Summary

Microbubbles are tiny gas bubbles (~2.5 μm), originally developed as contrast agents for echocardiography. When microbubbles are exposed to ultrasound, they start oscillating. They expand and shrink according to the negative and positive pressure differences of an ultrasonic wave. These acoustic properties lead to the development of microbubbles as a therapeutic modality. As it appeared that cells surrounding oscillating or bursting microbubbles showed enhanced uptake of drugs or genes. This creates the opportunity to develop a targeted and local therapy using ultrasound and microbubbles, increasing efficacy of current medications. Although it is known that ultrasound and microbubbles cause increased uptake of therapeutics, it is still not fully understood how exactly these cells internalize the molecules, and which biological effects are induced. The research in this thesis describes the biological effects evoked by ultrasound and microbubbles at the level of individual cells, as well as the mechanisms behind increased uptake of extracellular molecules.

In detail: in **chapter 3** and **4** we studied the biological effects in cardiomyoblast cells, because ultrasound contrast agents are mainly used for contrast-enhanced echocardiography. In **chapter 3** we found that ultrasound and microbubbles caused an increase in intracellular levels of H_2O_2. This H_2O_2 was generated outside the cells, demonstrated by the addition of catalase, an extracellular scavenger of H_2O_2. Furthermore, in this chapter we demonstrated that ultrasound and microbubbles caused formation of transient pores in the cell membrane, termed sonoporation. Sonoporation is proposed to be the mechanism by which ultrasound and microbubbles cause increased permeability of the cell membrane for extracellular molecules. We demonstrated the occurrence of sonoporation by the influx of calcium ions. Although the size of calcium ions is not in proportion to the size of drugs or genes, it did demonstrate the increased permeability of the cell membrane caused by microbubbles exposed to ultrasound at low acoustic pressures. It also demonstrated that resealing of the cell membrane does occur, as the increased level of calcium ions rapidly declined back to basal levels, indicating that sonoporation of the cell membrane is a transient phenomenon. Finally, in this chapter we showed a causal relationship between the generation of H_2O_2 and the influx of calcium ions, as scavenging H_2O_2 with catalase significantly diminished the calcium influx.

In **chapter 4** we further explored the effect of the calcium influx on the membrane potential. We found that the calcium influx activated the large-conductance, outwardly rectifying potassium channels (BK_{Ca} channels). These channels over compensated the influx of positive calcium ions with an efflux of positive potassium ions, thereby causing a hyperpolarization of the cell membrane.

In **chapter 5** we switched from cardiomyoblast cells to endothelial cells, as endothelial cells are more representative cells when it comes to local drug and gene delivery using

vehicles in the bloodstream such as microbubbles, which will first encounter the endothelium after intravenous injection. The endothelial lining of the vessel wall is an important target for different therapeutic strategies, for instance delivery of vascular endothelial growth factor (VEGF) to stimulate angiogenesis or nitric oxide for vasodilatation. Furthermore, the endothelial lining is a barrier that has to be crossed when targeting the myocardium for transgene expression. It is therefore of particular interest to investigate the interaction of ultrasound and microbubbles with endothelial cells. In this chapter we cultured human umbilical cord endothelial cell (HUVECs) monolayers, and studied ultrasound and microbubble-induced effects on cellular and intercellular permeability. Also in these endothelial cells, we demonstrated a calcium influx to show increased intracellular permeability, again with a causal relationship between the generation of H_2O_2 and calcium influx. Specifically, we investigated the generation of reactive oxygen species (ROS) in three different manners. We found increased production of H_2O_2, increased nitrosylation of protein residues, an established marker for ROS, and decreased gluthatione levels (an endogenous H_2O_2-scavenger). Changes in intercellular permeability were demonstrated by formation of intercellular gaps in the monolayer, which was accompanied by rearrangement of the F-actin cytoskeleton and an increased number of individual stress fibers after exposure to ultrasound and microbubbles. Importantly, these gaps were actively resealed within 30 minutes, which we demonstrated in real-time. The fact that cells exposed to ultrasound and microbubbles cells displayed active calcium handling and monolayer restoration, indicated that cell viability was not affected. This was confirmed by flow-cytometry analysis.

The results of these three chapters demonstrate that microbubbles insonified with low-intensity ultrasound, which causes microbubbles to oscillate but not to burst, already cause a whole range of microscale bioeffects and cellular responses without affecting cell viability.

In **chapter 6** we more specifically studied the mechanisms underlying enhanced uptake of extracellular macromolecules in bovine aortic endothelial cells after exposure to ultrasound and microbubbles. The general assumption is that enhanced uptake of macromolecules occurs via sonoporation. Indeed, in this chapter we found that internalization did take place via transient pores in the membrane, but this is not the only route of entry. As a model for drug delivery we used fluorescent dextrans ranging in size from 4.4 to 500-kDa. This covers approximately the whole range of therapeutic compounds; pharmaceutical drugs are generally smaller than 4.4-kDa, proteins may range between 4 and 500-kDa, and plasmid DNA often exceeds 500-kDa. The first indication of enhanced uptake via endocytosis was shown by a difference in cellular distribution of the small and large dextrans. The 4.4-kDa dextran showed a homogenous distribution throughout the cytosol, indicating uptake via transient pores in the cell membrane. The 500-kDa, in contrast, showed a clear localization in vesicle-like structures, indicating that it might be taken up via endocytosis. The role of endocytosis in ultrasound and microbubble-enhanced uptake was further confirmed by repeating the dextran-delivery experiments with cells

deprived from ATP, or in the presence of inhibitors of the three main routes of endocytosis, i.e. clathrin-mediated, caveolin-mediated endocytosis and macropinocytosis, and by demonstrating co-localization of the 500-kDa dextran with particularly clathrin, a marker for clathrin-mediated endocytosis. These results were obtained *in vitro*, but were extended to the *in vivo* situation. We were able to deliver 500-kDa dextran to the endothelium of the rat femoral artery. The dextran molecules were again located in vesicle-like structures, and a part of these dextran-positive vesicles co-localized with clathrin. Finally, we also demonstrated the formation of transient pores in the cell membrane *in vitro*, again by the influx of calcium ions, as well as by the cellular release of pre-loaded dextrans after ultrasound and microbubble exposure. The contribution of either endocytosis or pore formation in the uptake of extracellular molecules could not be expressed in absolute numbers, but it is clearly dependent on the size of the molecule. The smaller the molecule the greater the contribution of pore formation, and the larger the molecule, the greater the contribution of endocytosis, especially when the size of the molecule exceeds the size of the transient pores. The size of the ultrasound and microbubble-induced pores in the cell membrane is determined by the parameters of the applied ultrasound. In this study we employed ultrasound with a relative low acoustic pressure (0.22 MPa), which is not likely to cause large pores in the cell membrane, and therefore the role of endocytosis became evident. When ultrasound is applied at somewhat higher intensities and/or duty cycles, larger pores may be formed and the contribution of endocytosis decreases.