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**General introduction and  
outline of the thesis**

Microbubbles, this thesis is about millions of tiny bubbles. About their initial purpose, the variety of emerging applications, but especially about the effects microbubbles have on cells when they are subjected to ultrasound. First, a brief introduction into the remarkable world of microbubbles. Microbubbles are encapsulated gas-filled bubbles (1-10  $\mu\text{m}$  in diameter), initially developed as ultrasound contrast agents. When subjected to ultrasound microbubbles start oscillating at the frequency of the ultrasound, under influence of positive and negative pressure differences in the ultrasonic wave. The size of microbubbles causes them to resonate at frequencies normally used in echocardiography. Resonating microbubbles re-emit harmonics of the transmitted frequency, and generate a high-intensity signal, whereas tissue signals at harmonic frequencies are low. Therefore, contrast-enhanced echocardiography greatly increases signal-to-noise ratio in detection of microbubbles, improving diagnosis of several cardiac disorders, such as perfusion defects, wall motion disorders, or localizing ischemic areas where there is no blood flow.

As described above, microbubbles were originally designed to improve conventional ultrasound scanning. However, recent discoveries opened up promising emerging applications. Due to their acoustic behavior, microbubbles cause increased permeability of the surrounding cells for therapeutic compounds. Moreover, microbubbles only cause increased permeability when they are insonified, this opens a window for ultrasound-targeted local delivery and enhanced cellular uptake of drugs or genes. Although it is clear that only ultrasound-activated microbubbles cause increased permeability, it is not known how exactly these cells internalize therapeutic compounds, and which cellular responses they evoke.

### **Outline of the thesis**

The main objective of this thesis is to investigate the biological effects and cellular responses induced by ultrasound and microbubbles. An extensive introduction into the field of ultrasound contrast agents is given in **chapter 2**.

**Chapters 3 and 4** describe the biological effects in cardiomyoblast cells, because US contrast agents are mainly used for imaging the heart. In order to be able to study the cellular responses of cells subjected to ultrasound and microbubbles, we mounted an ultrasound transducer on the ZEISS Axiovert Marianas<sup>TM</sup> inverted microscope (Intelligent Imaging Innovations (I.I.I.)). This microscope is a fully computerized, widefield fluorescence microscopy, i.e. no confocal laser microscope, and is equipped with a 175 Watt Xenon light source, which is less phototoxic to cells, compared to a scanning laser, allowing extended live-cell imaging. By mounting the ultrasound transducer on the microscope, we were able to image the cellular responses in detail

during ultrasound exposure, both in time and 3D. **Chapter 3** investigated the formation of hydrogen peroxide ( $H_2O_2$ ) induced by ultrasound and microbubbles, and the role of  $H_2O_2$  in ultrasound and microbubble-evoked calcium influx. The effect of a sudden rise in intracellular calcium levels on the cell membrane potential was further explored in **chapter 4**.

As intravenously injected microbubbles first come into contact with the endothelial lining of the blood vessel, the following effects were studied in human primary endothelial cells in **chapter 5**. The effects of ultrasound and microbubbles on several relevant parameters for cellular and intercellular permeability changes, i.e. reactive oxygen species (ROS) homeostasis, calcium influx, F-actin cytoskeleton, monolayer integrity and cell viability, were studied.

**Chapter 6** describes more specifically the mechanisms underlying increased uptake of macromolecules. The generally accepted idea is that ultrasound and microbubbles induce formation of transient pores in the cell membrane, i.e. sonoporation, being the mechanism underlying increased uptake. In this chapter we test out hypothesis that another mechanism, namely endocytosis, is also involved.

Finally, in **chapter 7** the results are discussed in the context of previous and recent developments in the field of ultrasound contrast agents. In addition, a brief overview of future developments is provided.

