Abstract The development of ultrasound contrast agents, containing encapsulated microbubbles, has increased the possibilities for diagnostic imaging. Ultrasound contrast agents are currently used to enhance left ventricular opacification, increase Doppler signal intensity, and in myocardial perfusion imaging. Diagnostic imaging with contrast agents is performed with low acoustic pressure using non-linear reflection of ultrasound waves by microbubbles. Ultrasound causes bubble destruction, which lowers the threshold for cavitation, resulting in microstreaming and increased permeability of cell membranes. Interestingly, this mechanism can be used for delivery of drugs or genes into tissue. Microbubbles have been shown to be capable of carrying drugs and genes, and destruction of the bubbles will result in local release of their contents. Recent studies demonstrated the potential of microbubbles and ultrasound in thrombolysis. In this article, we will review the recent advances of microbubbles as a vehicle for delivery of drugs and genes, and discuss possible therapeutic applications in thrombolysis.

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Introduction

For the past decades ultrasound has been an imaging modality with multiple applications. Ongoing developments improved diagnostic possibilities remarkably whereas ultrasound also became of
interest as a therapeutic tool in interventional cardiology e.g. for intravascular sonotherapy of coronary arteries and non-invasively for induction of thrombolysis.  

The first use of contrast agents in echocardiography was already reported by Gramiak and Shah in 1968. Intracardiac injection of saline improved aortic delineation, probably by reflection of ultrasound by minibubbles present in the contrast medium. Since then, microbubbles have rapidly developed to an ultrasound contrast agent in echocardiography. Microbubbles are small gas-filled microspheres that have specific acoustic properties that make them useful as a contrast agent in ultrasound imaging. First-generation microbubbles are room air microspheres. However, air microbubbles disappear in a few seconds after intravenous administration as the solubility of air in blood is high and the lungs filter microbubbles, especially those with higher diameters. Therefore, they are not useful to opacify the left cardiac chamber. Improved stability and survival was reached by stabilizing microbubbles with a thin shell, like albumin (Albunex) or galactose palmitic acid (Levovist). These microbubbles are capable of passing the pulmonary capillary bed, but cannot resist arterial pressure gradients. To increase stability of microbubbles further, second-generation contrast agents are filled with a heavy-molecular-weight gas like e.g. sulphur hexafluoride, which decreases solubility, thus improving survival and stability under higher pressure. Surfactants, sonicated albumin and (phospho)lipids are used to improve stabilization of the shell. For example, Sonovue, a second-generation contrast agent, is a phospholipid coated, sulphur hexafluoride gas containing microbubble used in diagnostic imaging. These microbubbles have much smaller diameters than room air-filled bubbles (about 2.5 μm), which improve passage of the pulmonary capillary bed.

Low acoustic pressure, i.e. a mechanical index (MI) lower than 0.05, causes linear oscillations of microbubbles and the frequency of ultrasound reflection is equal to the transmitted frequency. Increase of acoustic pressure (MI > 0.05) causes non-linear expansion and compression of microbubbles, resulting in emission of non-linear harmonic signals at multiples of the transmitted frequency. Diagnostic imaging with contrast agents uses acoustic pressure higher than MI 0.05 and the harmonic imaging modality, which improves signal—noise ratio by transmitting a fundamental frequency and receiving multiples of this frequency. This creates an acoustic impedance mismatch between body tissue and fluids containing microbubbles and makes them useful in diagnostic ultrasound imaging.

Microbubbles and their bioeffects: potential use for drug delivery

Microbubbles have been proposed as a new vehicle for delivery of drugs and genes. Several properties of microbubbles make them a promising tool for drug and gene delivery to cells. Microbubbles have specific acoustic properties that make them useful for this goal. Whereas low and intermediate acoustic pressure results in linear and non-linear oscillations of microbubbles, respectively, high pressure ultrasound (MI > 1.0) causes forced expansion and compression of microbubbles, leading to bubble destruction. Several studies have shown that destruction of microbubbles leads to permeability of cell membranes. Skyba et al. demonstrated that microbubble destruction during ultrasound exposure caused rupture of microvessels with extravasation of red blood cells. Price et al. showed that microbubble destruction with ultrasound followed by infusion of 200 nm and 500 nm polymer microspheres resulted in red blood cells and polymer microspheres moving into the interstitium. Although various mechanisms by which microbubbles increase cell permeability have been proposed, the exact mechanism remains to be resolved. Ultrasound applied to fluid causes cavitation, i.e. the creation, vibration and collapse of small gas-filled bodies by the ultrasound beam. The effect of ultrasound alone has been studied and has been shown to increase cell permeability on its own, without the addition of microbubbles. However, microbubbles in the presence of ultrasound with high acoustic pressure has an additional effect in increasing cell permeability. First, microbubbles, by acting as cavitation nuclei, can lower the threshold for cavitation. Stride and Saffari made an analysis of the conditions in the shell of the microbubble under influence of ultrasound and concluded that extremely high shell stresses and ‘bubble like behaviour’, including cavitation may be expected. In body tissue or blood, cavitation sets fluid in motion and creates small shock waves that give rise to microstreaming along the endothelial cell. Destruction of microbubbles may cause high-energy microstreams, or microjets, that will cause shear stress on the membrane of an endothelial cell and increase its permeability (Fig. 1). This increase in permeability is probably due to transient holes in the plasma membrane and possibly the nuclear membrane. A second proposed mechanism, the generation of reactive oxygen species in endothelial cells under influence of ultrasound, was investigated by Basta et al.
A significant, time-dependent increase in intracellular radical production after exposure to ultrasound was demonstrated. As the use of microbubbles together with ultrasound lowers the threshold for cavitation, this could possibly result in an increased production of free radicals, which are associated with cell killing in vitro and, as a consequence, may be also involved in enhancement of permeability of endothelial cell layers. 

A third interesting aspect is the rise in temperature in tissue following the application of high pressure ultrasound. Bubble collapse following high energetic ultrasound can create high velocity jet streams that may cause a local, transient increase in temperature. As a rise in temperature influences the fluidity of phospholipid bilayer membranes, cell membrane permeability could possibly be changed directly as a consequence of the increased bilayer fluidity. Fourth, endocytosis or phagocytosis, active membrane transport mechanisms, may also be involved in the uptake of the bubble, bubble fragments or material entrapped in microbubbles. Preliminary studies of our group showed increased uptake of fluorescent 20 nm nanospheres in myocardial cells exposed to microbubbles loaded with these nanospheres. Nanospheres were dissolved in 5 ml NaCl 0.9%. Sonovue microbubbles were prepared with NaCl 0.9% containing the same concentration of nanospheres. Cultured myocardial H9C2 cells, placed in a live-cell observation chamber, were exposed to 0.5 ml nanospheres or to 0.5 ml Sonovue microbubbles loaded with nanospheres for 5 min at a temperature of 37 °C. After 5 min all solutions were removed from the cell cultures and residual nanospheres or microbubbles were washed out. Even in absence of ultrasound, myocardial cells exposed to microbubbles loaded with nanospheres showed a significantly higher uptake of nanospheres per cell, as measured by the product of mean intensity of fluorescence and area of fluorescence, in comparison with exposure to nanospheres alone ($p = 0.002$) (Fig. 2). This suggests that active processes like endocytosis or phagocytosis may be enhanced when microbubbles are used as a vehicle for nanospheres. A fifth mechanism by which the use of microbubbles could facilitate the deposition of drugs or genes in a cell is exchange or fusion of the phospholipid microbubble coating with the phospholipid bilayer of a cell membrane. This could result in delivery of the cargo of the microbubble directly into the cytoplasm of the cell with the possibility of further uptake in endosomes or delivery to the cell nucleus. Unger et al. described that drugs and genes can be attached to microbubbles (Fig. 3). Since microbubble shell constituents mainly exist from protein-, polymer- or lipid-based coatings, several ways of attachment of drugs to a microbubble have become available. Drugs can be conjugated to the microbubble membrane with the use of a charge dependent, noncovalent binding. Especially DNA that is a large negatively charged molecule is suitable for attachment to a positively charged membrane. Another mechanism to load a bubble with drugs is to incorporate the drug in the shell. This process is importantly influenced by the nature of the drug, in terms of lipophilicity and hydrophilicity. Although it seems difficult to enclose material within the microbubble itself, as it is a gas-filled microsphere, theoretically a drug containing powder may be attached to the inside of the bubble wall. Furthermore, drugs can be bound by ligands that are embedded in the membrane. With respect to this type of conjugation, a specific technique which is based on an avidin–biotin model, used for binding of antibodies in other studies, may be of importance for the binding of certain drugs. Finally, even microbubbles with multiple layers can be constructed, in which drugs can be
dissolved. In conclusion, loading of microbubbles with a certain drug depends on several important factors like molecular weight, lipophilicity and charge. Recent experiments have shown that it is possible to create targeted microbubbles by incorporating monoclonal antibodies into the membrane. Microbubbles targeted to GPIIbIIIa receptors and to ICAM-1 have already been used in experimental studies. These studies clearly suggest that microbubbles can be used as a vehicle for drugs, and bubble destruction by high acoustic pressure ultrasound can be used for local delivery of materials attached to or entrapped in the bubble. As mentioned earlier, cavitation is probably the mechanism by which destruction of microbubbles enables extravasation of red blood cells and increases cell permeability; however, the exact process is not clear and needs further investigation. Adverse effects like a rise in blood temperature or hemolysis when using ultrasound and microbubbles, have been a cause of concern for several investigators. In theory, microbubbles exposed to ultrasound could cause mechanical stress to act on cells and consequently lead to cell injury. Bioeffects are influenced importantly by factors like ultrasound frequency and amplitude, and although these factors are generally different in diagnostic imaging than in experimental testing, temporary and persistent effects on cell function may not be ruled out and deserve attention.

Delivery of drugs and genes with microbubbles is a promising technique; however, possible side effects on tissue and organ level in humans—as well

Figure 3 Several mechanisms to attach drugs to microbubbles. a: Incorporation in the bubble; b: incorporation in the bubble membrane; c: attachment to the membrane; d: attachment to a ligand; e: incorporation in multilayer microbubble.

Figure 2 Uptake of red fluorescent nanospheres by cultured H9C2 myocardial cells after exposure to (a) nanospheres and (b) microbubbles loaded with nanospheres. Nucleus: blue staining; nanospheres: red staining.
as their clinical importance—will remain a relevant issue and need further investigation.

**Delivery of genes and drugs**

As a consequence of the identification of thousands of genetic factors, in the near future gene therapy will become an important factor in treatment of several (cardiovascular) diseases. A few genes that could be important in the management of cardiovascular disease, e.g. the gene for VEGF, have already been studied in animals and in humans.\(^{31,32}\) To be effectively expressed a gene must be delivered in tissue, taken up by the cell and incorporated into the genome in the nucleus of the cell, without being digested. An important limitation in gene therapy is to find an adequate carrier to deliver genes into specific target cells. DNA consists of large molecules and direct intravascular injection will result in removal of the DNA from the blood. Furthermore, as genes are too large to enter the cell passively, they need an active carrier. Baumgartner et al. demonstrated that direct injection of VEGF in human skeletal muscle resulted in transient increase in serum level of VEGF and induced arteriogenesis in patients with critical limb ischemia.\(^{31}\) However, direct injection of DNA in myocardial tissue of the human ischemic heart would require a possibly dangerous procedure with the subsequent risk of complications. Viral vectors, particularly lentiviruses, retroviruses and adeno-associated viruses have been extensively used as a vehicle for gene delivery.\(^{33}\) However, viral vectors have the disadvantage of being antigenic and may cause an immune response.

A few years ago an 18-year old patient died as a direct consequence of severe complications caused by an adenoviral vector used in gene therapy.\(^{33}\) Another way of non-viral gene delivery is the use of DNA plasmids, that can carry relatively large DNA molecules. Nevertheless, intramuscular injections of plasmids are known to cause side effects like local inflammation and are still a cause of concern.\(^{34}\)

Ultrasound in combination with microbubbles can provide a new, safe method of delivering genes into the cell. Microbubbles are metabolically inert, i.e. they do not cause an immune response of the host. Unlike naked DNA that is injected directly intravascularly, genes that are bound to microbubbles can be carried to the tissue without being digested. Using gel electrophoresis, it was demonstrated that DNA carried by microbubbles was stable and intact after DNA release by destruction of the microbubbles with ultrasound.\(^9\) Several studies have shown the effectiveness of gene delivery enhanced by ultrasound and microbubbles\(^{20,35-38}\) (Table 1). Shohet et al. demonstrated that albumin-coated microbubbles in combination with ultrasound destruction can be used to effectively deliver an adenoviral transgene to rat myocardium. Microbubbles with β-galactosidase transgene attached to their surface were infused in the jugular vein of rats and underwent ultrasound-mediated destruction. Nuclear staining showed that in the group with β-galactosidase containing microbubbles, rat hearts over which ultrasound destruction was applied, had a 10-fold higher β-galactosidase activity than control groups. Interestingly, in one of five control groups, microbubble destruction followed by infusion of

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**Table 1**  
**Studies assessing the delivery of genes by microbubbles**

<table>
<thead>
<tr>
<th>Author(s)/reference</th>
<th>Microbubbles</th>
<th>Tissue</th>
<th>Gene</th>
<th>US frequency</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Porter et al.(^{37})</td>
<td>PESDA</td>
<td>Carotid artery restenosis</td>
<td>Antisense oligodeoxynucleotide Adenoviral, β-galactosidase transgene</td>
<td>20 kHz—2 MHz</td>
<td>Significantly less restenosis</td>
</tr>
<tr>
<td>Shohet et al.(^{38})</td>
<td>Perfluoro-propane microbubble</td>
<td>Rat myocardium</td>
<td>Adenoviral, β-galactosidase transgene</td>
<td>1.3 MHz</td>
<td>10-Fold higher β-galactosidase activity in group MB + US</td>
</tr>
<tr>
<td>Vannan et al.(^{39})</td>
<td>Perfluorobutane, phospholipid shell</td>
<td>Canine heart</td>
<td>Plasmid chloramphenicol acetyltransferase</td>
<td>1.3 MHz, MI 1.5–1.7</td>
<td>In contrast to control groups, expression of pCAT with MB and US</td>
</tr>
<tr>
<td>Frenkel et al.(^{36})</td>
<td>PESDA</td>
<td>293 Cell lines</td>
<td>Luciferase reporter gene</td>
<td>1.3 MHz, MI 1.6</td>
<td>Optimal gene expression with DNA-loading dose of 4000 μg DNA/ml</td>
</tr>
</tbody>
</table>

PESDA, perfluorocarbon-exposed sonicated dextrose albumin; MB, microbubble; US, ultrasound.
the transgene, β-galactosidase activity was 2-fold higher than in other control groups, indicating that disruption of the membrane is an important factor in viral transduction. Similar results were obtained by Bekeredjian et al. with the use of microbubbles containing CMV-luciferase plasmids and ultrasound. Luciferase activity was seen in rat hearts after application of ultrasound, with negligible expression in other organs. Porter et al. demonstrated that perfluorocarbon-exposed sonicated dextrase albumin (PESDA) microbubbles can target the delivery of synthetic antisense oligodeoxynucleotides to the carotid artery, an antisense nucleotide preventing restenosis. The concentration of oligodeoxynucleotides taken up within the carotid vascular wall was found to be significantly increased when they were administered bound to PESDA-bubbles and while transtranjective ultrasound was applied over the carotid artery, which resulted in a significant lower rate of restenosis.

Another study, performed by Vannan et al., showed the expression of plasmid chloramphenicol acetyltransferase (pCAT) in canine hearts after intravenous injection of a relatively low concentration of pCAT. An interesting point is that this study used positively charged phospholipid bilayer vesicles, called liposomes that bind the negatively charged DNA.

During the last decades, much attention has been focused on the use of liposomes as a vehicle for drug and gene delivery. Liposomes were a promising tool as they can be loaded with material and injected intravenously to deliver their contents in target tissue. Especially DNA, that is negatively charged, can bind to positively charged liposomes, forming a complex called lipoplex. These lipoplexes are thought to bind specific receptors on the cell membrane, which subsequently can facilitate endocytosis. To promote local release of drugs or genes carried by liposomes, several techniques like acidification, enzymatic, thermal or photochemical release were used. However, although studies in an in vitro setting showed good results, in vivo studies were disappointing. Microbubbles create new options. Several studies demonstrated that microbubbles alone in combination with ultrasound can be used as an effective vehicle for gene delivery and could possibly take over the role of liposomes. A major advantage is the possibility of a targeted microbubble, as local release of material is dependent on local destruction by ultrasound. Another challenging option may consist of a combination of these two techniques. Co-administration of liposomes with microbubbles creates the possibility to increase cell permeability with ultrasound in target regions, which could prepare specific tissue for the uptake of liposomes. Furthermore, liposomes could be directly conjugated to microbubbles.

All forms of drug therapy in which drugs are administered systemically require plasma concentrations within the therapeutic range. Although many diseases such as cancer, inflammatory diseases or thrombo-embolic processes may require higher concentrations of certain drugs, plasma concentrations are limited by occurrence of potentially dangerous side effects. After systemic administration, (targeted) microbubbles loaded with drugs can rupture under influence of localized ultrasound and drug release will result in higher local concentrations in comparison with systemic administration. Besides a higher availability of the drug for receptor binding, the drug may be pushed directly into the cell as a consequence of higher membrane permeability.

Recent studies performed by Li et al. and Pislaru et al. investigated the effect of different microbubbles on the enhancement of ultrasound-mediated gene transfer. Unexpectedly, these studies demonstrated that different transfection rates were reached when using other microbubbles. These findings show that microbubbles existing from different constituents do not have equal capacities for drug and gene delivery, which suggests that it could be useful to develop a gene-delivery-specific microbubble.

Designing ‘smart’ microbubbles: targeted microbubbles

A recent and intriguing issue in contrast agents is the development of targeted microbubbles. In combination with ultrasound, microbubbles have shown to be capable of delivering genes to specific tissue. As described earlier, microbubbles can be loaded with genes and injected into a vein, followed by localized ultrasound. In this way, microbubbles are aspecific and local delivery is controlled by the local application of ultrasound. However, as currently used microbubbles are relatively stable and circulate through the whole body, delivery of material could partly result in deposition of the contents of the microbubble in tissue that is not the target tissue, e.g. in the chest wall, or in the lungs, in which microbubbles with higher diameters are filtered. Therefore, it would be greatly desirable to have a microbubble which can be targeted to a specific tissue by using ligands and receptors that are incorporated in the bubble shell (Fig. 4). This would enable active attachment of microbubbles to target tissue and create further
possibilities for diagnostic imaging and therapy like local drug delivery to target lesions. Targeted microbubbles have been developed into various types of tissue and processes, e.g., endothelial cells, thrombi, inflamed tissue and angiogenesis. Villanueva et al. tested a new perfluorocarbon gas-filled microbubble prepared with a monoclonal antibody on the shell as a ligand for endothelial cell binding (Table 2). It was demonstrated that these microbubbles with intercellular adhesion molecule-1 (ICAM-1) antibodies, in contrast to control bubbles, bind to endothelial cells expressing ICAM-1. As expression of ICAM-1 by endothelial cells is associated with early atherosclerosis, this could have major consequences for diagnosis of preclinical atherosclerosis. Another important application of this technique in vivo was the non-invasive identification of acute cardiac transplant rejection in mice.

Schumann et al. explored a method to visualize vascular clots, known to be associated with cardiovascular diseases as stroke and myocardial infarction. As the GPIIbIIIa receptors play a key role in the formation of vascular clots, a lipid-coated perfluorocarbon gas containing microbubble with bioconjugated ligands inserted into the membrane, was developed (Fig. 5). In contrast to untargeted control bubbles, targeted bubbles showed improved binding to vascular thrombi in vitro and in vivo (mouse cremasteric muscle). This finding creates great opportunities for identification and treatment of vascular clots in humans. Clot lysis is known to be improved by ultrasound, and the presence of microbubbles further stimulates this effect by cavitation. The finding of microbubbles targeted to vascular clot creates the possibility of clot lysis by ultrasound in combination with targeted microbubbles loaded with thrombolytics. Another way to target microbubbles has been investigated by Lindner et al. Lipid microbubbles are retained within inflamed tissue because of complement-mediated attachment to leukocytes. Incorporation of phosphatidylserine into the bubble shell enhances this process by amplifying complement activation and creates the possibility of ultrasound imaging of inflammation. The extent of inflammation determined by microbubble ultrasound imaging correlated closely with tissue myeloperoxidase activity, indicating inflammation. Using this technique, postischemic myocardial inflammation was also characterized.

Table 2  Studies assessing the possibility of targeted microbubbles

<table>
<thead>
<tr>
<th>Author(s)/reference</th>
<th>Microbubbles</th>
<th>Target tissue</th>
<th>Receptor</th>
<th>Ligand</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Villanuevea et al. 28</td>
<td>Perfluorobutane, lipid derived MB</td>
<td>Coronary artery endothelial cells</td>
<td>ICAM-1</td>
<td>Monoclonal antibody to ICAM-1</td>
<td>Significantly increased binding of MB with anti-ICAM-1</td>
</tr>
<tr>
<td>Schumann et al. 27</td>
<td>Lipid-coated perfluorocarbon containing MB</td>
<td>Thrombus</td>
<td>GPIIbIIIa-receptor</td>
<td>Bioconjugate ligands</td>
<td>Higher affinity of targeted MB versus untargeted MB</td>
</tr>
<tr>
<td>Lindner et al. 26</td>
<td>Lipid MB containing decafluorobutane</td>
<td>Inflamed tissue</td>
<td>Complement-mediated attachment to leucocytes</td>
<td>PS</td>
<td>Greater attachment of PS containing bubbles</td>
</tr>
<tr>
<td>Klibanov 49</td>
<td>Perfluorobutane containing MB</td>
<td>Avidin coated culture plates</td>
<td>Avidin</td>
<td>Biotin</td>
<td>Increased attachment in contrast to control MB</td>
</tr>
</tbody>
</table>

MB, microbubble; ICAM-1, intercellular adhesion molecule-1; PS, phosphatidylserine.
Klibanov studied an avidin–biotin model to study the possibility of targeting. Biotin was attached to the microbubble whereas avidin was adsorbed on the surface of culture plates. In contrast to the control surfaces, microbubbles with biotin attached firmly to avidin surfaces. A higher content of biotin in the bubble shell was more efficient than lower concentrations, which suggests that binding is regulated by a number of attachment points. Flow along the target surface was not able to dislocate the bubble from its target. This is an important finding for in vivo applications whereby a targeted microbubble is attached to an endothelial cell, which is thereby continuously exposed to capillary blood flow. Stability despite shear stress on the bubble would probably favor uptake of entrapped material in the cell.

These studies have described the first results in the assessment of targeted microbubbles. Identification of inflammation, preclinical atherosclerosis, angiogenesis and vascular clots or thrombi is promising and creates options for diagnosis and treatment of cardiovascular diseases.

**Microbubbles and thrombolysis: a test case**

Several serious cardiovascular diseases like myocardial infarction and non-hemorrhagic stroke result from thrombo-embolic processes. Although rapid thrombolysis improves morbidity and mortality from these diseases by degrading fibrinogen and fibrin in blood clots, recanalization of the occluded vessel is not reached in a substantial part of patients and systemic administration of thrombolysis can be complicated, especially by bleeding. However, in most hospitals invasive revascularization therapies are limited. For the last decade, several studies have investigated the influence of ultrasound on thrombolysis. A wide range of ultrasound frequencies, from 26 kHz to 1.03 MHz were assessed in absence or presence of fibrinolytic therapy in vitro and in vivo. Although not all studies showed a positive correlation between the use of ultrasound and thrombolysis, the presence of ultrasound was found to increase clot lysis in most studies both in vitro and in vivo. Enhancement of thrombolysis was seen particularly in low-frequency ultrasound with high power. Ultrasound, existing from positive and negative pressure waves, causes cavitation and motion of fluid. These mechanisms are thought to induce penetrating forces and shear stress on the surface of the vascular clot and could play a role in microfragmentation which makes the clot more susceptible for fibrinolytic agents. The studies mentioned above did not assess the influence of microbubbles on thrombolysis. As described before, microbubbles lower the energy needed for cavitation. Whereas linear oscillations in a low acoustic field may cause microstreams around the bubble, destruction by high power ultrasound can create

![Figure 5: Thrombus specific targeted microbubble.](image_url)
powerful microjets that accelerate the thrombus-dissolving effect of ultrasound alone. Tachibana and Tachibana were one of the first who described the effect of an echo-contrast agent on fibrinolysis in vitro. The combination of urokinase, microbubbles (Albunex) and ultrasound (170 kHz) resulted in significant increase in fibrinolysis after 60 min of incubation, in contrast to ultrasound and urokinase or urokinase alone. This result was confirmed by another study (Table 3). Birnbaum et al. were one of the first to describe the effect of microbubbles in combination with ultrasound without the use of fibrinolytics on thrombolysis in vivo. In a rabbit study it was shown that in contrast to ultrasound or contrast agent alone, dissolution of a vascular clot in the iliofemoral artery could be achieved by intravenous administration of perfluorocarbon-exposed sonicated dextrose albumin (PESDA) microbubbles and transcutaneous ultrasound (37 kHz). This result was confirmed in other studies also using rabbit iliofemoral arteries and dodecafluoropentane microbubbles.

A number of factors may influence clot lysis by microbubbles and ultrasound. First, the stability of the contrast agent. Room air containing microbubbles are less stable in blood and cavitation may be enhanced more by PESDA-bubbles. Mizushige et al. showed that thrombolysis using a dodecafluoropentane emulsion was significantly enhanced when compared with a control group, air-filled albumin microbubbles or galactose coated air-filled microbubbles, which indicates that stability of the bubble plays an important role. Another explanation for this effect is possibly the microbubble concentration, which is significantly higher in PESDA-bubbles. The observation that thrombolysis by microbubbles was accompanied by characteristic increase in echo intensity of the surface layer of the thrombus and the histological presence of numerous microcavities, specific for microbubble treated thrombi, suggests that the echo-contrast agent directly harms the clot surface. Therefore, a higher concentration of microbubbles could give rise to a higher concentration of cavitation nuclei.

### Table 3  Studies assessing the influence of microbubbles on thrombolysis

<table>
<thead>
<tr>
<th>Author(s)/ reference</th>
<th>Microbubble</th>
<th>Target in vivo/ in vitro</th>
<th>TL, US, MB</th>
<th>US frequency</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tachibana and Tachibana&lt;sup&gt;60&lt;/sup&gt;</td>
<td>Albumin MB (Albunex)</td>
<td>In vitro thrombus</td>
<td>Urokinase, US and MB</td>
<td>170 kHz, 0.5 W/cm</td>
<td>Significantly increased thrombolysis with TL, US and MB</td>
</tr>
<tr>
<td>Kondo et al.&lt;sup&gt;61&lt;/sup&gt;</td>
<td>Air-filled MB with galactose/ palmitic acid shell</td>
<td>In vitro white thrombus</td>
<td>TPA, US and MB</td>
<td>10 MHz, 0.5—1.0 W/cm</td>
<td>Significant enhancement of thrombus reduction by MB</td>
</tr>
<tr>
<td>Birnbaum et al.&lt;sup&gt;62&lt;/sup&gt;</td>
<td>PESDA MB</td>
<td>In vivo canine iliofemoral artery</td>
<td>US and MB</td>
<td>Up to 160 W/cm</td>
<td>Significant higher recanalization rate with MB</td>
</tr>
<tr>
<td>Nishioka et al.&lt;sup&gt;63&lt;/sup&gt;</td>
<td>DDFP</td>
<td>In vitro and in vivo canine iliofemoral arteries</td>
<td>MB and US</td>
<td>20 kHz, 1.5 W/cm</td>
<td>Increased clot disruption and recanalization with US and MB</td>
</tr>
<tr>
<td>Porter et al.&lt;sup&gt;64&lt;/sup&gt;</td>
<td>Air-filled MB/ PESDA MB</td>
<td>In vitro thrombus</td>
<td>Urokinase, US and MB</td>
<td>20 kHz, 40 W/cm</td>
<td>Significant better thrombolysis of PESDA than air-MB. Optimal thrombolysis with UK and MB</td>
</tr>
<tr>
<td>Mizushige et al.&lt;sup&gt;65&lt;/sup&gt;</td>
<td>Albumin shell, air-filled/ galactose shell air-filled/ DDFP-filled MB</td>
<td>In vitro thrombus</td>
<td>TPA, US, MB</td>
<td>10 MHz, 1.02 W/cm</td>
<td>Thrombolysis was greatest in DDFP-MB-group</td>
</tr>
</tbody>
</table>

MB, microbubble; US, ultrasound; TL, thrombolysis; TPA, tissue plasminogen activator; PESDA, perfluorocarbon-exposed sonicated dextrose albumin; DDFP, dodecafluoropentane.
Although improvement of clot lysis with the use of ultrasound, thrombolytics or microbubbles was seen in many in vitro and in vivo models, it is uncertain whether the same effects can be reached in humans. Earlier studies reported endothelial cell injury of microvessels, which could be a potential danger in clinical applications. Diagnostic imaging uses frequencies of around 1.5 MHz. Possibly, thrombolysis by ultrasound would require lower frequencies with higher acoustic pressure. Enhancement of thrombolysis by ultrasound is a promising technique and microbubbles would be an interesting option in treatment of cardiovascular disease; however, the bioeffects of low-frequency ultrasound with high power and the safety of these techniques in humans need further investigation.

**Conclusion**

In this article we described the most important issues in the development of contrast agents. Firstly, specific acoustic and biological properties make microbubbles a promising tool as a vehicle for drug and gene delivery. In vivo studies have been performed and showed the expression of genes delivered by microbubbles in combination with ultrasound. Although results were positive, more in vivo animal studies are needed to investigate possible future applications in humans. An interesting problem is the precise interaction of microbubbles with living cells. Although several options, like transient cell membrane holes, endocytosis, phagocytosis and fusion of microbubble shell components with the cell membrane have been proposed, the exact mechanism remains to be elucidated. Recent advances in live-cell imaging techniques (e.g. multidimensional digital imaging microscopy) offer excellent opportunities to study this process at the (sub)cellular level in real-time, thereby creating the possibility to visualize the interaction of fluorescent labeled microbubbles and myocardial or endothelial cells under high ultrasound pressure (Fig. 2).

Secondly, the use of targeted microbubbles has been a great step forward. Microbubbles have been targeted to receptors of leukocytes and blood clots, and may in the future probably be used in diagnostic imaging of thrombo-embolic or inflammatory processes. Targeted microbubbles create various challenging therapeutic options, not only in cardiovascular disease but also in treatment of inflammatory and malignant diseases. These microbubbles can be used as a vehicle for drugs or genes and local delivery can be achieved by destruction of microbubbles with ultrasound. The same mechanism could be used in treatment of thrombo-embolic processes. Enhancement of thrombolysis with the use of microbubbles and ultrasound looks promising in vitro and in vivo. The development of microbubbles targeted to GP-IIbIIIa receptors could be a step forward in treatment of vascular thrombi. Attachment of microbubbles loaded with thrombolytic agents and administration of ultrasound may in the future well be a therapeutic option.

In summary, over the past years, contrast agents in echocardiography have rapidly evolved from a diagnostic adjuvant to a possible therapeutic agent. In the coming years, this promising technique needs further development to make it available for clinical applications.

**References**


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