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Gene Delivery with Ultrasound and Microbubbles

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1. Introduction

Gene therapy and treatment with siRNA hold potential to treat a wide variety of different diseases. Genetic material is not usually stable and is generally hydrolyzed following intravascular administration. This makes the delivery of genes somewhat problematic since intact genetic material must usually be delivered intracellularly for therapeutic effect. For gene therapy, in most cases the gene construct must reach the cellular intranuclear sub-compartment in order to elicit the desired biological effect. Viruses have evolved to deliver DNA and RNA to cells but viral vector-based gene therapy has been associated with effects inherent in biological systems (Marshall, Muruve, Thomas). Non-viral based systems afford the potential to deliver genetic materials without the adverse biological effects of viral-based systems. In most cases, however, non-viral based gene delivery systems have been less effective than viral based systems, yielding lower levels of gene expression (Litzinger). Microbubbles, e.g. acoustically active carriers, in concert with ultrasound (Unger, Zhou) may afford potential for highly effective site directed gene therapy and delivery of other genetic materials such as siRNA (Zhigang).

2. Summary of gene delivery with microbubbles and ultrasound

The basic outline of a microbubble is shown in Figure 1. Microbubbles are composed of gas with a stabilizing shell material oftentimes consisting of lipids, albumin, or biocompatible polymers. For biomedical application they range in size from several microns in diameter to several hundred nanometers in diameter. The original biomedical application was as ultrasound contrast agents for echocardiography. Two agents, Definity®, phospholipid-coated perfluoropropane microbubbles (Lantheus, Billerica, MA) and Optison®, albumin-coated perfluoropropane microbubbles, are approved by the FDA in the US and are sold as contrast agents with approved claims for echocardiography.

Because of the large impedance mismatch between liquid and gas, when sound waves strike a microbubble, the waves are efficiently scattered back (microbubbles are excellent acoustic reflectors) and this is the basis for the use of microbubbles as ultrasound contrast agents. They are excellent reflectors of sound energy and hence are outstanding contrast agents for biomedical ultrasound imaging. Furthermore, the design of special ultrasound pulse sequences as 2nd harmonic imaging and phase inversion harmonic imaging has helped to increase ultrasound imaging by eliminating significant amounts of noise from tissue reflection.

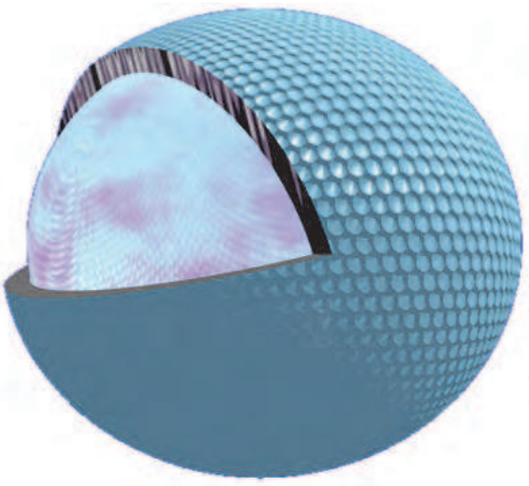

Fig. 1. Depicts a microbubble coated with a film of stabilizing material.

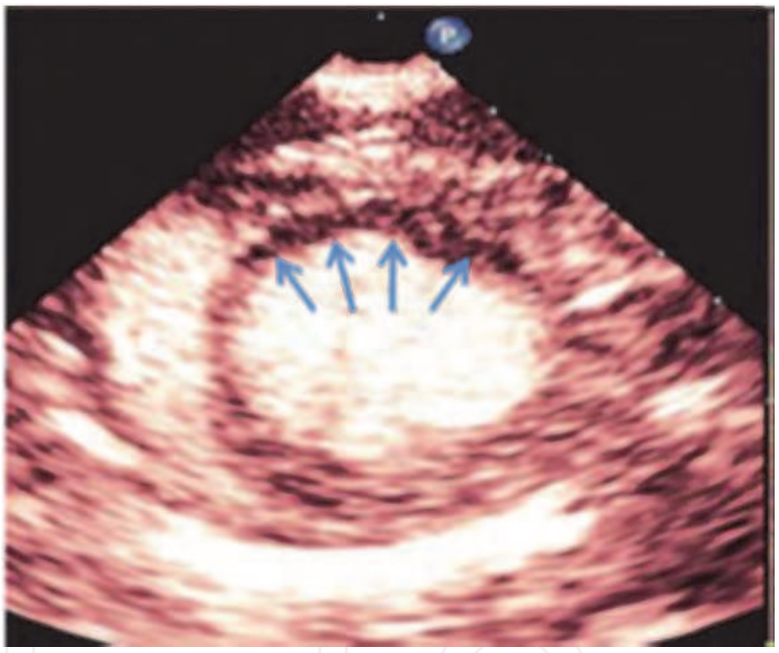

Fig. 2. Shows a contrast-enhanced echocardiogram image of a porcine heart after occlusion of the left anterior descending coronary artery (model of acute myocardial infarction). The area of decreased perfusion in the left ventricular wall is clearly seen on the post contrast images but was not detectable without contrast.

When ultrasound encounters the acoustic interface of a microbubble not only may the ultrasound be scattered, but also, because of their size and the fortuitous insonation frequencies used clinically, the microbubbles also can oscillate (stable cavitation). Depending upon the acoustic pressure, oscillating microbubbles may rupture (cavitate – described further in section 4, inertial cavitation). On the basis of cavitation it was discovered that microbubbles had therapeutic applications for gene and drug delivery and treatment of vascular thrombosis. Ultrasound effects on cell membranes may be two-fold; 1) ultrasound itself can enhance membrane permeability, thereby allowing more diffusion from the extracellular milieu (sonoporation); or, 2) can be used in conjunction with

microbubbles to enhance localized delivery via microbubble bursting and subsequent radiation force induced particle penetration through the membrane surface. Cavitation can be used to increase cell permeability and local delivery of materials such as DNA for gene therapy.

A number of preclinical studies have been performed for gene (described further below) and drug delivery, but phospholipid-coated microbubbles have entered human clinical trials for treatment of vascular thrombosis. In these studies microbubbles have been administered intravenously and been shown to permeate a thrombus. Ultrasound energy has then been applied to the site of the clot to cavitate the microbubbles and dissolve the thrombus.

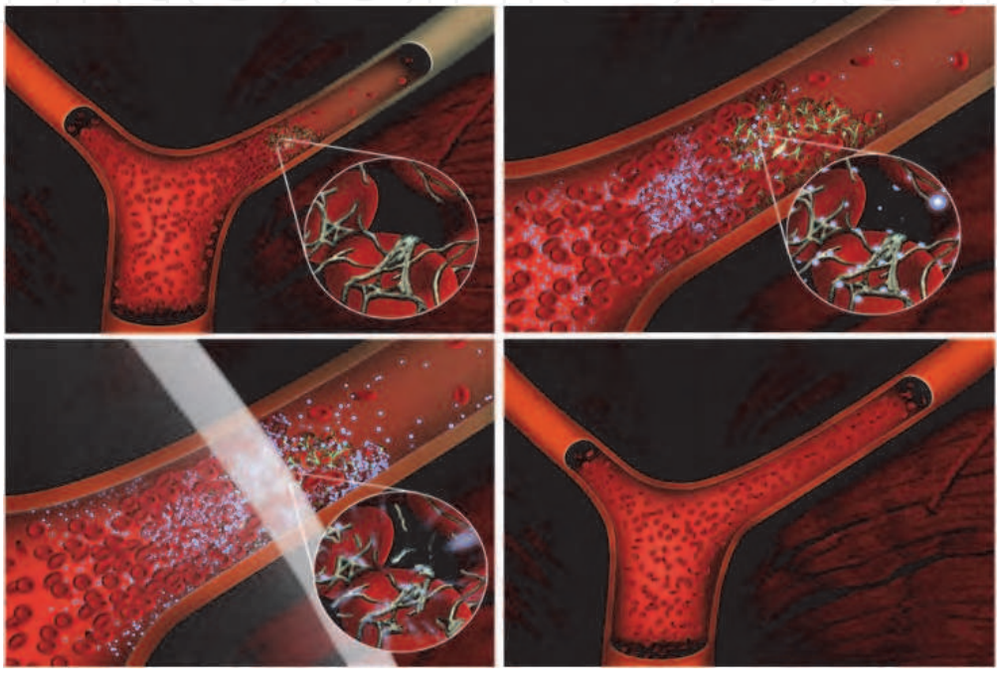

Fig. 3. Depicts and occlusive thrombus in an arterial blood vessel (upper left). Microbubbles are infused IV and permeate the clot (upper right). This can be seen on ultrasound imaging. High energy ultrasound is focused on the site of thrombus (lower left) and the microbubbles cavitate, dissolving the clot and restoring blood flow.

3. Details of microbubbles for gene delivery

Microbubbles are mainly composed of fluorinated gases. Air and nitrogen are relatively water-soluble and hence will diffuse into the blood and the bubbles will then rapidly shrink and eventually collapse from Laplace pressures. This is not to say that air and nitrogen cannot be used to make microbubbles for biomedical ultrasound applications, but that stabilizing materials will need to be more robust to preserve the microbubbles.

As opposed to oxygen and nitrogen being relatively soluble in water, perfluorocarbons are virtually insoluble in the aqueous milieu. In fact perfluorocarbons are amphiphobic. The higher the molecular weight of the fluorinated compound the less water soluble and we would predict that microbubbles prepared from that gas should be correspondingly more stable in the blood stream (all else equal). Note that perfluoropentane has a boiling point of 29^{o}C and that perfluorohexane volatilizes at 56.6^{o}C and therefore will be a liquid at biological temperature (presumably due to van der Waal's attractions).

Compound	Molecular Weight	Aqueous Solubility (Ostwald's Coefficient)	Boiling Point °C
Nitrogen	28	18071	-196
Oxygen	32	4865	-183
Sulfur Hexafluoride	146	5950	-64
Perfluoropropane	188	583	-36.7
Perfluorobutane	238	<500	-1.7
Perfluoropentane	288	>24 and <500	29
Perfluorohexane	338	24	56.6

Table 1. Potential Compounds for Making Gaseous Cores of Microbubbles

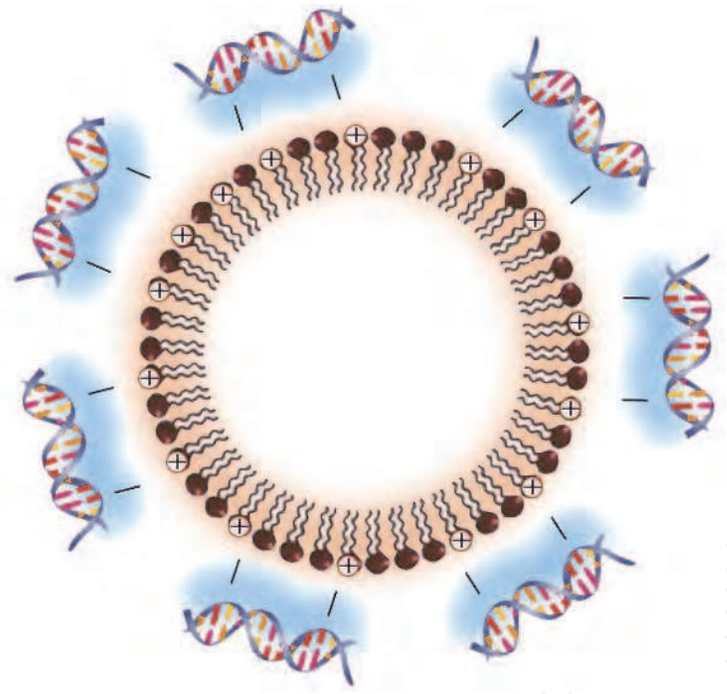

Fig. 4. Depicts a microbubble for gene delivery. The gaseous core is coated by a monolayer of phospholipid containing cationic lipid imparting a net positive charge to the microbubble. DNA, as a polyanion, is adsorbed electrostatically to the exterior surface of the microbubble.

With respect to the microbubble membrane, the above design demonstrates how DNA is adsorbed to the surface via electrostatic adhesion with cationic lipids inserted in the membrane. However, note also that there is no steric protection of the DNA, making it susceptible to biodegradation/hydrolysis. As shown below in Figure 5, microbubbles can be designed to incorporate PEG'ylated lipid (e.g. 8-10 mole percent PEG'ylated lipid) and we have still found that cationic PEG'ylated microbubbles will still adsorb useful payloads of DNA.

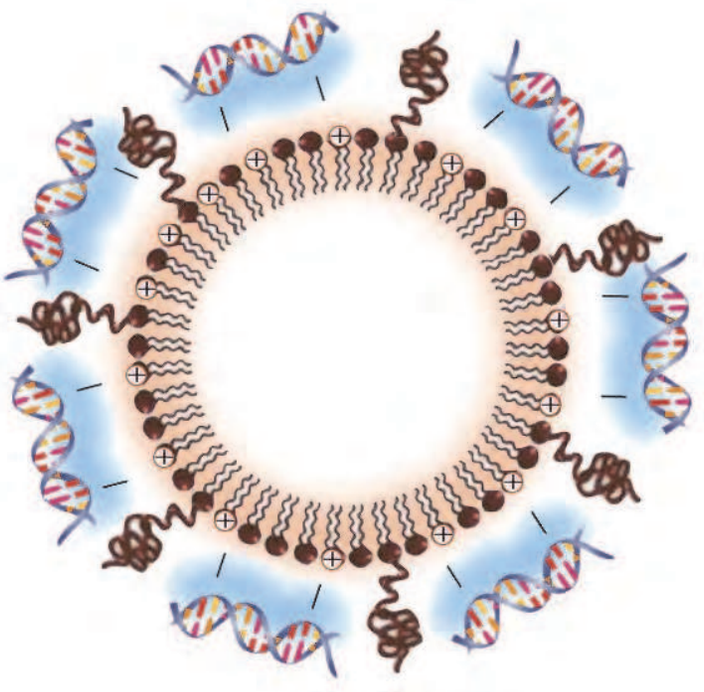

Fig. 5. Depicts a PEG'ylated cationic microbubble binding DNA.

In vivo studies with plasmid DNA using the construct depicted in Figure 5 have shown high levels of gene expression in the zones of insonation.

We predict, however, that targeted constucts that would bind to cellular targets should be more effective for gene delivery. Since microbubbles are micron-sized structures, they are not expected to extravasate from the intravscular space. However, for the purposes of delivering genes to regions in close proximity to targeted tissues, they can be targeted to eptitopes expressed on endothelial cells. Because they can also be engulfed by phagocytic cells, such as immune cells, and targeted as intracellular passengers, the nanoparticles fused to microbubble surfaces as described below (Figure 8) can also be decorated with cell surface identifiers/targets, uptake enhancers, and even intracellular targets in order to provide additional selectivity. We have prepared targeted microbubbles to a variety of different targets. Depicted below is the design for a targeted microbubble to the integrin $\alpha_v\beta_{III,}$ Expressed on the endothelial surface in angiogenesis.

The microbubble construct depicted above combines several features of nanotechnology. The parent microbubble is PEG'ylated to impart stealth properties in order to prevent reticuloendothelial system elimination and includes targeting ligands directed to epitopes expressed on endothelial cells. It could be monitored as an ultrasound contrast agent and then activated with higher energy ultrasound using low MI ultrasound for imaging to monitor delivery to the target site and high MI ultrasound energy for cavitation. Upon activation the nanoparticles containing DNA would be expected to extravasate from the vasculature into the interstitial tissues. The nanoparticles could be constructed to contain targeting ligands and/or cell penetration-enhancing agents to facilitate uptake by target cells. A challenge is to design linkers to attach the nanoparticles to the surface of the parent microbubbles. One approach would be to biotinylate the nanoparticles and to have avidin tethers attached to the parent microbubbles taking advantage of the avidin/biotin interaction. This approach could be utilized in proof-of-principle studies but would not be

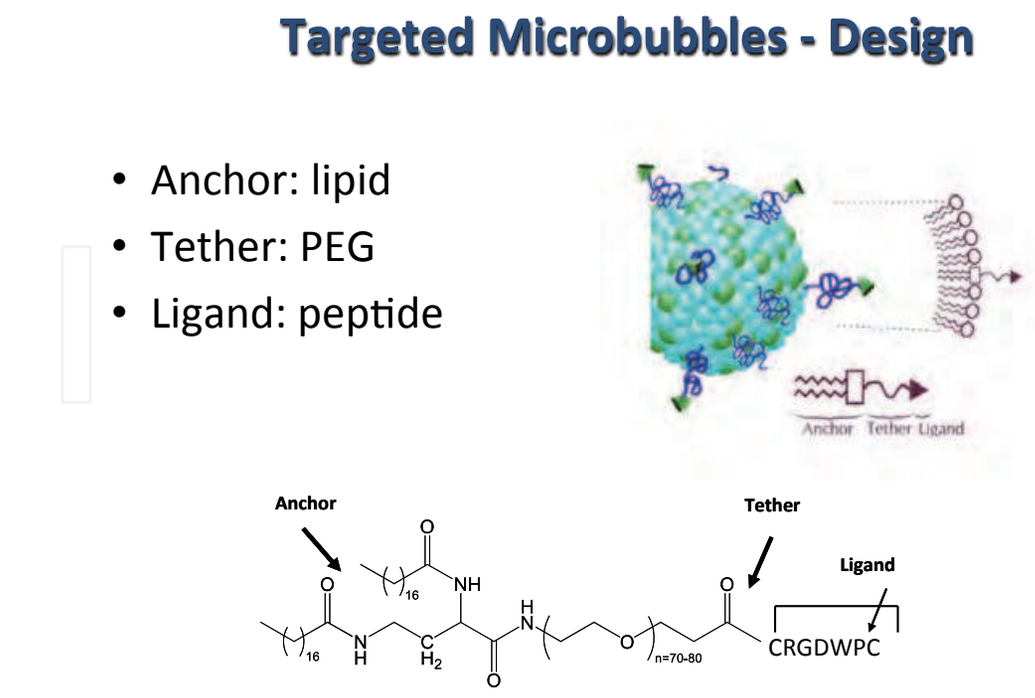

Fig. 6. Depicts a targeted microbubble. The cyclo-CRGDC analog is attached to a lipid anchor via a PEG spacer to form a bioconjugate. The bioconjugate comprises about from about 0.1 to 5 mole percent of the lipid coating the microbubble. There is additional PEG'ylated lipid from about 5-10 mole percent in the lipid coating the microbubble.

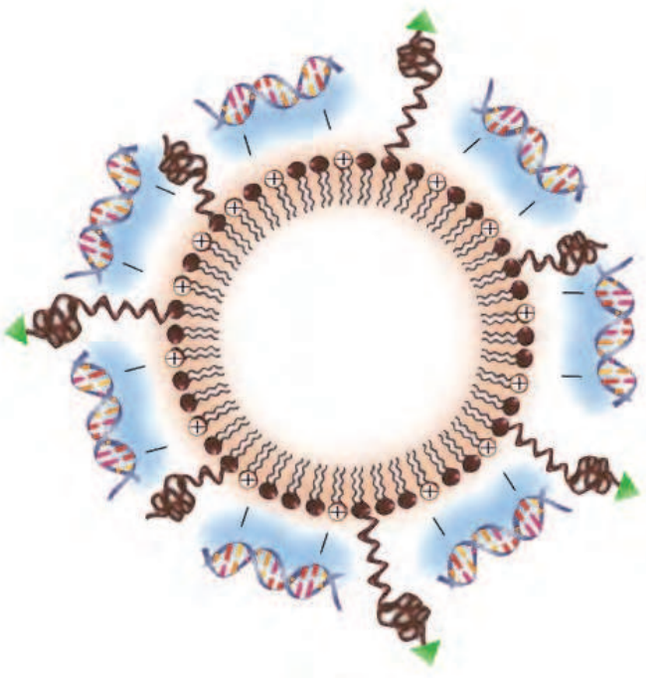

Fig. 7. Depicts a targeted cationic microbubble binding DNA. The bioconjugates are designed to bind to endothelial epitopes upregulated in disease. The microbubble can be followed and monitored by ultrasound imaging and activated with ultrasound energy for local delivery.

clinically translatable. A maleimide labeled spacer might be affixed to the microbubble and the nanoparticles might be thiolated to covalently bind the nanoparticle to the surface of the microbubbles in a potentially biocompatible manner. Another approach would be to use electrostatic interaction between the nanoparticle and the surface of the microbubbles but this must be optimized to ensure that the DNA is bound until it reaches the target site. Note also that if the bubble has excess cationic charge this may adversely affect biodistribution. Note also that it is more difficult to bind low molecular weight genetic material such as si-RNA using merely electrostatic interaction.

Fig. 8. Depicts a unique microbubble construct for gene delivery. The parent microbubble is PEG'ylated but the DNA (or siRNA) is condensed into nanoparticles that are bound to the surface of the microbubble. The nanoparticles have targeting ligands to bind to cell specific epitopes. Note also that the construct could be modified to comprise endothelial targeting moieties on the microbubble so that the microbubble could bind to endothelial epitopes.

4. Ultrasound – parameters and bioeffects

Ultrasound is a commonly used modality for biomedical imaging, only exceeded by X-rays in overall worldwide use. For medical imaging the power or intensity of ultrasound that can be used is limited by regulatory guidelines. The intensity of the ultrasound can be described by the mechanical index (MI) which is related to the peak negative pressure of the ultrasound wave divided by the square root of the center frequency of the ultrasound. The FDA-approved mechanical index for most body imaging ultrasound (e.g. cardiac and abdominal) is limited to an MI < 1.9. For neurovascular imaging it is limited to MI < 1.0 and for ophthalmic ultrasound to MI < 0.8.

Cavitation is a phenomenon in which ultrasound exposure at the resonance frequency of the microbubble will induce expansion and collapse of microbubbles (Apfel, Hallow). This can occur spontaneously at high acoustic pressure in the absence of exogenous microbubbles. Sufficiently high acoustic power is sufficient to create a microbubble nidus in situ and cause expansion and collapse, i.e. cavitation (Marmottant). Cavitation causes

acoustic streaming and local shock waves that may radiate on the order of microns or larger depending upon the acoustic intensity and other factors (Mehier-Humbert). Cavitation can be used to increase cell permeability (i.e. sonoporation)(Deng), open the blood brain barrier or destroy tissues (i.e. sonoablation)(Conger, Feril). The acoustic pressure can be controlled to create the desired effects (Forsberg). Within the ranges of allowable acoustic pressures, in the absence of exogenous microbubbles, biomedical ultrasound imaging does not generally cause violent cavitation and ultrasound imaging is generally quite safe, particularly compared to other technologies such as X-ray imaging that uses ionizing radiation.

As shown below in Figure 9, microbubbles lower the threshold of ultrasound energy necessary for cavitation to occur. The effect is frequency dependent with a greater proportional effect at 1 MHz than at 10 MHz. Note that at 1 MHz, cavitation occurs at MI < 0.5 within the allowable ultrasound power limits for biomedical imaging. Note that microbubbles in the size range of 0.5 to 2.0 microns are quite effective in lowering the thresh-hold of energy for cavitation. Stable microbubbles for gene delivery can certainly be made in this size range. If necessary, for therapeutic applications, higher levels of ultrasound energy can be employed. Higher levels of ultrasound energy are used therapeutically for hyperthermia and focused ultrasound surgery (Brujan).

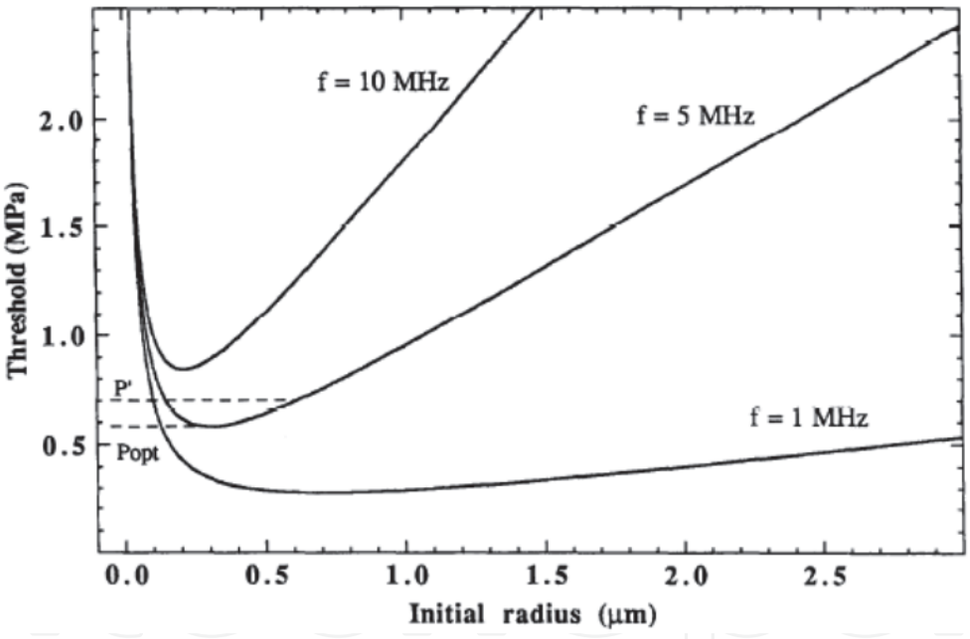

Fig. 9. Microbubbles Lower Thresh-hold of Ultrasound Energy for Cavitation (Apfel).

Figure 10, below, shows images from ultra-high speed videomicroscopy of a single bubble in response to a single high MI pulse of ultrasound energy. The microbubble expands, collapses, and fragments. The daughter fragments then undergo one additional cycle of expansion, collapse and disappear. In this process genetic materials might be released from a gene-carrying microbubble. The cavitation process will also create acoustic jets and streaming which might be used for delivering the genetic material to the target tissue or cells.

Figure 11 depicts a gene carrying microbubble in response to cavitation. The stabilizing wall material of the microbubble fragments and the genetic material is ejected with the cavitation ballistically, thereby extravasating from the vasculature to the target tissue.

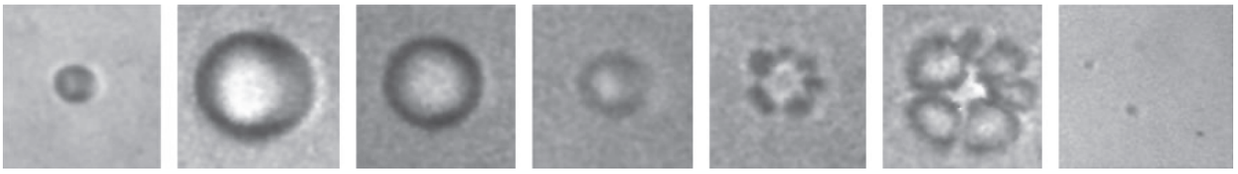

Fig. 10. Videomicroscopy images of a single bubble in response to a single ultrasonic wave.

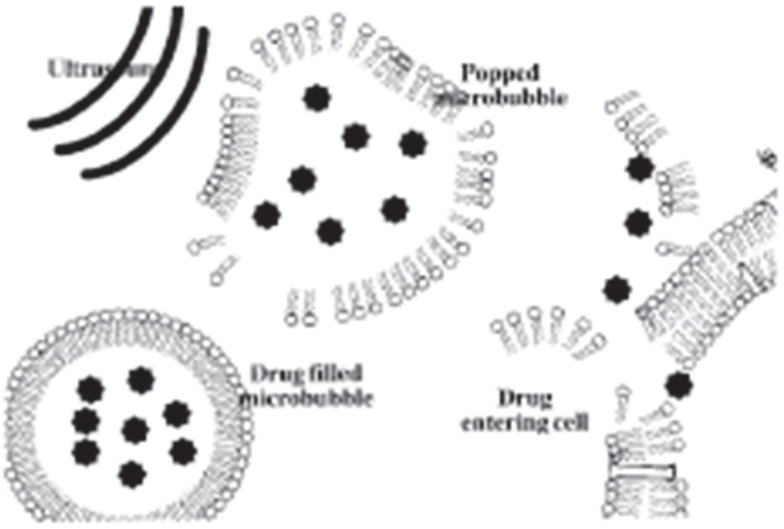

Fig. 11. A drug carrying-microbubble cavitating in response to ultrasound.

Another major mechanism in ultrasound that may be useful for drug and gene delivery is the mechanical force of microbubbles. Ultrasound exerts a radiation (pushing) force that can move microparticles and improve cellular delivery of biomaterials. Figure 12 below shows avidin-coated microbubbles flowing in blood over a biotinylated plate. The microbubbles flow and do not appear to bind but after application of relatively low MI ultrasound the microbubbles are pushed by the acoustic waves and bind to the surface of the plate. The same process can be used to improve cellular uptake of biomaterials such as DNA. The (pushing) radiation force of ultrasound occurs at lower energies than necessary for cavitation and can be used to increase efficacy from gene delivery.

An acoustic transducer that generally uses a piezoelectric material to convert electrical energy into ultrasound waves creates ultrasound waves. The transducer design varies depending upon the biomedical application. Figure 13 (below) shows the design of a three dimensional transducer for imaging and treating the heart. This transducer can be used to image the heart and visualize microbubble as they enter the myocardial circulation. Ultrasound energy can be applied to the heart to cavitate the microbubbles or for the radiation force to improve myocardial delivery. The transducer is currently being used in pre-clinical studies for microbubble enhanced sonothrombolysis to treat myocardial infarction and planned for use in clinical studies. The same transducer design could potentially be used in clinical studies for microbubble-mediated gene delivery to treat the heart.

Despite its ease of use, unwanted bioeffects can be experienced using ultrasound at high acoustic outputs. High levels of ultrasound energy with cavitation may cause cell damage, cell death and apoptosis (Miller). Ultrasound may also heat tissues and cause coagulative

necrosis at high temperatures (Ter Haar). We emphasize, however, that ultrasound power levels within the limits for diagnostic ultrasound are generally safe. Ultrasound power levels can be optimized for gene therapy to maximize gene expression while minimizing unwanted bioeffects (Chen, Rahim).

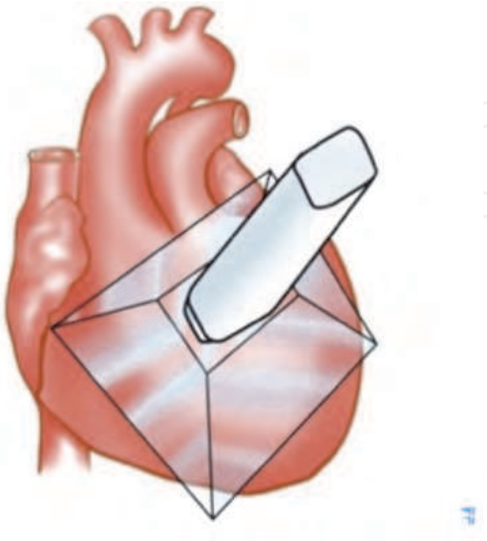

Fig. 12. Drawing of three-dimensional transducer for imaging and treating the heart.

5. Studies using microbubbles and ultrasound for gene delivery

In this section we summarize studies that have been performed using microbubbles and ultrasound for gene delivery (Klibanov, Liu, Newman).

Over the period of more that a decade a number of different studies have been performed, in vitro and in vivo. In vitro studies showed that ultrasound increased the efficacy of transfection with cationic liposomes in cell culture studies to express reporter genes. Saphenous vein grafts have been transfected with ultrasound and MBs (Kodama) to enhance graft survival after implantation (Akowuah). In vivo studies have been performed with reporter genes showing increased expression of the reporter gene after IV administration of microbubbles either binding or in association with the reporter genes. The zones of highest expression have been in the regions of tissues of insonation (except that cationic microbubbles have also been accumulated by liver, lungs, spleen and phagocytic organs). Most of the in vivo studies Have been performed with reporter genes. A few studies have performed with therapeutic genes. A wide variety of different tissues (Hauff) and cells have shown enhanced transfection with ultrasound including neuronal cells (Fischer) and skeletal muscle (Liang).

A number of groups have studied ultrasound and microbubbles to transfect tumors (Michel, Anwer). With collaborators we performed a study assessing tumor regression with transfection of the IL-12 gene with ultrasound and cationic liposomes (Anwer). In this study the liposomes were lyophilized and may have contained nitrogen gas. The tumors were insonated with 1 MHz ultrasound. Increased expression of IL-2 was observed in the insonated tumors and statistically significant tumor regression.

In addition to phospholipid coated microbubbles, MBs can be stabilizes with denatured serum albumin (e.g. Optison®, GE Healthcare Medical Diagnostics, Princeton, NJ). Albumin binds a variety of molecules and also appears to bind DNA. In one study the plasmid of AdCMV-b-Gal was attached to the microbubbles (Shohet). In these studies the solution of AdCMV-b-Gal

was added to the microbubble suspension and mixed for 2 hours at 4°C. The mixture was separated into 2 distinct layers. The upper layer consisted of microbubbles with attached virus; the bottom layer, which contained unattached virus, was discarded. The concentration of microbubbles with attached AdCMV-β-Gal was $1.2x10^9$ bubbles/mL; the mean diameter was 3.5 um. The viral titer of these microbubbles was determined. Rats were anesthetized, and MBs were administered IV. Echocardiography was performed at 1.3 MHz with a mechanical index of 1.5. Images were ECG-triggered to deliver a burst of 3 frames of ultrasound every 4 to 6 cardiac cycles. The hearts of all 6 rats in the experimental group showed blue staining with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. None of the control rats showed myocardial staining, which confirmed that the destruction of the microbubbles containing the virus was responsible for the observed β-galactosidase expression in the rat myocardium.

Initiated in 2001, researchers began using gas-filled, albumin-shelled MBs (Optison) and non-viral carriers to increase nascent serum HDL cholesterol in mice and Sprague-Dawley rats. Initial feasibility studies were performed using reporter genes, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside and GFP. Subsequent studies now use ApoA-I DNA plasmids in combination with Optison. In a series of rat studies performed with partial support from an NIH SBIR award (44HL095238-01), the results revealed that an average peak response of HDL-C elevation occurred within 24 hours of treatment and 88% of the responses occurred within 48 hours. The control animal group included ultrasound only, apoA-I DNA only, and microbubble only rats. These data demonstrated a rapid incorporation of the plasmid into the cell and efficient ApoA-I protein production that was later incorporated into serum HDL-C.

Summarized data revealed an average increase of 16.8% on day one post treatment. Due to wide normal variation in individual rat HDL cholesterol values, all data is plotted as a percentage from an individual animal's baseline. When examining the overall response to treatment in mg/dL, the average peak response to treatment was 14.0% (p-value <0.0001) as seen in Figure 1 (below). This response was observed uniformly across different animals and ranged from 40mg/dL HDL-C baseline to 100mg/dL HDL-C baseline.

Ongoing studies include additional design of experiments for the following: (1) optimization of sonoporation utility for raising HDL cholesterol, (2) efficient energy delivery algorithms, (3) modes and methods of delivery, and (4) mechanistic analyses of the intra-cellular plasmid location.

Ongoing studies have been performed with lipid coated MBs binding plasmid DNA with genes to treat diabetes. In vivo studies in rats have shown long-term normalization of blood glucose levels in diabetic rats. Studies are planned to test this system in primates.

Encouraging work has been performed in models of hypercholesterolemia showing the potential to perform gene therapy for H1Alpha to improve the capacity of the liver to produce HDL as treatment for atherosclerosis. Promising work continues to progress in the use of MBs binding genes to treat hemophilia. Cationic MBs binding DNA (similar to Figure 7) have been tested with ultrasound and compared to Definity^R MBs which do not bind DNA. Expression of Factor IX transgenes is more robust with the cationic MBs binding DNA than with MBs not binding DNA. In both the HI1Alpha/HDL and hemophilia treatment applications the target organ is the liver. The goal in both of these programs and in many others is to generate sustained transgene expression in the liver (Guo). Catheter mediated administration may prove attractive to maximize efficiency of delivery to hepatocytes and catheters can be deployed for delivery to other organs such as the kidneys, heart and blood vessels.

Ultrasound can be targeted to any organ for which it is possible to create an acoustic window. Most tissues are readily accessible to ultrasound. Ultrasound can be applied across

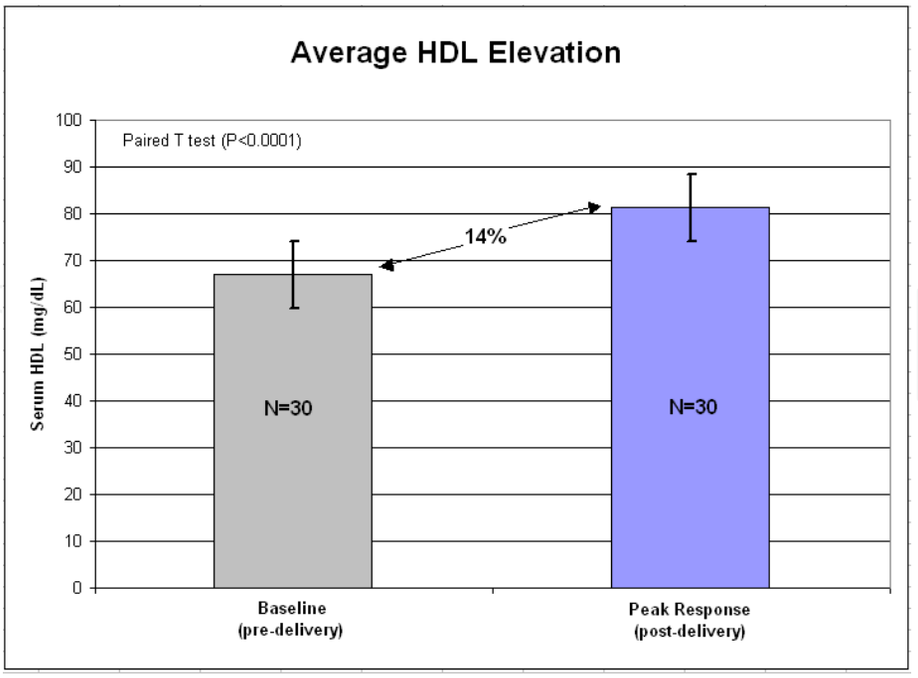

Fig. 13. Baseline to peak response measurement in 30 treated rats recorded in raw data values of mg/dL. A statically significant 14% increase was measured (p-value= 0.0001).

the intact skin or, if need be, via specialized probes. While ultrasound is blocked by air/tissue or air/fluid interfaces, even in the lung, it is possible to create acoustic windows to the bronchi with bronchoscopes and water filled balloons. Ultrasound can be targeted precisely to tissues to treat volumes of tissues ranging to more than 500 ml to less than a ml. Most studies have shown relatively low toxicity due to ultrasound and MBs. It also is possible to repeat treatment, potentially indefinitely.

Despite early studies in ultrasound and gene delivery with MBs being performed more than a decade ago, none of the ultrasound gene delivery programs with MBs, at the time of preparation of this chapter, have advanced to clinical trials. The field is still early in its development, but the tolerability, ease of use and high degrees of expression in the target tissue indicate that this technology merits serious consideration for clinical translation.

6. Conclusions

Ultrasound mediated gene delivery with acoustically active carriers (e.g. microbubbles) is a promising field that affords the potential for high levels of expression in the target tissue without the adverse bioeffects of viral-based carriers. The field is early in its development however as no clinical studies have yet been performed as of the time of preparation of this manuscript. Clinical development of this promising field will require identification of biological targets in areas of medical need and multi-disciplinary collaborations between material scientists, biologists and biopharma.

7. Acknowledgements

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