) low molecular weight drugs like some anticancer drugs, (b) large biomolecules like genetic drugs and proteins and, finally, (c) drugs encapsulated in

nanoparticles.

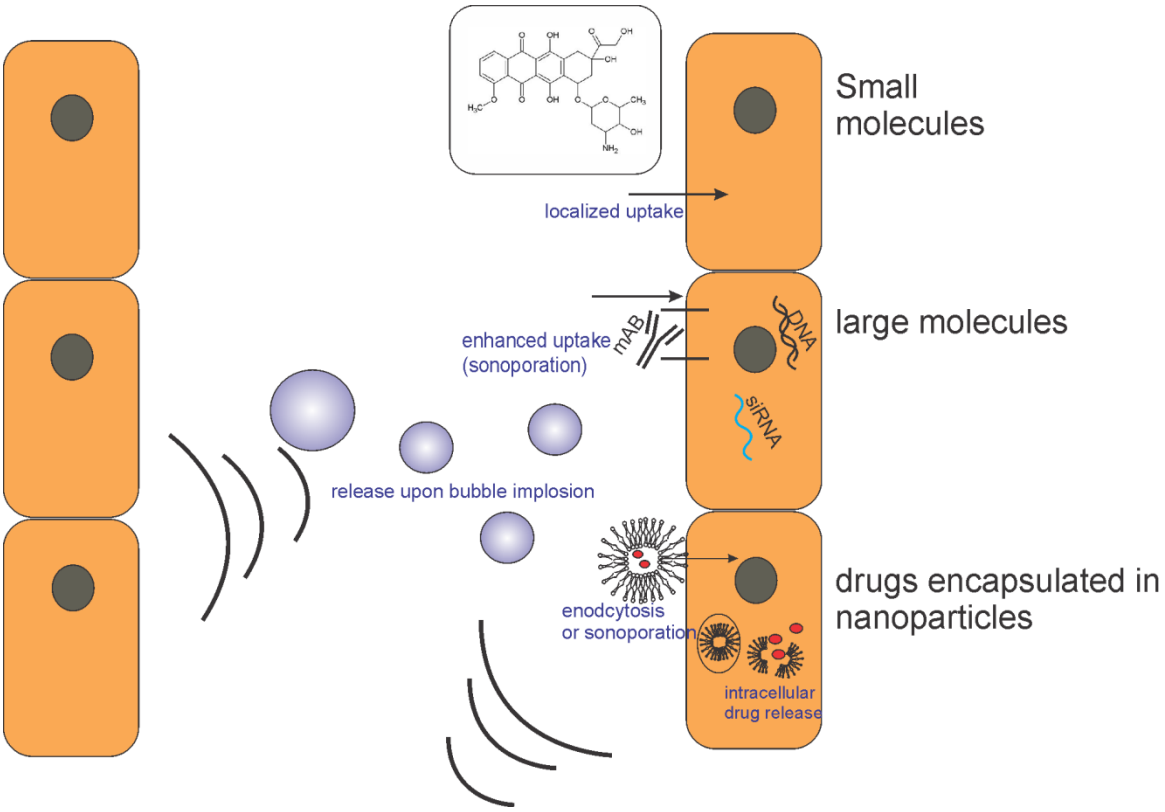

Figure 5: Schematic representation of different therapeutic moieties which can be delivered using microbubbles and ultrasound.

Depending on its characteristics a drug can benefit from ultrasound triggered drug delivery differently, as ultrasound and microbubbles will either enhance the uptake of a molecule or particle that shows limited uptake or it will localize their bioavailablity .

4.1 Small therapeutic molecules

The “small therpautics” used in ultrasonic drug delivery studies are mostly antineoplastic drugs like DOX or Paclitaxel. Upon injection such low molecular weight drugs will distribute throughout the body and easily accumulate in different cell types, causing cytotoxic (side) effects. The most important reason why these drugs would benefit from ultrasonic drug

delivery is the fact that drug uptake would become limited to the ultrasound treated tissue. A more efficient localized delivery to the tumor can substantially reduce the required dose and lower side effects.

4.2 Large therapeutic molecules

Large molecules like nucleic acids (pDNA, siRNA, mRNA) and proteins are under investigation for ultrasonic delivery as well. Unlike small molecules these macromolecules show inefficient uptake in target tissues. As Figure 5 envisions, ultrasound mediated microbubble destruction can be used to locally permeablize cell membranes which should enhance the uptake of large molecules.

The most important studies report on the delivery of genes into cells, *in vitro* and/or *in vivo*. Most studies involve pDNA, associated with a microbubble [47] or co-administred [48,49] with them. Due to its negative electrostatic loading pDNA will not penetrate into cells, but if it is located near a microbubble imploding in the vicinity of a cell it may profit from the temporal permeablization of the cell membrane (sonoporation) [50].

4.3 Drugs encapsulated in nanoparticles

Not only single molecules can benefit from ultrasonic delivery. Several publications have shown that microbubbles and ultrasound can be used to improve the extravasation of a variety of nanoparticles that can be loaded with a drug or have a therapeutic effect themselves. PLGA nanoparticles [51], magnetic nanoparticles [52], liposomes and lipoplexes [53], gold nanoparticles and silica nanoparticles [54] were used in combination with microbubbles and ultrasound. Through encapsulating the drug molecules in nanoscopic particles they become well protected against degradation, which is a major challenge, especially for biological drugs.

5 INFLUENCE OF ULTRASOUND PARAMETERS ON DRUG-DELIVERY EFFICIENCY

As schematically shown in Figure 6, an ultrasound wave basically has 3 characteristics that may play a role in ultrasonic drug delivery: (a) the number of cycles per ultrasound pulse, (b) the peak negative pressure and (c) the frequency. In most ultrasonic drug delivery related reports ultrasound waves with a frequency of around 1 Mhz are used, the major reason being that the frequency which allows the microbubbles to respond upon ultrasound exposure indeed depends on the size of the bubbles which, in most studies, is between 1 and 3 µm.

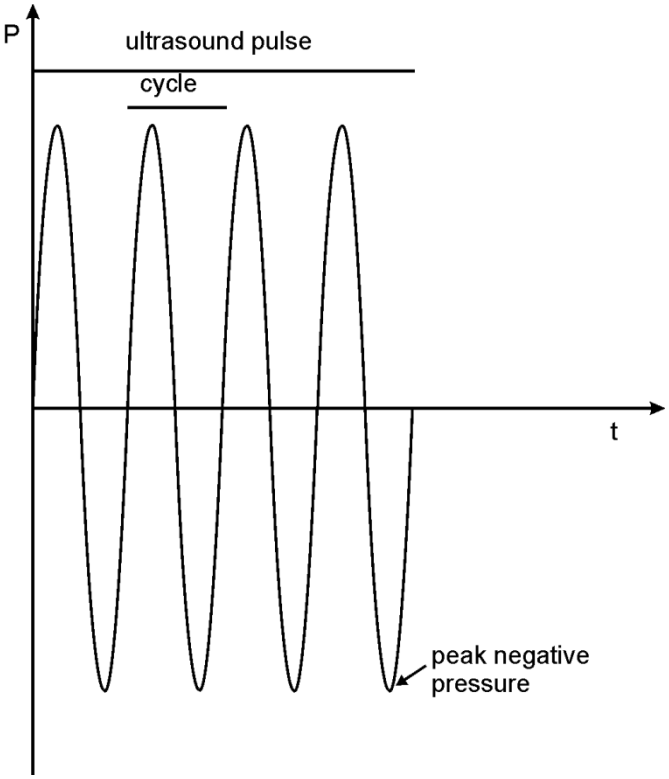

Figure 6: Schematic representation of the ultrasound pulse, cycle and peak negative pressure of an ultrasound wave.

In contrast to frequency, the variety of ultrasound pressures used in different studies is striking. At higher acoustic pressures (above a threshold of approximately 500 kPa [55]), the microbubbles will highly likely show inertial cavitation. This may result in the formation of shock waves and micro-jets [56,57]. Different studies report that such effects may porate cell membranes [55,58-60] and may facilitate the delivery of nanoparticles into the cytoplasm of cells [22,61]. In other studies lower acoustic pressures are used. Under these conditions, the bubble will "gently" oscillate and disturb its surroundings. If a microbubble is located near the cell membrane, these gentle oscillations may induce cell membrane instabilities which may stimulate endocytosis [55,62].

Another important variable if one compares ultrasound settings used in different studies, is the number of acoustic cycles (i.e. the number of acoustic oscillations per ultrasound pulse) applied to the samples. As shown by *Mannaris et al* [63], bubbles can oscillate at lower pressures when up to 100 cycles are used. However, when more cycles are applied combined with higher pressures, the bubbles become instantaneously destroyed. Subsequently, under such conditions one does no longer study microbubble related effects on drug delivery but rather effects from ultrasound forces. These forces may play an important role in *in vivo* drug and gene delivery, although this still has not been investigated in detail. Studies on ultrasonic drug delivery were indeed performed, reporting the use of 10000 cycles and high acoustic pressure [64]. This study clearly shows these settings do have an effect on drug delivery and the effects generated will not be caused by imploding microbubbles.

6 PRECLINICAL EVIDENCE

Searching for *in vivo* evidence for ultrasonic drug delivery with microbubbles, a difference is observed between the number of studies reporting the co-administration approach (i.e. the co-administration of drugs and microbubbles), and the number of reports in which drug-loaded microbubbles are studied.

There is clear preclinical evidence available in the literature with regard to the co-administration approach. Many studies, as e.g. performed by *Hynynen, McDannold* and co-workers, show enhanced drug delivery after injection of diagnostic microbubbles (Sonovue® or Definity®) and applying ultrasound allowing bubbles to implode at the target site. For example, enhanced drug [65] or Magnetic Resonance (MR) contrast-agent [66] delivery into the brain of rats has been shown. In these brain delivery studies, a transient disruption of the Blood Brain Barrier (BBB), induced by microbubble implosion has been reported. An enhanced delivery of Evans blue in muscles [67] has been shown as well following the co-administration approach. The progress in this particular field has been reviewed by *Vykhodtseva et al* [68].

The number of *in vivo* studies with drug loaded microbubbles is rather limited, is probably due to the fact that 'loading microbubbels with drugs' is a recent strategy in ultrasonic drug delivery. Indeed, while in co-administration approved commercial (clinically used) microbubbles can be used, custom-made (still often poorly characterized) drug loaded microbubbles need to be designed. *Rapoport et al* showed reduced tumor growth with Paclitaxel-loaded nanodroplets [69]. *Tinkov* [28] showed enhanced DOX-uptake in tumors with DOX-loaded microbubbles. Accoustically active pDNA bubble liposomes resulted in enhanced gene transfection in the mouse abdomen [70]. Finally, *Müller et al* [71] reported

an improved gene transduction in the heart of rats using microbubbles with adeno-associated viral vectors electrostatically attached to the surface of the bubbles.

7 EMERGING CONCEPTS AND APPLICATIONS

7.1 Theranostics

An elegant new approach would be the use of drug-loaded microbubbles as a theranostic tool. Theranostics is an emerging field that focuses on the combination of drug therapy and diagnosis via medical imaging techniques (e.g.: MRI, ultrasound) [72,73]. It involves the use of agents that are able (a) to visualize a specific pathological process and (b) simultaneously deliver a drug at this site. *Kiessling* et al[74] reported recently on the current status of different preclinical and clinical theranostic applications. It would be a breakthrough if one could design a drug loaded microbubble which specifically detects pathophysiological processes and which delivers its drug at the site where a diagnostic signal is observed. As shown above, various types of antibody loaded microbubbles have been described, however, so far targeted drug loaded microbubbles have not been reported.

Note that microbubbles can be used as contrast agents in Magnetic Resonance Imaging (MRI) as well, as they can be loaded with FeO_2 [75] or other magnetic nanoparticles providing a magnetic contrast in MRI. Intrinsically microbubbles can be visualized via 19F-MRI as well [76].

7.2 Temporal window upon sonoporation and two-step delivery protocols.

Several research groups have demonstrated the existence of cell membrane pores upon applying ultrasound lasting in the order of seconds to minutes [50,60,77,78]. However, Yudina and colleagues recently claimed pore opening lasting up to 24h [79]. This was

evidenced by evaluating the uptake of the small molecule Sytox® Green as a function of time in sonoporated glioma cells. Although a further confirmation of the observations of *Yudina et al* would be useful, these findings open up new perspectives for ultrasonic drug delivery .

The same group also proposed a two-step delivery protocol combining the benefits of temperature sensitive liposomes (being liposomes that release their content at temperatures around 41°C) and sonoporation [80]. A proof of concept paper was published using TO-PRO-3 loaded thermosensitive liposomes and diagnostic microbubbles. Upon heating by high intensity focused ultrasound (HIFU), TO-PRO-3 became released from the temperature sensitive liposomes while the membrane permeabilization promoted the uptake of TO-PRO-3 in the cancer cells. Based upon their findings that drug uptake can last for several hours after sonoporation, the authors also suggested that it could be even more advantageous first to sonoporate the tissue (taking advantage of the temporal window), followed by different applications of the nanoparticles.

7.3 Stem cell therapy

There are indications that stem cell therapy can be used to repair infarcted cardiac tissue and even improve cardiac function. However, current clinical studies are not convincing as only a small fraction of the injected stem cells is able to reach the ischemic heart region [81]. Recently, several research groups have shown that a combination of microbubbles and ultrasound [82-84] or focused ultrasound only [48] can be applied to enhance the migration or extravasation of stem cells. This is particularly interesting to enhance the homing of mesenchymal stem cells to ischemic heart tissue. An important consequence of the microbubble implosions is the creation of a local inflammation response and an enhanced expression of inflammatory cytokines (e.g. SDF-1) and adhesion receptors (VCAM-1). It has

been demonstrated that SDF-1 is a crucial factor to mediate the homing of stem cells to myocardial tissue [85] and that VCAM-1 is involved in the adhesion of stem cells to the damaged endothelium. These results indicate that a pre-treatment of the ischemic tissue with imploding microbubbles could be a very promising strategy to improve the outcome of stem cell therapy for myocardial infarctions.

7.4 Cancer vaccination

Another interesting emerging application is the use of microbubbles and ultrasound for cancer vaccination. Cancer vaccination strategies nowadays relies upon the *in vitro* manipulation of antigen-presenting cells (APC) like dendritic cells (DCs). APCs are able to capture and process antigens and present them to CD4+ or CD8+ T-cells. Activated T-cells are responsible for cellular immunity and can be used to eliminate cancerous cell lines before they can do any harm. In 2009, Un and colleagues [86] were the first to use "bubble liposomes" and ultrasound to pulse DCs with the model-antigen ovalbumin.

Recently, our group showed that nanoparticle loaded microbubbles (mRNA-lipoplex loaded microbubbles) can be used to transfect DC's as well, resulting in a significant expression of reporter genes. Sonoporation of mouse DCs leads to a slight shift in maturation status of DCs which could be interesting to obtain an efficient T-cell response. The concept of sonoporation based vaccination is shown in Figure 7.

The idea of microbubble and ultrasound induced vaccination is attractive as it has been shown that vaccination in the lymph nodes (intranodal vaccination) leads to a stronger immune response [87]. Sonoporation could be a valuable alternative to ex vivo electroporation as it could enable the direct intranodal transfection of DCs *in vivo,* thereby circumventing the expensive and time-consuming procedure of ex vivo transfection. In this

regard it has been shown that microbubbles are able to migrate to the lymph nodes after subcutaneous injection, a technique which is currently under investigation for sentinel lymph node detection [88].

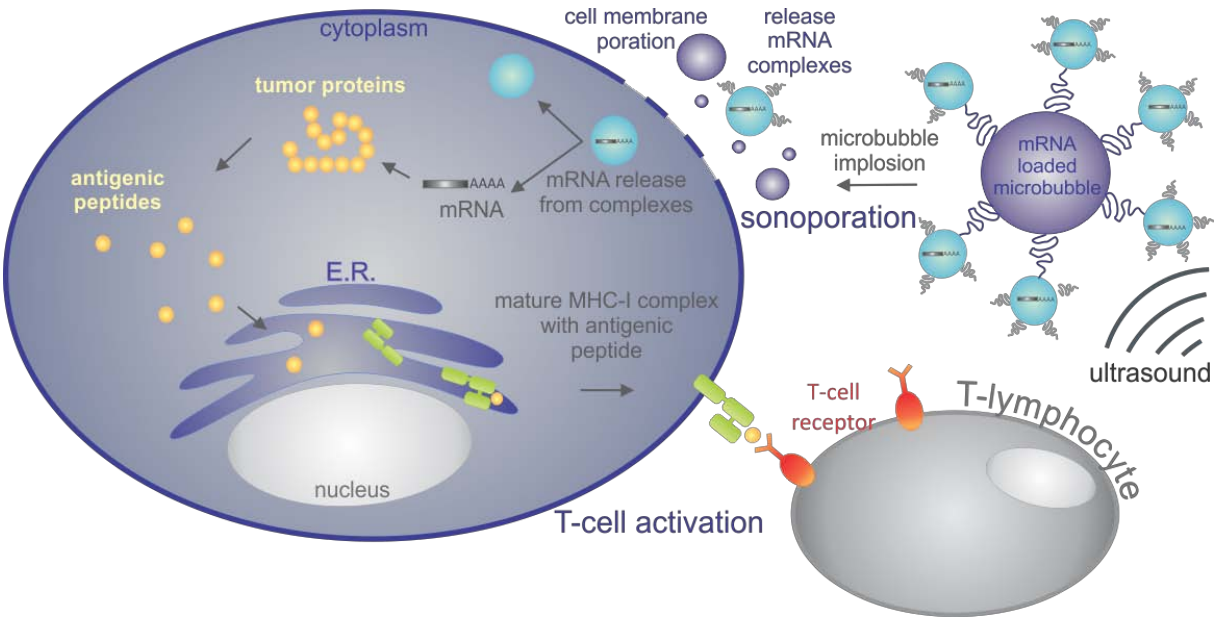

Figure 7: Schematic representation of the concept of ultrasound and microbubble enhanced cancer vaccination. Reprinted with permission from Elsevier (*De Temmerman et al* [21]).

8 PERSPECTIVES

Generally, we can conclude that ultrasonic drug delivery becomes an increasingly attractive technique in medicine, as reflected by the growing number of research groups being active in this field.

We need to emphasize, however, that the full potential of this technique is not yet met: (a) microbubble design can be further explored and perfected for new emerging applications like vaccination or theranostics; (b) there is no concensus on the exact ultrasound settings to be used in drug delivery and (c) new insights in sonoporation and cellular mechanisms (like

the temporal window and two-steps delivery) need to be further explored to allow optimal use of the biological implications of ultrasonic drug delivery.

9 ACKNOWLEDGMENTS

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10 REFERENCE LIST

[1] B.P. Timko, T. Dvir, and D.S. Kohane, Remotely triggerable drug delivery systems, Adv. Mater., 22 (2010) 4925-4943.

[2] S.L. Mulvagh, A.N. DeMaria, S.B. Feinstein, P.N. Burns, S. Kaul, J.G. Miller, M. Monaghan, T.R. Porter, L.J. Shaw, and F.S. Villanueva, Contrast echocardiography: current and future applications, J. Am. Soc. Echocardiogr., 13 (2000) 331-342.

[3] S. Qin, C.F. Caskey, and K.W. Ferrara, Ultrasound contrast microbubbles in imaging and therapy: physical principles and engineering, Phys. Med. Biol., 54 (2009) R27-R57.

[4] A. Bouakaz, M. Versluis, and J.N. de, High-speed optical observations of contrast agent destruction, Ultrasound Med. Biol., 31 (2005) 391-399.

[5] J. Wu and W.L. Nyborg, Ultrasound, cavitation bubbles and their interaction with cells, Adv. Drug Deliv. Rev., 60 (2008) 1103-1116.

[6] E. Quaia, Assessment of tissue perfusion by contrast-enhanced ultrasound, Eur. Radiol., 21 (2011) 604-615.

[7] Y. Qiu, Y. Luo, Y. Zhang, W. Cui, D. Zhang, J. Wu, J. Zhang, and J. Tu, The correlation between acoustic cavitation and sonoporation involved in ultrasound-mediated DNA transfection with polyethylenimine (PEI) in vitro, J. Control Release, 145 (2010) 40-48.

[8] I. Lentacker, S.C. De Smedt, and N.N. Sanders, Drug loaded microbubble design for ultrasound triggered delivery, Soft Matter, 5 (2009) 2161-2170.

[9] S. Tinkov, R. Bekeredjian, G. Winter, and C. Coester, Microbubbles as ultrasound triggered drug carriers, J. Pharm. Sci., 98 (2009) 1935-1961.

[10] P.A. Frenkel, S. Chen, T. Thai, R.V. Shohet, and P.A. Grayburn, DNA-loaded albumin microbubbles enhance ultrasound-mediated transfection in vitro, Ultrasound Med. Biol., 28 (2002) 817-822.

[11] M.A. Borden, C.F. Caskey, E. Little, R.J. Gillies, and K.W. Ferrara, DNA and polylysine adsorption and multilayer construction onto cationic lipid-coated microbubbles, Langmuir, 23 (2007) 9401-9408.

[12] S.R. Sirsi, S.L. Hernandez, L. Zielinski, H. Blomback, A. Koubaa, M. Synder, S. Homma, J.J. Kandel, D.J. Yamashiro, and M.A. Borden, Polyplex-microbubble hybrids for ultrasound-guided plasmid DNA delivery to solid tumors, J. Control Release, 157 (2012) 224-234.

[13] L.C. Phillips, A.L. Klibanov, B.R. Wamhoff, and J.A. Hossack, Targeted gene transfection from microbubbles into vascular smooth muscle cells using focused, ultrasound-mediated delivery, Ultrasound Med. Biol., 36 (2010) 1470-1480.

[14] S. Tinkov, G. Winter, C. Coester, and R. Bekeredjian, New doxorubicin-loaded phospholipid microbubbles for targeted tumor therapy: Part I--Formulation development and in-vitro characterization, J. Control Release, 143 (2010) 143-150.

[15] C.Y. Ting, C.H. Fan, H.L. Liu, C.Y. Huang, H.Y. Hsieh, T.C. Yen, K.C. Wei, and C.K. Yeh, Concurrent blood-brain barrier opening and local drug delivery using drug-carrying microbubbles and focused ultrasound for brain glioma treatment, Biomaterials, 33 (2012) 704-712.

[16] A.C. Camarozano, Garcia de Almeida Cyrino FZ, D.A. Bottino, and E. Bouskela, Effects of microbubbles and ultrasound on the microcirculation: observation on the hamster cheek pouch, J. Am. Soc. Echocardiogr., 23 (2010) 1323-1330.

[17] K. Kooiman, M.R. Bohmer, M. Emmer, H.J. Vos, C. Chlon, W.T. Shi, C.S. Hall, S.H. de Winter, K. Schroen, M. Versluis, J.N. de, and W.A. van, Oil-filled polymer microcapsules for ultrasound-mediated delivery of lipophilic drugs, J. Control Release, 133 (2009) 109-118.

[18] M.S. Tartis, J. McCallan, A.F. Lum, R. LaBell, S.M. Stieger, T.O. Matsunaga, and K.W. Ferrara, Therapeutic effects of paclitaxel-containing ultrasound contrast agents, Ultrasound Med. Biol., 32 (2006) 1771-1780.

[19] S. Hernot and A.L. Klibanov, Microbubbles in ultrasound-triggered drug and gene delivery, Adv. Drug Deliv. Rev., 60 (2008) 1153-1166.

[20] I. Lentacker, B. Geers, J. Demeester, S.C. De Smedt, and N.N. Sanders, Design and evaluation of doxorubicin-containing microbubbles for ultrasound-triggered doxorubicin delivery: cytotoxicity and mechanisms involved, Mol. Ther., 18 (2010) 101-108.

[21] M.L. De Temmerman, H. Dewitte, R.E. Vandenbroucke, B. Lucas, C. Libert, J. Demeester, S.C. De Smedt, I. Lentacker, and J. Rejman, mRNA-Lipoplex loaded

microbubble contrast agents for ultrasound-assisted transfection of dendritic cells, Biomaterials, 32 (2011) 9128-9135.

[22] I. Lentacker, N. Wang, R.E. Vandenbroucke, J. Demeester, S.C. De Smedt, and N.N. Sanders, Ultrasound exposure of lipoplex loaded microbubbles facilitates direct cytoplasmic entry of the lipoplexes, Mol. Pharm., 6 (2009) 457-467.

[23] R.E. Vandenbroucke, I. Lentacker, J. Demeester, S.C. De Smedt, and N.N. Sanders, Ultrasound assisted siRNA delivery using PEG-siPlex loaded microbubbles, J. Control Release, 126 (2008) 265-273.

[24] P. Caliceti, M. Chinol, M. Roldo, F.M. Veronese, A. Semenzato, S. Salmaso, and G. Paganelli, Poly(ethylene glycol)-avidin bioconjugates: suitable candidates for tumor pretargeting, J. Control Release, 83 (2002) 97-108.

[25] B. Geers, I. Lentacker, N.N. Sanders, J. Demeester, S. Meairs, and S.C. De Smedt, Self-assembled liposome-loaded microbubbles: The missing link for safe and efficient ultrasound triggered drug-delivery, J. Control Release, 152 (2011) 249-256.

[26] Y.C. Barenholz, Doxil(R) - The first FDA-approved nano-drug: Lessons learned, J. Control Release, (2012).

[27] A. Gabizon, R. Catane, B. Uziely, B. Kaufman, T. Safra, R. Cohen, F. Martin, A. Huang, and Y. Barenholz, Prolonged circulation time and enhanced accumulation in malignant exudates of doxorubicin encapsulated in polyethylene-glycol coated liposomes, Cancer Res., 54 (1994) 987-992.

[28] S. Tinkov, C. Coester, S. Serba, N.A. Geis, H.A. Katus, G. Winter, and R. Bekeredjian, New doxorubicin-loaded phospholipid microbubbles for targeted tumor therapy: in-vivo characterization, J. Control Release, 148 (2010) 368-372.

[29] R. Suzuki, E. Namai, Y. Oda, N. Nishiie, S. Otake, R. Koshima, K. Hirata, Y. Taira, N. Utoguchi, Y. Negishi, S. Nakagawa, and K. Maruyama, Cancer gene therapy by IL-12 gene delivery using liposomal bubbles and tumoral ultrasound exposure, Journal of Controlled Release, 142 (2010) 245-250.

[30] Y. Wang, X. Li, Y. Zhou, P. Huang, and Y. Xu, Preparation of nanobubbles for ultrasound imaging and intracelluar drug delivery, International Journal of Pharmaceutics, 384 (2010) 148-153.

[31] M.S. Tartis, D.E. Kruse, H. Zheng, H. Zhang, A. Kheirolomoom, J. Marik, and K.W. Ferrara, Dynamic microPET imaging of ultrasound contrast agents and lipid delivery, J. Control Release, 131 (2008) 160-166.

[32] F.Y. Yang, T.T. Wong, M.C. Teng, R.S. Liu, M. Lu, H.F. Liang, and M.C. Wei, Focused ultrasound and interleukin-4 receptor-targeted liposomal doxorubicin for enhanced targeted drug delivery and antitumor effect in glioblastoma multiforme, J. Control Release, (2012).

[33] Y. Wang, X. Li, Y. Zhou, P. Huang, and Y. Xu, Preparation of nanobubbles for ultrasound imaging and intracelluar drug delivery, International Journal of Pharmaceutics, 384 (2010) 148-153.

[34] Z. Gao, A.M. Kennedy, D.A. Christensen, and N.Y. Rapoport, Drug-loaded nano/microbubbles for combining ultrasonography and targeted chemotherapy, Ultrasonics, 48 (2008) 260-270.

[35] N. Rapoport, K.H. Nam, R. Gupta, Z. Gao, P. Mohan, A. Payne, N. Todd, X. Liu, T. Kim, J. Shea, C. Scaife, D.L. Parker, E.K. Jeong, and A.M. Kennedy, Ultrasound-mediated tumor imaging and nanotherapy using drug loaded, block copolymer stabilized perfluorocarbon nanoemulsions, J. Control Release, 153 (2011) 4-15.

[36] P.S. Sheeran, S. Luois, P.A. Dayton, and T.O. Matsunaga, Formulation and acoustic studies of a new phase-shift agent for diagnostic and therapeutic ultrasound, Langmuir, 27 (2011) 10412-10420.

[37] J. Fang, H. Nakamura, and H. Maeda, The EPR effect: Unique features of tumor blood vessels for drug delivery, factors involved, and limitations and augmentation of the effect, Advanced Drug Delivery Reviews, 63 (2011) 136-151.

[38] P.S. Sheeran, V.P. Wong, S. Luois, R.J. McFarland, W.D. Ross, S. Feingold, T.O. Matsunaga, and P.A. Dayton, Decafluorobutane as a phase-change contrast agent for low-energy extravascular ultrasonic imaging, Ultrasound Med. Biol., 37 (2011) 1518-1530.

[39] X. Wang, H. Chen, Y. Chen, M. Ma, K. Zhang, F. Li, Y. Zheng, D. Zeng, Q. Wang, and J. Shi, Perfluorohexane-encapsulated mesoporous silica nanocapsules as enhancement agents for highly efficient high intensity focused ultrasound (HIFU), Adv. Mater., 24 (2012) 785-791.

[40] C.E. Ashley, E.C. Carnes, G.K. Phillips, D. Padilla, P.N. Durfee, P.A. Brown, T.N. Hanna, J. Liu, B. Phillips, M.B. Carter, N.J. Carroll, X. Jiang, D.R. Dunphy, C.L. Willman, D.N. Petsev, D.G. Evans, A.N. Parikh, B. Chackerian, W. Wharton, D.S. Peabody, and C.J. Brinker, The targeted delivery of multicomponent cargos to cancer cells by nanoporous particle-supported lipid bilayers, Nat. Mater., 10 (2011) 389-397.

[41] B.A. Kaufmann, J.M. Sanders, C. Davis, A. Xie, P. Aldred, I.J. Sarembock, and J.R. Lindner, Molecular imaging of inflammation in atherosclerosis with targeted ultrasound detection of vascular cell adhesion molecule-1, Circulation, 116 (2007) 276-284.

[42] S. Hernot, S. Unnikrishnan, Z. Du, T. Shevchenko, B. Cosyns, A. Broisat, J. Toczek, V. Caveliers, S. Muyldermans, T. Lahoutte, A.L. Klibanov, and N. Devoogdt, Nanobody-coupled microbubbles as novel molecular tracer, J. Control Release, 158 (2012) 346-353.

[43] E.A. Ferrante, J.E. Pickard, J. Rychak, A. Klibanov, and K. Ley, Dual targeting improves microbubble contrast agent adhesion to VCAM-1 and P-selectin under flow, J. Control Release, 140 (2009) 100-107.

[44] L. Yang, X. Zhang, M. Ye, J. Jiang, R. Yang, T. Fu, Y. Chen, K. Wang, C. Liu, and W. Tan, Aptamer-conjugated nanomaterials and their applications, Adv. Drug Deliv. Rev., 63 (2011) 1361-1370.

[45] C.H. Wang, S.T. Kang, Y.H. Lee, Y.L. Luo, Y.F. Huang, and C.K. Yeh, Aptamer-conjugated and drug-loaded acoustic droplets for ultrasound theranosis, Biomaterials, 33 (2012) 1939-1947.

[46] K. Kooiman, M. Foppen-Harteveld, A.F. van der Steen, and J.N. de, Sonoporation of endothelial cells by vibrating targeted microbubbles, J. Control Release, 154 (2011) 35-41.

[47] I. Lentacker, B.G. De Geest, R.E. Vandenbroucke, L. Peeters, J. Demeester, S.C. De Smedt, and N.N. Sanders, Ultrasound-responsive polymer-coated microbubbles that bind and protect DNA, Langmuir, 22 (2006) 7273-7278.

[48] A. Ghanem, C. Steingen, F. Brenig, F. Funcke, Z.Y. Bai, C. Hall, C.T. Chin, G. Nickenig, W. Bloch, and K. Tiemann, Focused ultrasound-induced stimulation of microbubbles augments site-targeted engraftment of mesenchymal stem cells after acute myocardial infarction, Journal of Molecular and Cellular Cardiology, 47 (2009) 411-418.

[49] H. Leong-Poi, M.A. Kuliszewski, M. Lekas, M. Sibbald, K. Teichert-Kuliszewska, A.L. Klibanov, D.J. Stewart, and J.R. Lindner, Therapeutic arteriogenesis by ultrasound-mediated VEGF(165) plasmid gene delivery to chronically ischemic skeletal muscle, Circulation Research, 101 (2007) 295-303.

[50] W.A. van, K. Kooiman, M. Harteveld, M. Emmer, F.J. ten Cate, M. Versluis, and J.N. de, Vibrating microbubbles poking individual cells: drug transfer into cells via sonoporation, J. Control Release, 112 (2006) 149-155.

[51] M. Figueiredo and R. Esenaliev, PLGA Nanoparticles for Ultrasound-Mediated Gene Delivery to Solid Tumors, J. Drug Deliv., 2012 (2012) 767839.

[52] T.B. Brismar, D. Grishenkov, B. Gustafsson, J. Harmark, A. Barrefelt, S.V. Kothapalli, S. Margheritelli, L. Oddo, K. Caidahl, H. Hebert, and G. Paradossi, Magnetite Nanoparticles Can Be Coupled to Microbubbles to Support Multimodal Imaging, Biomacromolecules., (2012).

[53] I. Lentacker, R.E. Vandenbroucke, B. Lucas, J. Demeester, S.C. De Smedt, and N.N. Sanders, New strategies for nucleic acid delivery to conquer cellular and nuclear membranes, J. Control Release, 132 (2008) 279-288.

[54] M. Seo, I. Gorelikov, R. Williams, and N. Matsuura, Microfluidic assembly of monodisperse, nanoparticle-incorporated perfluorocarbon microbubbles for medical imaging and therapy, Langmuir, 26 (2010) 13855-13860.

[55] M. Afadzi, C.d. Davies, Y.H. Hansen, T. Johansen, O.K. Standal, R. Hansen, S.E. Masoy, E.A. Nilssen, and B. Angelsen, Effect of Ultrasound Parameters on the Release of Liposomal Calcein, Ultrasound in Medicine and Biology, 38 (2012) 476-486.

[56] C.D. Ohl and R. Ikink, Shock-wave-induced jetting of micron-size bubbles, Phys. Rev. Lett., 90 (2003) 214502.

[57] C.D. Ohl, M. Arora, R. Ikink, J.N. de, M. Versluis, M. Delius, and D. Lohse, Sonoporation from jetting cavitation bubbles, Biophys. J., 91 (2006) 4285-4295.

[58] R. Karshafian, P.D. Bevan, R. Williams, S. Samac, and P.N. Burns, Sonoporation by ultrasound-activated microbubble contrast agents: effect of acoustic exposure parameters on cell membrane permeability and cell viability, Ultrasound Med. Biol., 35 (2009) 847-860.

[59] R. Karshafian, S. Samac, P.D. Bevan, and P.N. Burns, Microbubble mediated sonoporation of cells in suspension: clonogenic viability and influence of molecular size on uptake, Ultrasonics, 50 (2010) 691-697.

[60] S. Mehier-Humbert, T. Bettinger, F. Yan, and R.H. Guy, Plasma membrane poration induced by ultrasound exposure: implication for drug delivery, J. Control Release, 104 (2005) 213-222.

[61] B. Geers, I. Lentacker, A. Alonso, N.N. Sanders, J. Demeester, S. Meairs, and S.C. De Smedt, Elucidating the mechanisms behind sonoporation with adeno-associated virus-loaded microbubbles, Mol. Pharm., 8 (2011) 2244-2251.

[62] B.D. Meijering, L.J. Juffermans, W.A. van, R.H. Henning, I.S. Zuhorn, M. Emmer, A.M. Versteilen, W.J. Paulus, W.H. van Gilst, K. Kooiman, J.N. de, R.J. Musters, L.E. Deelman, and O. Kamp, Ultrasound and microbubble-targeted delivery of macromolecules is regulated by induction of endocytosis and pore formation, Circ. Res., 104 (2009) 679-687.

[63] C. Mannaris and M.A. Averkiou, Investigation of microbubble response to long pulses used in ultrasound-enhanced drug delivery, Ultrasound Med. Biol., 38 (2012) 681-691.

[64] R. Seip, C.T. Chin, C.S. Hall, B.I. Raju, A. Ghanem, and K. Tiemann, Targeted ultrasound-mediated delivery of nanoparticles: on the development of a new HIFU-based therapy and imaging device, IEEE Trans. Biomed. Eng, 57 (2010) 61-70.

[65] L.H. Treat, N. McDannold, N. Vykhodtseva, Y. Zhang, K. Tam, and K. Hynynen, Targeted delivery of doxorubicin to the rat brain at therapeutic levels using MRI-guided focused ultrasound, Int. J. Cancer, 121 (2007) 901-907.

[66] F. Marquet, Y.S. Tung, T. Teichert, V.P. Ferrera, and E.E. Konofagou, Noninvasive, transient and selective blood-brain barrier opening in non-human primates in vivo, PLoS. One., 6 (2011) e22598.

[67] M.R. Bohmer, C.H. Chlon, B.I. Raju, C.T. Chin, T. Shevchenko, and A.L. Klibanov, Focused ultrasound and microbubbles for enhanced extravasation, J. Control Release, 148 (2010) 18-24.

[68] N. Vykhodtseva, N. McDannold, and K. Hynynen, Progress and problems in the application of focused ultrasound for blood-brain barrier disruption, Ultrasonics, 48 (2008) 279-296.

[69] N. Rapoport, Z. Gao, and A. Kennedy, Multifunctional nanoparticles for combining ultrasonic tumor imaging and targeted chemotherapy, J. Natl. Cancer Inst., 99 (2007) 1095-1106.

[70] K. Un, S. Kawakami, M. Yoshida, Y. Higuchi, R. Suzuki, K. Maruyama, F. Yamashita, and M. Hashida, The elucidation of gene transferring mechanism by ultrasound-responsive unmodified and mannose-modified lipoplexes, Biomaterials, 32 (2011) 4659-4669.

[71] O. Mueller, S. Schinkel, J. Kleinschmidt, H. Katus, and R. Bekeredjian, Augmentation of AAV-mediated cardiac gene transfer after systemic administration in adult rats, Gene Therapy, 15 (2008) 1558-1565.

[72] T. Lammers, S. Aime, W.E. Hennink, G. Storm, and F. Kiessling, Theranostic nanomedicine, Acc. Chem. Res., 44 (2011) 1029-1038.

[73] T. Lammers, F. Kiessling, W.E. Hennink, and G. Storm, Nanotheranostics and image-guided drug delivery: current concepts and future directions, Mol. Pharm., 7 (2010) 1899-1912.

[74] F. Kiessling, S. Fokong, P. Koczera, W. Lederle, and T. Lammers, Ultrasound microbubbles for molecular diagnosis, therapy, and theranostics, J. Nucl. Med., 53 (2012) 345-348.

[75] Z. Liu, T. Lammers, J. Ehling, S. Fokong, J. Bornemann, F. Kiessling, and J. Gatjens, Iron oxide nanoparticle-containing microbubble composites as contrast agents for MR and ultrasound dual-modality imaging, Biomaterials, 32 (2011) 6155-6163.

[76] Y.T. Lim, M.Y. Cho, J.H. Kang, Y.W. Noh, J.H. Cho, K.S. Hong, J.W. Chung, and B.H. Chung, Perfluorodecalin/[InGaP/ZnS quantum dots] nanoemulsions as 19F MR/optical imaging nanoprobes for the labeling of phagocytic and nonphagocytic immune cells, Biomaterials, 31 (2010) 4964-4971.

[77] R.K. Schlicher, J.D. Hutcheson, H. Radhakrishna, R.P. Apkarian, and M.R. Prausnitz, Changes in cell morphology due to plasma membrane wounding by acoustic cavitation, Ultrasound Med. Biol., 36 (2010) 677-692.

[78] W.A. van, K. Kooiman, M. Emmer, F.J. ten Cate, M. Versluis, and J.N. de, Ultrasound microbubble induced endothelial cell permeability, J. Control Release, 116 (2006) e100-e102.

[79] A. Yudina, M. Lepetit-Coiffe, and C.T. Moonen, Evaluation of the temporal window for drug delivery following ultrasound-mediated membrane permeability enhancement, Mol. Imaging Biol., 13 (2011) 239-249.

[80] A. Yudina, S.M. de, M. Lepetit-Coiffe, S. Langereis, R.L. Van, P. Smirnov, V. Bouchaud, P. Voisin, H. Grull, and C.T. Moonen, Ultrasound-mediated intracellular drug delivery using microbubbles and temperature-sensitive liposomes, J. Control Release, 155 (2011) 442-448.

[81] E. Chavakis, M. Koyanagi, and S. Dimmeler, Enhancing the outcome of cell therapy for cardiac repair: progress from bench to bedside and back, Circulation, 121 (2010) 325-335.

[82] S.M. Herbst, M.E. Klegerman, H. Kim, J. Qi, H. Shelat, M. Wassler, M.R. Moody, C.M. Yang, X. Ge, Y. Zou, J.A. Kopechek, F.J. Clubb, D.C. Kraemer, S. Huang, C.K. Holland, D.D. McPherson, and Y.J. Geng, Delivery of stem cells to porcine arterial wall with echogenic liposomes conjugated to antibodies against CD34 and intercellular adhesion molecule-1, Mol. Pharm., 7 (2010) 3-11.

[83] X. Song, H. Zhu, L. Jin, J. Wang, Q. Yang, P. Jin, and X. Li, Ultrasound-mediated microbubble destruction enhances the efficacy of bone marrow mesenchymal stem cell transplantation and cardiac function, Clin. Exp. Pharmacol. Physiol, 36 (2009) 267-271.

[84] S. Zhong, S. Shu, Z. Wang, J. Luo, W. Zhong, H. Ran, Y. Zheng, Y. Yin, and Z. Ling, Enhanced homing of mesenchymal stem cells to the ischemic myocardium by ultrasound-targeted microbubble destruction, Ultrasonics, 52 (2012) 281-286.

[85] A.T. Askari, S. Unzek, Z.B. Popovic, C.K. Goldman, F. Forudi, M. Kiedrowski, A. Rovner, S.G. Ellis, J.D. Thomas, P.E. DiCorleto, E.J. Topol, and M.S. Penn, Effect of stromal-cell-derived factor 1 on stem-cell homing and tissue regeneration in ischaemic cardiomyopathy, Lancet, 362 (2003) 697-703.

[86] K. Un, S. Kawakami, R. Suzuki, K. Maruyama, F. Yamashita, and M. Hashida, Enhanced Transfection Efficiency into Macrophages and Dendritic Cells by a Combination Method Using Mannosylated Lipoplexes and Bubble Liposomes with Ultrasound Exposure, Human Gene Therapy, 21 (2010) 65-74.

[87] S. Kreiter, A. Selmi, M. Diken, M. Koslowski, C.M. Britten, C. Huber, O. Tuereci, and U. Sahin, Intranodal Vaccination with Naked Antigen-Encoding RNA Elicits Potent Prophylactic and Therapeutic Antitumoral Immunity, Cancer Research, 70 (2010) 9031-9040.

[88] A. Sever, S. Jones, K. Cox, J. Weeks, P. Mills, and P. Jones, Preoperative localization of sentinel lymph nodes using intradermal microbubbles and contrast-enhanced

ultrasonography in patients with breast cancer, British Journal of Surgery, 96 (2009) 1295-1299.