Toward increased applicability of ultrasound contrast agents

Malin Larsson
Preface

This thesis is submitted to the KTH Royal Institute of Technology in partial fulfillment of the requirements for the Doctoral degree in Technology. The work has been performed with Professor Lars-Åke Brodin as the main supervisor, and Doctor Anna Bjällmark and Professor Kenneth Caidahl as co-supervisors. The research project was partly conducted as a part of the 3MiCRON (245572) project, that was funded by the European commission within the Seventh Framework Program.

The thesis will be publicly defended at 10 am, 28 April 2015, in the lecture hall 3-221, Alfred Nobels Allé 10, Huddinge, Sweden.
Abstract

Ultrasound is one of the most widely used modalities in medical imaging because of its high cost-effectiveness, wide availability in hospitals, generation of real-time images, and use of nonionizing radiation. However, the image quality can be insufficient in some patients. Introducing a contrast agent (CA), which comprises a suspension of 2–6 μm-sized microbubbles, improves the image quality and thus the image analysis. At present, contrast-enhanced ultrasound is frequently used during standard clinical procedures such as kidney, liver, and cardiac (echocardiography) imaging. Multimodality and targeted imaging are future areas for ultrasound CAs. Multimodality imaging may improve diagnostics by simultaneously providing anatomical and functional information. Targeted imaging may allow for identification of particular diseases.

The work within this thesis focused mainly on a novel multimodal polymer-shelled CA with the potential to be target specific. In Study I, the acoustic response was determined in a flow phantom by evaluating the contrast-to-tissue-ratio when using contrast sequences available in clinical ultrasound systems. This study showed that a high acoustic pressure is needed for optimal visualization of the polymer-shelled CA. In Study II, the in vivo performance of this CA was evaluated in a rat model, and the blood elimination time and subcellular distribution were determined. In Study III, the efficiency in endocardial border delineation was assessed in a pig model. The polymer-shelled CA had a significantly longer blood circulation time than the commercially available CA SonoVue, which is favorable for target-specific CA, in which a long circulation time increases the probability of target-specific binding. Transmission electron microscopic analysis of tissue sections from liver, kidney, spleen and lungs, obtained at different time points after CA injection showed that macrophages were responsible for the elimination of the polymer-shelled CA. A higher dose of the polymer-shelled CA was needed to obtain similar endocardial border delineation efficiency as that obtained using SonoVue. The results of Studies I–III demonstrate that the polymer-shelled CA has potential applicability in medical imaging.

Current guidelines for contrast-enhanced echocardiography are limited to cases of suboptimal image quality or when there is a suspicion of structural abnormalities within the left ventricle. It may be hypothesized that the wider use of contrast-enhanced echocardiography may help to detect some diseases earlier. Study IV assessed the diagnostic outcomes after contrast administration in patients without indications for CA use. The myocardial wall motion score index and ejection fraction were evaluated by experienced and inexperienced readers, and a screening for left ventricular structural abnormalities was performed. More cases of wall motion and structural abnormalities were detected in the contrast-enhanced analysis. Intra- and interobserver variability was lower with the use of CAs. This study suggests that the more widespread use of CAs instead of the current selective approach may contribute to earlier detection of cardiovascular disease.

Keywords: Contrast agent, Contrast-to-tissue-ratio, Echocardiography, Endocardial border, Microbubbles, Multimodal, Phantom, Polymer, Ultrasound, Wall motion score index.
Sammanfattning


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Many people have supported and inspired me during the work on this thesis. I wish to give special thanks to some of them.

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I would also like to express my gratitude to my family, relatives, and friends; to my parents, Kersti and Per-Arne, thanks for always being there when I need you; to my brother Martin, who taught me that everything in life can be a competition; till världens bästa mormor och morfar för att ni alltid engagerar er i allt jag gör; to all the dear members of “Syjuntan”, Isabel, Frida, Lollo, Ingrid, and Emelie, for all the laughs and inspiring projects you brought me; and to my dear friends Asa, Britta, and Larsa for all the encouraging words that have made this work much easier.

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Malin Larsson
Stockholm, March 2015
### Abbreviations

<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>CA</td>
<td>Contrast agent</td>
</tr>
<tr>
<td>ch</td>
<td>Chamber</td>
</tr>
<tr>
<td>CT</td>
<td>Computed tomography</td>
</tr>
<tr>
<td>CTR</td>
<td>Contrast to tissue ratio</td>
</tr>
<tr>
<td>CPS</td>
<td>Contrast pulse sequence</td>
</tr>
<tr>
<td>2D</td>
<td>Two-dimensional</td>
</tr>
<tr>
<td>3D</td>
<td>Three-dimensional</td>
</tr>
<tr>
<td>EF</td>
<td>Ejection fraction</td>
</tr>
<tr>
<td>ECG</td>
<td>Electrocardiogram</td>
</tr>
<tr>
<td>EDV</td>
<td>End-diastolic volume</td>
</tr>
<tr>
<td>ESV</td>
<td>End-systolic volume</td>
</tr>
<tr>
<td>FDA</td>
<td>The Food and Drug Administration</td>
</tr>
<tr>
<td>ICC</td>
<td>Intraclass correlation coefficient</td>
</tr>
<tr>
<td>LV</td>
<td>Left ventricular</td>
</tr>
<tr>
<td>MB</td>
<td>Microbubble</td>
</tr>
<tr>
<td>MI</td>
<td>Mechanical index</td>
</tr>
<tr>
<td>MR</td>
<td>Magnetic resonance</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
</tr>
<tr>
<td>PI</td>
<td>Pulse inversion</td>
</tr>
<tr>
<td>PM</td>
<td>Power modulation</td>
</tr>
<tr>
<td>PPI</td>
<td>Power pulse inversion</td>
</tr>
<tr>
<td>PVA</td>
<td>Polyvinyl alcohol</td>
</tr>
<tr>
<td>PVC</td>
<td>Polyvinyl chloride</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of interest</td>
</tr>
<tr>
<td>SPION</td>
<td>Superparamagnetic iron oxide nanoparticle</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TI</td>
<td>Thermal index</td>
</tr>
<tr>
<td>WMSI</td>
<td>Wall motion score index</td>
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1 Introduction

Ultrasound contrast agents (CAs) are pure blood pool agents that are frequently used in ultrasound imaging because they enhance the echo from relatively weak echogenic regions such as the vascular lumen. The introduction of a CA during image acquisition enables visualization of blood flow in the microcirculation and throughout the vasculature. A CA comprises gas-filled microbubbles (MBs) that are stabilized with a shell whose size distribution is limited by the diameter of the capillary, which is 5–10 µm. To be an ideal CA, its MBs must comprise a biocompatible material, be stable during image acquisition, circulate without causing obstructions or negative physiological effects, and be able to increase the backscattering efficiency when exposed to ultrasound.

The first application of an ultrasound CA was reported in 1968 when Gramiak and Shah demonstrated the contrast effect of injecting an agitated solution of saline containing free gas MBs [1]. However, the practical use was limited by the instability of these free gas MBs, with a lifetime of only a few seconds, in combination with their relatively large size distribution, which obstructed their passage through the pulmonary capillaries. In 1984, a CA that could pass through the pulmonary circulation was developed [2]. This CA comprised MBs that were stabilized with a thin shell. However, the thin shell of these MBs allowed air diffusion through the shell, and hence, the lifetime of these MBs was limited. To increase the MB stability of the second-generation CAs, a low-solubility gas was introduced inside the MBs, which prevented gas leakage. At present, CAs comprising MBs with improved stability are widely used during standard ultrasound procedures [3-5]. For example, the use of CAs with improved MB stability enables the accurate assessment of left ventricular (LV) volume, ejection fraction (EF), and LV structural abnormalities, such as thrombosis and noncompaction, because the endocardial border definition is improved after contrast administration [6].

Although the present commercially available ultrasound CAs are relatively stable and can be applied in various diagnostic procedures, there remains a need for improvements and extended applicability. For instance, it would be beneficial to have a more stable CA, which would allow for a longer examination time for a specific injection and prolonged shelf life. It would also be beneficial to be able to use a CA comprising MBs with a narrow size distribution, which would increase the image sensitivity. In addition to a more stable and monodispersed CA, a future application for the next generation of CAs is targeted imaging. Attaching specific antibodies or ligands to the shell surface could allow the visualization of specific inflammatory diseases such as inflammation, cancer, and atherosclerosis. Moreover, local drug delivery may be possible by incorporating a drug into a target-specific CA. This method would involve attachment of MBs into a specific region, followed by transmission of a high-pressure ultrasound pulse to disrupt the MBs in that region, resulting in local drug release. Finally, introduction of magnetic or ionizing particles into the MBs would allow for multimodality imaging, which may increase the clinical value by allowing the simultaneous viewing of both anatomical and functional images. The prospects for the next generation of CAs (i.e., third-generation CAs) are therefore considerable.
This thesis focuses on the performance of a novel polymer-shelled CA, that has been developed within the European FP7 research project 3MiCRON (245572). The polymer-shelled CA may fulfill the criteria for the third-generation ultrasound CAs. In addition to this, the potential clinical value of CA usage beyond current recommendation for echocardiography was investigated in this thesis.

1.1 Thesis outline

This thesis is based on the work described in four papers, three of which focused on the performance of a new polymer-shelled CA, and the fourth of which investigated the clinical value of increased CA usage during echocardiography. The thesis is organized into 10 chapters, starting with an introduction and followed by the specific aims for each study, lists of the included papers, and other scientific contributions. The basic principles of medical ultrasound and the general aspects of ultrasound CAs are described in Chapter 5. Chapter 6 presents the methods used in this thesis. The following three chapters (Chapters 7-9), provide a summary of the results obtained from the four studies, a general discussion and a conclusion. The future aspects are discussed in Chapter 10. Finally, the references are listed. The full versions of the papers are provided in the appendixes.
2 Aims

The general aim of this thesis was to investigate the in vitro and in vivo performance of a novel polymer-shelled CA, with target and multimodality potential, from an ultrasound prospective. In addition to this, the potential clinical value of CA usage beyond current recommendation for echocardiography was investigated. The specific aim of each study was as follow.

Study I  To determine the acoustic response of three types of the polymer-shelled CA in a tissue-mimicking flow phantom setup using different clinical ultrasound systems with optimized sequences for contrast-enhanced imaging.

Study II  To assess the blood elimination time of three types of the polymer-shelled CA in a rat model by studying the ultrasound image intensity over time. The subcellular distribution of the CA in the liver, spleen, lung and kidney was also studied by transmission electron microscopy (TEM).

Study III  To study the diagnostic features of the polymer-shelled CA by evaluating the efficiency for endocardial border delineation in a pig model. In addition, the effect of the CA injection on physiological variables was studied.

Study IV  To investigate whether contrast-enhanced echocardiography beyond the current recommendations for CA usage affects the diagnostic outcomes with respect to: 1) assessment of regional wall motion abnormalities, 2) EF classification, and 3) detection of LV structural abnormalities. A secondary aim was to evaluate whether the outcomes from the image analysis varied between users.
3 List of included papers

The thesis is based on the four papers listed below. Full versions of the papers are attached as appendixes at the end of the thesis.


3.1 Division of work between authors

Paper I: **MKL**, LÅB, and AB participated in the initiation and design of the study. LO, SM and GP produced the polymer-shelled CAs used in the study. **MKL**, ML, JN, KC, and AB participated in data collection and analysis of the results. All authors read and approved the final manuscript.

Paper II: **JH**, **MKL**, LÅB, KC, and AB participated in the initiation and design of the study. **MKL** and GP produced the polymer-shelled CAs used in the study. **JH**, **MKL**, AR, PJBK, KC, HH, and AB participated in data collection and analysis of the results. All authors read and approved the final manuscript.

Paper III: **MKL**, ML, LÅB, and AB participated in the initiation and design of the study. **MKL** and GP produced the polymer-shelled CA used in the study. **MKL**, ML, GN, KC, and AB participated in data collection and analysis of the results. All authors read and approved the final manuscript.
Paper IV: MKL, RW, and AB participated in the initiation and design of the study. MKL, CDS, EG, AABI, KS, and RW performed the ultrasound analysis. MKL processed all data and prepared the manuscript. All authors read and approved the final manuscript.
4 Other scientific contributions

4.1 Manuscripts submitted to peer-reviewed journals


4.2 Conference proceedings

4.3 Conference contributions


5 Background

5.1 Basic principles of ultrasound
Ultrasound consists of high-frequency (>20 kHz) longitudinal sound pressure waves that are used in different applications such as sonar systems, material testing, and as a communication and localization tool in bats and dolphins. Ultrasound is also commonly used in medical imaging to visualize and diagnose different diseases. Typically, medical ultrasound systems operate at the frequency range of 1-15 MHz. Compared with other imaging methods, ultrasound is inexpensive, operates without emitting ionizing radiation, provides real-time images, and allows for bedside examinations.

In medical ultrasound systems, the ultrasound wave is generated and detected by a transducer comprising piezoelectric crystals that convert voltage into pressure waves and vice versa. An ultrasound wave is a longitudinal wave with alternating pressure that induces refraction and compression in the medium through which it travels. The ultrasound image is built up from the reflected echoes produced when the ultrasound wave passes boundaries between two media with different acoustic impedance ($Z$). The acoustic impedance of a media is a measure of the particle response to a pressure wave and is determined as:

$$Z = \sqrt{\rho k}$$

where $\rho$ is the density and $k$ is the stiffness of the medium. The degree of reflection is determined by the difference in acoustic impedance between the two materials, where a large difference in acoustic impedance results in large reflection, and a low difference in acoustic impedance results in low reflection. The speed of sound in a specific media is determined by its stiffness and density. For soft tissues, the speed of sound is approximated to 1540 m/s [7]. Because the distance that the sound wave travels is proportional to time, the depth of the reflector can be determined. When the ultrasound wave propagates within the body, it is attenuated because of scattering and absorption. Scattering arises when the sound wave interacts with an irregular surface or when the size of an object is smaller than the transmitted wavelength (wavelength=speed of sound/frequency), whereas absorption occurs constantly during ultrasound wave propagation through the medium. The degree of absorption is affected by the attenuation coefficient of the propagating medium and the frequency of the incident ultrasound wave, where a high attenuation coefficient and a high frequency result in high absorption. For human soft tissues, the attenuation coefficient is approximated to 0.5 dB/cm/MHz [8].

Present medical ultrasound transducers are constructed of arrays with several piezoelectric crystals. During the transmission phase, the crystals can be activated either in groups (linear transducer) or all at the same time (phased array transducer). The linear transducer normally operates at a high frequency range (4-15 MHz), and the phased array transducer at a low frequency range (1-3 MHz). An ultrasound system can operate in several different imaging modes, providing both two-
dimensional (2D) and three-dimensional (3D) images. B-mode (brightness mode) imaging, in which the echo signal is transformed into bright spots, is the most commonly used imaging mode. The higher magnitude of the received echo is related to the brightness displayed in the image, where a high amplitude value corresponds to a brighter spot from that specific area. Other examples of imaging modes for medical ultrasound are M-mode (motion mode), Doppler mode and contrast mode; the latter is explained in more detail in Section 5.3 Ultrasound contrast-enhanced imaging techniques.

To be able to detect small and fast moving objects, high temporal and spatial resolution is needed. The temporal resolution of an ultrasound system refers to the number of images that can be acquired in 1 s, i.e. the frame rate. To increase the frame rate, a lower imaging depth can be chosen or the number of scan lines per frame can be decreased. The spatial resolution refers to the ability to resolve two adjacent objects in the lateral (perpendicular to the beam), axial (along the axis of the beam), and elevational (perpendicular to the image plane) directions. In general, high frequencies result in an increased spatial resolution. The lateral and elevational resolution can further be improved by narrowing the beam width, and the axial resolution can be improved by shortening the transmitted pulse. The length of the transmitted pulse is dependent on the wavelength (wavelength=speed of sound/frequency) and the number of cycles, implying that low number of cycles and high frequency result in a short ultrasound pulse.

In general, ultrasound is considered to be a safe imaging method. However, some aspects should be considered. A certain proportion of the energy from the ultrasound wave is constantly absorbed by the medium throughout the wave propagation. The combination of a high absorption coefficient and the high transmitted frequency of the ultrasound wave results in more energy deposition to the medium. The absorbed energy is transferred into heat, resulting in a temperature increase, which can affect chemical processes within the body. To estimate the probability of thermal effects caused by ultrasound exposure, a thermal index (TI) can be calculated. TI is an estimation of the temperature increase in the scanned volume. For example, a TI-value of 2 indicates a possible temperature increase of 2°C in the imaged area. To inform the sonographer about the thermal effect during scanning, the TI is calculated based on the ultrasound settings and is displayed on the ultrasound image. The Food and Drug Administration (FDA) in the United States limits the TI to 6.0 for medical ultrasound imaging, with the exception of prenatal and ophthalmology examinations, where a lower TI is needed [7].

In addition to the thermal effect caused by ultrasound exposure, a nonthermal effect also exists. The nonthermal effect corresponds to the cavitation phenomenon of free gas bubbles, such as CAs, within the body, which can cause cell damage. To estimate the risk of the nonthermal effect, a mechanical index (MI) can be estimated as:

\[ MI = \frac{p}{\sqrt{f}} \]  

where \( p \) is the peak negative pressure and \( f \) is the frequency of the ultrasound system applied. A higher MI corresponds to a higher risk of biological damage. To avoid biological damage by the nonthermal effect, the FDA has stated that the MI must be set below 1.9 [7]. To reduce the potential risk of cavitation when using CAs, the lowest possible MI should be considered. As discussed in Section 5.2.3 The acoustic principles of ultrasound contrast agents, the cavitation effect is dependent on the shell properties of the CA. During acquisition, the MI is displayed on the image.

5.2 Ultrasound contrast agents
Introducing CAs into diagnostic ultrasound can improve the diagnostics because the contrast enhancement caused by CAs enables detection of the microcirculation, highlights borders between
different structures, and increases the reproducibility of image analysis [6, 9, 10]. A CA comprises a suspension with gas-filled MBs entrapped in a stabilized shell. The CA suspension is administered intravenously via either a bolus or a continuous injection. The size of the MBs is limited by the diameter of the capillaries (5–10 µm). An ideal ultrasound CA must also be biocompatible, easy to inject, stable during image acquisition, and able to change its acoustic properties when exposed to an ultrasound field and flow without causing obstructions or negative physiological effects.

The first application of CAs during ultrasound examinations was described in 1968 [1]. However, the instability of these free gas MBs, with a lifetime of only a few seconds, limited their practical use. In addition, because of their relatively large size, the MBs obstructed the pulmonary capillaries. To increase the diagnostic use of ultrasound CAs, the stability and size distribution of MBs were improved by encapsulating air within a thin shell [2]. Although the air was encapsulated by a shell, air diffusion still occurred. To optimize further the use of CA in diagnostic ultrasound, a low solubility gas was introduced inside the MB cavity instead of air, which minimized gas leakage. At present, several CAs with improved stability are approved for diagnostic ultrasound (Table 5.1) [5, 11, 12]. One of the most frequently used CAs in Europe is SonoVue (Bracco Imaging, Milan, Italy). SonoVue contains the low solubility gas sulfur hexafluoride encapsulated by a phospholipid shell [13, 14].

Although the present CAs are applicable for diagnostic ultrasound, research on further improvements and extended applicability, such as multimodal and targeted imaging, is currently under way to produce the next generation of CAs. These new diagnostic features can be achieved by introducing antibodies and ligands into the shell of the MBs. Multimodality imaging may improve the clinical use of contrast-enhanced imaging by allowing both anatomical and functional images to be reviewed at the same time, either separately or by fused images. Targeted imaging can be adapted to visualize specific areas within the body, using antibodies on the shell surface, which can attract the MBs to specific regions. The next generation of ultrasound CAs could also be used as a carrier of drugs that could be released locally at chosen target sites by disruption of the MBs with high-pressure ultrasound pulses.

Table 5.1 List of the contrast agents that are approved for diagnostic ultrasound [5, 11, 12, 15].

<table>
<thead>
<tr>
<th>Name</th>
<th>Gas</th>
<th>Shell material</th>
<th>Areas of use</th>
<th>Countries</th>
</tr>
</thead>
<tbody>
<tr>
<td>SonoVue*</td>
<td>Sulfur hexafluoride</td>
<td>Phospholipid</td>
<td>LVO, EBD, breast, liver, carotid, peripheral arteries</td>
<td>USA, EU, Norway, Switzerland, Iceland, China, Singapore, South Korea, Canada, India</td>
</tr>
<tr>
<td>Definity</td>
<td>Octafluoropropane</td>
<td>Phospholipid</td>
<td>LVO, EBD, liver, kidney</td>
<td>USA†, EU†, Mexico, New Zealand, India, Australia, Korea, Singapore, United Arab Emirates</td>
</tr>
<tr>
<td>Optison</td>
<td>Octafluoropropane</td>
<td>Albumin</td>
<td>LVO, EBD</td>
<td>USA, EU</td>
</tr>
<tr>
<td>Levovist</td>
<td>Air</td>
<td>Lipid</td>
<td>LVO</td>
<td>EU, Canada, some Latin American and Asian countries</td>
</tr>
<tr>
<td>Sonazoid</td>
<td>Perfluorocarbon</td>
<td>Lipid</td>
<td>Liver, breast</td>
<td>Japan</td>
</tr>
</tbody>
</table>

LVO= left ventricular opacification, EBD= endocardial border delineation, *=Lumason in USA, †=only approved for LVO and EBD.
5.2.1 A novel polymer-shelled contrast agent
Research on a novel ultrasound CA with target-specific and multimodality potential was performed within the European research project 3MiCRON (245572), which was supported by the European Commission within the Seventh Framework Program [16-18]. This research project is a direct continuation of a previously European research project, SIGHT. The CA developed within 3MiCRON comprises monodisperse air-filled MBs with a mean diameter of 3.8 µm±0.6 µm [16]. In this CA, the MBs are stabilized with a polymeric shell, with a thickness of about 700 nm, comprising polyvinyl alcohol (PVA) [19]. Entrapping particles, such as superparamagnetic iron oxide nanoparticles (SPIONs) and technetium, either inside the shell or attached to the shell surface, makes multimodality imaging possible. Magnetic resonance (MR) imaging and emission imaging are also possible using this CA [16, 20, 21].

Before initiation of the 3MiCRON project, the ultrasound performance of this polymer-shelled CA had been tested in an in vitro setup using single-element ultrasonic transducers [22, 23]. The possibility of introducing other molecules relevant to targeted and local drug delivery has also been tested [24]. Although these experiments demonstrated that the polymer-shelled CA has potential for extended use in medical imaging, extensive studies are still needed before its regular use in clinical practice. For example, the in vivo interactions and diagnostic features for the different modalities must be identified. The work within this thesis focused on the in vivo interaction and the diagnostic features of the polymer-shelled CA during ultrasound imaging.

5.2.2 Safety of ultrasound contrast agents
In general, ultrasound CAs are considered to be safe for use in clinical practice [25, 26]. Common minor adverse effects such as headache, a warm sensation, and flushing have been observed. Some people may develop allergic reactions to CAs.

In 2007, fatal accidents in patients with acute coronary syndrome or acute myocardial infarction were reported after CA use. This led to a black-box warning (i.e., a warning that appears on the package insert) issued by the FDA [27]. Nevertheless, the numbers of adverse effects reported for CA use in diagnostic ultrasound are low compared with other imaging methods [27]. Additionally, complications after a medical procedure might not strictly relate to the procedure itself but to the health status of the patient. Several ultrasound CA safety studies have been published after 2007, and these indicate no increased mortality for contrast-enhanced ultrasound compared with non-enhanced ultrasound [28-30]. The FDA now recommends monitoring of vital signs, electrocardiogram (ECG) and oxygen saturation during at least the first 30 min after CA administration in patients with pulmonary hypertension or an unstable cardiopulmonary condition but not in every patient as required before [31].

At high pressures, cavitation of gas bubbles such as CAs within the body occurs, and this can cause vessel rupture [32]. To minimize the risk of cavitation effects during image acquisition, moderate pressure of the ultrasound wave and limited exposure time should be considered. The pressure level is however dependent on the elasticity profile of the CA.

5.2.3 The acoustic principles of ultrasound contrast agents
The MBs have high echogenicity (i.e., high ability to reflect the ultrasound wave) because of the considerable difference in acoustic impedance between the blood and the MBs. As a consequence, a large proportion of the transmitted ultrasound wave will be reflected when interacting with the MBs. However, the reflected ultrasound wave will scatter in all directions because the MB size is smaller than the wavelength of the ultrasound wave, and therefore, only a portion of the ultrasound echo will be detected by the transducer. By contrast, CAs are highly compressible when exposed to an ultrasound pressure wave because the MBs can change size when located in an altering pressure
Figure 5.1 A) Linear oscillation of a microbubble (MB) (solid line) at low acoustic pressures. B) Nonlinear oscillation of an MB (solid line) at moderate acoustic pressure. MB size during absence of acoustic pressure is illustrated with a dashed line.

field, resulting in MB oscillation. Consequently, the MBs can generate sound and be more than a passive reflector. MBs expand when located in a negative pressure field but compress in a positive pressure field. The degree of MB oscillation is dependent on the ultrasound pressure, the frequency applied, and the properties of the encapsulating gas and shell material [33-35].

At low acoustic pressures (\(<100 \text{ kPa}\)), the MBs will oscillate linearly because they expand as much as they compress, and thus the returning echoes from the MBs have the same frequency as the transmitted ultrasound wave, see Figure 5.1 A. When increasing the acoustic pressure (\(\sim 100 \text{ kPa-1 MPa}\)), the MBs will start to oscillate in a nonlinear regime, because the MBs’ expansion phase is longer than the compression phase, see Figure 5.1 B. This will result in multiples of the fundamental frequency (f₀), which are referred to as harmonics (2f₀, 3f₀ etc.) and subharmonics (f₀/2, f₀/3 etc.). The second harmonic (i.e., double the transmitted frequency) is usually the most dominant harmonic because higher frequencies are attenuated to a greater extent, and the limitation in the bandwidth of the transducer. With further increases in the acoustic pressure (\(\sim >1 \text{ MPa}\)), violent expansion and compression will cause MB destruction, which will be either complete or partial, by fragmentation of the MB shell. This will result in formation of unstable free gas bubbles, which increase the acoustic scattering within a few milliseconds [36]. The MB destruction also generates a wide band of harmonic signals.

Common to all CAs is the fact that each CA type has a resonance frequency at which the MB oscillation is preeminent. The resonance frequency for an MB that is stabilized with a thin elastic shell is defined as:

\[
 f_0 = \frac{1}{2\pi} \sqrt{\frac{3\gamma P}{\rho R_0^2} + \frac{S_e}{m}}
\]  

(3)

where \(R_0=MB\) diameter, \(\gamma\)ideal adiabatic constant of the encapsulating gas, \(P=\text{ambient fluid pressure}\), \(\rho=\text{density of the shell material}\), \(S_e=\text{shell elasticity parameter}\), and \(m=\text{mechanical resistance of the surrounding media}\) [34, 35]. The \(S_e\) is determined as:

\[
 S_e = \frac{8\pi E_l}{(1-\nu)}
\]

(4)
where $E=$ shell elasticity, $t=$ shell thickness, and $v=$ Poisson’s ratio. According to equation 4, the higher the shell elasticity, the higher the resonance frequency of the CAs and vice versa. Moreover, the larger the MB diameter, the lower the resonance frequency. Typically, the resonance frequency of ultrasound CAs is in the range of the frequencies used in clinical ultrasound systems.

The elastic properties of the MB shell are also an important factor for the oscillation profile of the MBs. An MB with a thick shell in combination with a low flexibility shell material oscillates less than an MB with a thinner and more flexible shell [33]. Stiff MBs need a higher acoustic pressure to produce the same large radius change compared with more elastic MBs [37].

5.3 Ultrasound contrast-enhanced imaging techniques
The nonlinear response obtained from the MB oscillation in an acoustic field can be used when constructing an ultrasound contrast sequence that is optimized for contrast-enhanced imaging. The nonlinear response includes mainly the second harmonic, and the bandwidth of the transducer must be optimal for both the fundamental frequency used during the transmission phase and twice the transmitted frequency obtained during the receiving phase. Common to all ultrasound contrast sequences is the fact that they are based on multipulse transmission. Examples of ultrasound contrast sequences used in clinical ultrasound systems are pulse inversion (PI), power modulation (PM), contrast pulse sequence (CPS) and power pulse inversion (PPI). Compared with conventional grayscale (B-mode) imaging, a lower frame rate is obtained for the multipulse contrast sequences because several pulses are transmitted for each scan line instead of only one. Most of the ultrasound contrast sequences used in the clinical ultrasound systems operate at low acoustic pressures, which minimize the nonlinear response from tissue and MB destruction.

5.3.1 Pulse inversion
During the transmission phase in PI, every second transmitted pulse is an inverted copy of the previous one [15], see Figure 5.2 A. Because tissue generates mainly linear response when exposed to an ultrasound field, the reflected echoes from tissue includes two identical inverted pulses. On the other hand, MBs display a nonlinear response when exposed to an ultrasound field, and therefore, the reflected echoes from the MBs will not be two identical inverted pulses (Figure 5.2 A). By adding the response from two subsequent echoes, the tissue signal can be canceled out, while the echoes from the MBs will remain. The added signal is then used for image reconstruction.

5.3.2 Power modulation
In PM, two pulses are transmitted in phase but with different amplitudes, and the second pulse is twice the amplitude of the first pulse [15], see Figure 5.2 B. The echoes from the transmitted pulses are received by the transducer, whereupon the first echo is amplified by a factor of two before being subtracted from the second echo. The difference between the reflected echoes from tissues will result in signal cancellation, whereas the echoes obtained from the MBs are not canceled out because of the nonlinear oscillation (Figure 5.2 B).

For a high-frequency ultrasound system developed for small-animal imaging, a different approach of the PM technique is applied compared with that used in the clinical ultrasound systems. Instead of detecting the second harmonic signal, the high-frequency transducers detect the subharmonic signal [38, 39]. This is needed because tissue generates a significant nonlinear response at high frequencies in combination with the high attenuation of high frequencies [40].
Figure 5.2 The pulse scheme for pulse inversion (A), power modulation (B), contrast pulse sequence (C), and power pulse inversion (D). Note that the illustration describes only the principle behind each contrast sequence.

5.3.3 Contrast pulse sequence
CPS is the general name for an imaging technique in which several pulses with both phase and amplitude modification are emitted for each scan line [41]. Transmission of three pulses for each scan line, with a 1:2:1 ratio of peak amplitudes, and phase shifts of 0°, 180°, and 0°, is a combination that is used often in present clinical ultrasound systems that use the CPS technique. The received echoes are then summed and used during image reconstruction. This allows for detection of nonlinear responses from the MBs, see Figure 5.2 C.

5.3.4 Power pulse inversion
When applying PPI, three pulses are transmitted for each scan line [15]. The first and the third transmitted pulses are identical, whereas the second transmitted pulse is an inverted replica, see Figure 5.2 D. An advantage of using a contrast sequence based on three pulses instead of two pulses is that the decorrelation caused by tissue movements can be minimized.

5.4 Clinical application of ultrasound contrast agents
One of the most frequently used clinical applications for contrast-enhanced ultrasound is cardiac imaging (echocardiography). Abdominal diagnostics, such as kidney and liver imaging, are other types of contrast-enhanced ultrasound commonly used in clinical practice [3, 4]. In this section, applications for contrast-enhanced ultrasound are explained with the focus on cardiac imaging.

5.4.1 Endocardial border delineation
Reliable diagnostic procedures in patients with cardiovascular disease require accurate assessment of LV volume (end-diastolic volume (EDV) and end-systolic volume (ESV)), EF, and myocardial wall
motion and thickening pattern. Obesity, chronic lung disease, and ventilator support are examples of factors that can cause insufficient image quality and impede correct image analysis [42]. The use of CA during echocardiography examination has been shown to improve LV opacification and endocardial border definition compared with standard echocardiography in patients whose conditions lead to suboptimal images [42, 43]. Contrast-enhanced images in patients with poor image quality allow for a more correct assessment of LV volumes, EF, and myocardial wall motion and thickening pattern, which are important factors for the characterization and prognosis of cardiac diseases such as myocardial ischemia and myocardial infarction [44, 45]. Additionally, identification of LV structural abnormalities, such as thrombus, apical aneurysm, ventricular noncompaction and hypertrophy, can be improved by CA administration [5]. The improved endocardial border delineation when using contrast-enhanced echocardiography instead of standard echocardiography is illustrated in Figure 5.3.

EF measurement and myocardial wall motion evaluation can be adapted during both standard echocardiography (at rest) and stress echocardiography. The increased heart rate obtained during stress can be induced by having the patient perform supine bicycle exercise or by intravenous injection of dobutamine or vasodilator (so-called 'pharmacological stress') [46]. It has been shown that the diagnostic accuracy in patients with suspected acute myocardial infarction (e.g., complete and stable occlusion of a coronary artery) is improved by using standard contrast-enhanced imaging [47, 48]. In a patient with suspected coronary artery disease (e.g., incomplete occlusion of a coronary artery), the wall motion and thickening pattern at rest are usually seen as normal. To detect abnormalities caused by the coronary occlusion, increased oxygen consumption is needed. This can be obtained during stress echocardiography and is therefore preferable in patients with suspected coronary artery disease.

According to guidelines, CAs should be adapted only during standard echocardiographic examination in patients with suboptimal image quality (i.e., ≥2 contiguous segments not visualized on a grayscale image) or when the LV volumes, EF, and myocardial wall motion must be assessed accurately [5, 49]. CAs can also be used during standard echocardiography when there is suspicion of LV structural abnormalities, such as apical hypertrophic cardiomyopathy, ventricular noncompaction, thrombus, or ventricular pseudoaneurysm. By contrast, the guidelines for CA use during stress echocardiography suggest that CA should be used for the diagnostic assessment of segmental wall motion and thickening pattern [5]. Because the major reason to use stress echocardiography is to diagnose patients with suspected myocardial ischemia, the stress examination routinely includes CA administration.
Assessment of left ventricular volumes and ejection fraction

To assess EDV and ESV, manual tracing of the endocardial border is typically performed on apical long-axis ultrasound images. End diastole is visually defined as the maximal cavity area in adjunction to mitral valve closing, and end systole as the minimal cavity area in adjunction to mitral valve opening. In accordance with the recommendations of the European Association of Cardiovascular Imaging and the American Society of Echocardiography, papillary muscles and trabeculations should be excluded from the cavity during tracing. After tracing EDV and ESV in both the 2 chamber (ch) and 4ch views, the volume is calculated using Simpson’s biplane method [50]. The principle aim of this method is to summarize the volume from a number of elliptical discs. The cross-sectional area of the discs is based on the diameters obtained from the two imaging planes, and the height of each disc is calculated as a fraction (usually 1/20) of the longest chamber length (i.e., the distance between the atrioventricular plane and the apex) of the two imaging planes. The EF (i.e., the fraction of blood ejected from the ventricle during a heartbeat) is then calculated from the EDV and ESV as:

$$EF = \frac{(EDV-ESV)}{EDV} \times 100$$  \hspace{1cm} (5)

According to the reference values, patients with an EF <55% have systolic dysfunction, which is classified as 45–54% mildly abnormal, 30–44% moderately abnormal, and <30% severely abnormal [50].

Evaluation of myocardial thickening and movement pattern

To evaluate myocardial thickening and movement pattern, the myocardium is divided into different segments that are attributed to the three major coronary arteries, see Figure 5.4. Each segment should be analyzed separately in the context of its movement pattern. A normal contracting segment is graded as 1, hypokinesis as 2, akinesis as 3, and dyskinesis as 4. After completing the segment evaluation, a patient wall motion score index (WMSI) can be determined by calculating the sum of all wall motion scores divided by the number of segments visualized [50].

![Figure 5.4 Illustration of the coronary artery (right coronary artery (RCA), left anterior descending (LAD), and circumflex (CX) coronary arteries) blood supply to different segments of the myocardium. Some of the segments have variable coronary perfusion. Reprinted with permission [50].]
5.4.2 Myocardial perfusion imaging

Another diagnostic tool to identify myocardial ischemia and infarction is to study myocardial perfusion. Because CAs are pure blood pool agents, their distribution within the myocardium will reflect the blood perfusion volume and flow rate.

At low and mediate CA concentrations, the relationship between brightness in an ultrasound image, and the backscattered signal obtained from the CA is linear [51]. Consequently, the perfusion in different tissues can be determined either visually or by calculating the image intensity over time. During the initial phase of perfusion, the number of MBs in a specific area will increase, and thus the image intensity will increase. When the number of MBs entering the area is equal to the number of MBs leaving the area, the image intensity will remain constant. The shift in the image intensity over time can be represented by a wash-in curve, where the slope ($\beta$) of the curve represents the blood flow, and the plateau (A) represents the blood volume in the investigated area [45], see Figure 5.5. Normally, the steady state of contrast-enhancement is obtained within 5-6 heartbeats. A continuous intravenous inflow of CA is preferable during perfusion imaging as the steady state time period increases. A continuous inflow of CA enables for flash replenishment studies because high acoustic pressure pulses can be transmitted, causing MB destruction in the image plane. The reperfusion of MBs into the imaged area can then be monitored when lowering the acoustic pressure. Figure 5.6 shows an example of an image from patient with defect myocardial perfusion in the apical segments. It can be clearly seen that there is a limited amount of MBs in the diseased area.

![Figure 5.5 Wash-in curve represents the shift in image intensity over time after replenishment of MBs in a specific area. The slope ($\beta$) represents the blood flow, and the plateau (A) represents the blood volume.](image1.png)

![Figure 5.6 Apical ultrasound image of the left ventricle. Perfusion defect is observed in the apical segments.](image2.png)
6 Materials and methodology

6.1 Contrast agents
Combinations of the commercially available ultrasound CA SonoVue (Bracco Imaging, Milan, Italy) and different types of the polymer-shelled CA (Plain PVA, PVA Type A, and PVA Type B), developed within the European FP7 research project 3MiCRON, were used in Studies I-IV. Characteristics of the CAs are shown in Table 6.1. Except for Study IV, SonoVue was used as the reference when evaluating the in vitro and in vivo performance of the polymer-shelled CA. In Study IV, the clinical benefit of CA use beyond the current recommendations for CA use during echocardiography examinations was studied.

Plain PVA is an unmodified version of the polymer-shelled CA that can be visualized with ultrasound, whereas PVA Type A and PVA Type B are two shell-modified versions designed for both ultrasound and MR imaging. The Plain PVA was produced during high-shear stirring with an Ultra-Turrax (Wilmington, NC, USA) at 8000 rpm for 2 h by cross-linking synthesized PVA at the air-water interface [17]. When producing PVA Type A and PVA Type B, SPIONs were either covalently linked to the shell surface via chitosan molecules (PVA Type A) or embedded within the shell (PVA Type B) of the CA during MB formation [16]. An illustration of the shell structure of the different types of the polymer-shelled CA is shown in Figure 6.1.

Table 6.1 Overview of the characteristics of the contrast agents used in Study I-IV [13, 16, 19, 37].

<table>
<thead>
<tr>
<th>Contrast agent</th>
<th>Shell material</th>
<th>Gas</th>
<th>Diameter and shell thickness</th>
<th>Study</th>
<th>Dose (ml)</th>
<th>Concentration (MBs/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SonoVue</td>
<td>Phospholipid</td>
<td>Sulfur hexafluoride</td>
<td>2.5 (1-10) µm, 4 nm</td>
<td>I</td>
<td>-</td>
<td>10^4/10^5/10^6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>II</td>
<td>-</td>
<td>10^4/10^5/10^6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>II</td>
<td>0.4</td>
<td>2.5 × 10^8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>III</td>
<td>1.5, 3.5</td>
<td>5 × 10^8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>IV</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Plain PVA</td>
<td>Polymer</td>
<td>Air</td>
<td>3.8±0.6 µm, 700 nm</td>
<td>I</td>
<td>-</td>
<td>10^4/10^5/10^6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>II</td>
<td>-</td>
<td>10^4/10^5/10^6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>II</td>
<td>0.4</td>
<td>7.5 × 10^7/6.6 × 10^8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>III</td>
<td>1.5, 3.5</td>
<td>5 × 10^8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>PVA Type A</td>
<td>Polymer + SPIONs</td>
<td>Air</td>
<td>3.8±0.6 µm, 700 nm</td>
<td>I</td>
<td>-</td>
<td>10^4/10^5/10^6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>II</td>
<td>0.4</td>
<td>7.5 × 10^7/6.6 × 10^8</td>
</tr>
<tr>
<td>PVA Type B</td>
<td>Polymer + SPIONs</td>
<td>Air</td>
<td>3.8±0.6 µm, 700 nm</td>
<td>I</td>
<td>-</td>
<td>10^4/10^5/10^6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>II</td>
<td>0.4</td>
<td>7.5 × 10^7/6.6 × 10^8</td>
</tr>
</tbody>
</table>

MB= microbubble, PVA=polyvinyl alcohol, SPION=superparamagnetic iron oxide nanoparticle, *= no information available, †= used for histological analysis, §= used for ultrasound imaging.
**Figure 6.1** Schematic illustration (A) and transmission electron micrographs (B) of the different types of the polymer-shelled contrast agent (Plain PVA (left), PVA Type A (middle), and PVA Type B (right)). The dark dots indicate superparamagnetic iron oxide nanoparticles either covalently bound to the shell surface (PVA Type A) or embedded inside the shell (PVA Type B). Scale bar represents 500 nm.

### 6.2 Phantom design

A tissue-mimicking flow phantom including a vessel with a lumen size similar to that of the human carotid artery was designed for Studies I and II, see Figure 6.2. The vessel phantom was constructed by heating a mixture of 15% (w/w) PVA (Sigma-Aldrich, St. Louis, MO, USA), 3% (w/w) graphite powder (particle size <50 µm, Merck KGaA, Darmstadt, Germany), and deionized water to 90°C. The graphite powder was added as an acoustic scatterers [52]. The mixture was then poured into a cylindrical vessel mold of acrylic plastic (diameter 12 mm, length 100 mm), and the vessel lumen was created by inserting a metal rod (diameter 6 mm) into the center of the vessel mold. Thereafter, the vessel phantom mold was stored at -20°C for 12 h and at room temperature (20°C) during the subsequent 12 h. The number of freeze-thaw cycles affects the stiffness of the phantom because crystallites are formed through the freeze-thaw process. To achieve similar acoustical properties as in human tissue (speed of sound=1540 m/s), the phantom underwent three freeze-thaw cycles [53].

The vessel phantom was then attached in a polyvinyl chloride (PVC) box (100 mm x 60 mm) using plastic connectors. To minimize reflections, a 3 mm thick rubber layer was placed at the bottom of the PVC box. The tissue-mimicking material was constructed by heating a solution of 3% (w/w) agar (Merck KGaA) and 4% (w/w) graphite powder (particle size <50 µm, Merck KGaA) to 85°C. To avoid dissolving the vessel phantom, the tissue-mimicking solution was cooled to about 55°C before it was poured into the PVC box. To prevent the vessel lumen from collapsing, the vessel was filled with deionized water before the tissue-mimicking solution was poured into the PVC box. The height of tissue-mimicking material covering the vessel varied according to the ultrasound system used. When a high-frequency ultrasound system was applied (frequency=18 MHz, Study II), the tissue-mimicking layer covered a few millimeters above the vessel, whereas in studies involving a clinical ultrasound system, a layer of 3 cm (frequency=4-5 MHz, Study I) or 6 cm (frequency=1.3-1.5 MHz, Study I) was used.
Figure 6.2 A) Schematic illustration of the phantom setup. B) The cross-sectional vessel dimensions of the tissue mimicking flow phantom, C) Ultrasound B-mode image of the phantom obtained in the short-axis view.

6.3 Study subjects

In vivo experiments were conducted in Studies II, III, and IV. Before initiation of the experiments, the study protocols were approved by the regional ethical committee for animals (Study II in rats, N372-10, Stockholm, Sweden; Study III in pigs, S 38-12, Huddinge, Sweden) and humans (Study IV- 2014/805-32, Stockholm, Sweden) experiments. An overview of the characteristics of the animals and humans is shown in Table 6.2.

6.3.1 Animals

Standardized protocols were used to induce anesthesia [54, 55]. In brief, rats were anesthetized utilizing 3-4% isoflurane, and 1-2% isoflurane was ventilated to maintain anesthesia. Temgesic (0.01 mg/kg) was given before and 12 h after surgery to the rats used for histology analysis 24 h after CA injection. In the pigs, anesthesia was initiated by an intramuscular injection of fentanyl and atracurium, and was maintained with 1-2% isoflurane ventilation. To prevent pulmonary and systemic hypertension, an intravenous injection of liometacen (10 mg/kg, 5 mg/kg/h) was given to the pigs before CA injection [56].

Table 6.2 Overview of the study subjects in Studies II-IV.

<table>
<thead>
<tr>
<th>Study II - rats</th>
<th>Study III - pigs</th>
<th>Study IV - humans</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of subjects : ultrasound analysis</td>
<td>16*</td>
<td>8</td>
</tr>
<tr>
<td>Number of subjects : histology analysis</td>
<td>9†</td>
<td>-</td>
</tr>
<tr>
<td>Female/male</td>
<td>0/25</td>
<td>8/0</td>
</tr>
<tr>
<td>Body weight/ mass index</td>
<td>~450 g</td>
<td>35±3 kg</td>
</tr>
</tbody>
</table>

* = 4 for each CA type, † = 3 rats, euthanized either at 10 min, 40 min or 24 h after CA injection, for each CA type, CA = contrast agent.
6.3.2 Humans
A retrospective search of patients who underwent stress echocardiography at Karolinska University Hospital, Huddinge, Sweden, from January 2013 to February 2014 was performed in Study IV. Stress echocardiography patients were chosen because both grayscale and contrast-enhanced images at rest are included in the imaging protocol (Table 6.2). This enabled a comparison of the diagnostics between grayscale and contrast-enhanced images in the subset of patients outside the current recommendations for CA use in standard echocardiography. Patients were therefore excluded if their grayscale images at rest fulfilled the criteria for CA use during standard echocardiography (i.e., if ≥2 contiguous segments were not visualized on any of the apical grayscale images or if there was suspicion of LV structural abnormalities, such as apical hypertrophic cardiomyopathy, ventricular noncompaction, thrombus, or ventricular pseudoaneurysm [5, 49]. Note that the grayscale images are acquired routinely with optimal image quality, as deformation analysis using speckle tracking is performed at rest and stress. Experienced sonographers (n=4, 3-15 years of experience) visually evaluated whether patients should or should not be included. Patients were also excluded if apical 2ch, 3ch, or 4ch images in either grayscale or contrast mode at rest were missing.

6.4 Experimental procedures

6.4.1 In vitro ultrasound imaging
Phantom imaging was performed in Studies I and II. Before image acquisition was initiated, dilution series of each CA were prepared by diluting a stock solution with deionized water to a final volume of 500 ml, see Table 6.1. As shown in Figure 6.2, the phantom was connected to a peristaltic pump (Watson Marlow, Falmouth, UK) that circulated the CA solution within a closed system at a speed of 8.8 cm/s (Study I) or 1.8 ml/s (Study II). Before image acquisition, the transducer was fixed over the phantom vessel lumen by using a tripod holder. In addition, the focus point was adjusted to the center of the phantom vessel lumen.

Thereafter, ultrasound image sequences of the vessel phantom were acquired for different clinical ultrasound systems (Study I) and for a high-frequency ultrasound system (Study II). Detailed information about the ultrasound systems and image settings is shown in Table 6.3. The image acquisition started with the lowest concentration (Study I: 10^4 MBs/ml, Study II: 10^2 MBs/ml) and was then increased gradually. A circulating solution of pure deionized water was imaged before each dilution series in Study II, as a control sequence. To minimize the influence of possible MB bursting because of ultrasound exposure, the order of the image acquisition with the different contrast sequences in Study I was determined by a random number generator in MATLAB (MathWorks, Natick, MA, USA). After image acquisition of a specific dilution series was completed, a cleaning procedure was performed to rinse the plastic tubes from MBs. During the cleaning procedure, pure water was circulated within the closed system.

6.4.2 In vivo ultrasound imaging
In vivo ultrasound imaging was performed in rats (Study II), pigs (Study III), and humans (Study IV). In Study II and III, the ultrasound image settings such as contrast sequence, MI, focus depth, and gain were individually optimized for each CA type before image acquisition, see Table 6.4. All patients in the retrospective study (Study IV) had undergone the standard procedure for stress echocardiography, in which apical grayscale and contrast-enhanced images were obtained at rest and stress.

In Studies II and III, the CA bolus was injected manually through a venous catheter into the vena jugularis at a speed of ~0.03 ml/s (Study II) and ~0.75 ml/s (Study III). Before the subsequent CA injection, the catheter was washed with a flush of 0.9% saline. The different CA types used in Study
III were injected in a randomized order in every subject, and two repeated injections of one particular CA type were given in the same subject for the ultrasound imaging part in Study II.

The transducer was fixed in a tripod holder to minimize movements of the transducer during image acquisition of the carotid artery in Study II. Ultrasound long-axis image sequences (1.5-2 min) were acquired before and during the subsequent 10 min after CA injection. The image acquisition stopped earlier than 10 min after CA injection if no contrast enhancement was observed visually in the vessel lumen. To ensure that no contrast enhancement from the previous CA injection interacted with the subsequent CA injection, a waiting period of 30 min was inserted between repeated injections.

In Study III, ECG-triggered ultrasound image sequences of the left ventricle from the apical 2ch view were acquired by experienced sonographers at end systole once in every cardiac cycle. The image acquisition continued until no or very low contrast enhancement was observed visually in the left ventricle. The subsequent CA injection started about 10 min after the previous CA injection.

6.4.3 Histology
To assess the interactions of the CA at the subcellular level, rat tissue samples of liver, spleen, kidney, and lung were collected at 10 min, 40 min, and 24 h after the CA injection in Study II (Table 6.1 and Table 6.2). The organs were cut into 1 mm thick slices and immediately immersed into a fixation buffer (2% glutaraldehyde, 1% paraformaldehyde in 0.1 M phosphate buffer) and placed in a refrigerator until further preparation. Sections of 1 mm² were randomly selected for liver, spleen

| Table 6.3 | Characteristics of the ultrasound image settings used in the in vitro studies. |
|-----------|----------------------------------|----------|--------|---------|
| Ultrasound system | Transducer | Contrast sequence | Frequency (MHz) | Frame rate (frames/s) | MI |
| Study I | | | | |
| Vivid7 (GE Healthcare) | M12L | Pulse inversion | 5/10 | 32.7 | 0.2-1.2 |
| Vivid7 (GE Healthcare) | M3S | Pulse inversion | 1.5/3.1 | 25.7 | 0.2-0.8 |
| iE33 (Philips Healthcare) | S5-1 | Power modulation | 1.5/3.2 | 39.0 | 0.2-1.2 |
| iE33 (Philips Healthcare) | S5-1 | Power pulse inversion | 1.3/2.6 | 39.0 | 0.2-1.0 |
| Acuson Sequoia 512 (Siemens Healthcare) | 9L4 | Contrast pulse sequence | 4/8 | 17.0 | 0.2-1.0 |
| Study II | | | | |
| VisualSonics (Fujifilm, VisualSonics) | MS250 | Power modulation | 18 | 15.0 | 4 % |

| Table 6.4 | Characteristics of the ultrasound image settings used in the in vivo studies. |
|-----------|----------------------------------|----------|--------|---------|
| Ultrasound system | Transducer | Contrast sequence | Frequency (MHz) | Frame rate (frames/s) | MI |
| Study II | | | | |
| VisualSonics (Fujifilm, VisualSonics) | MS250 | Power modulation | 18 | 15 | 4 % |
| Study III | | | | |
| iE33 (Philips Healthcare) | S5-1 | Power pulse inversion | 1.6/3.2 | - | 0.89-0.93 |
| iE33 (Philips Healthcare) | S5-1 | Power modulation | 1.6/3.2 | - | 0.39 |
and lung tissue because these tissues are relatively homogenous, and 1 mm² tissue sections from the filtration barrier between the blood and urine in the glomerulus were chosen for the kidney. The tissue sections were then immediately transferred to 0.1 M phosphate buffer and stored at 7-8°C overnight. To increase the tissue contrast during TEM imaging, samples were placed in an osmium fixation buffer (2% OsO₄ in 0.1 M phosphate buffer). The tissue sections were then prepared for TEM by following the protocol described by Hansson et al. [57]. In brief, the tissue sections were dehydrated in ethanol followed by acetone and embedded in Epoxy resin (LX 112) (Ladd, Williston, NH, USA). Thereafter, 50 nm thick tissue slices were cut using a Leica Ultracut UCT (Leica, Vienna, Austria) and placed on formvar coated copper grids coated with a thin carbon film and imaged using a Philips CM 120 transmission electron microscope (FEI, Hillsboro, OR, USA). Before tissue sectioning of the kidney, semithin sections of ~0.5 µm were examined in a light microscope to identify the areas of interest. The electron microscope was operated at an accelerating voltage of 80 kV and the micrographs were recorded onto Kodak SO-163 films and digitized using an Epson Perfection V600 photo scanner (Epson, Long Beach, CA, USA). In total, 144 tissue sections (4 sections/rat x 9 rats x 4 tissue types) were prepared for each CA type. Defective tissue sections were excluded from the analysis.

6.5 Image analysis

The ultrasound images were analyzed offline using custom-made workstations such as EchoPAC for Vivid7 acquisition (GE Healthcare, Little Chalfont, United Kingdom), QLAB for iE33 acquisition (Philips Healthcare, Best, The Netherlands), Syngo for Acuson Sequoia 512 acquisition (Siemens Healthcare, Erlangen, Germany), and Vevo LAB for VisualSonics acquisition (Fujifilm, VisualSonics, Toronto, Canada).

6.5.1 Contrast-to-tissue-ratio

The visualization capability for the different CAs in Study I was investigated by calculating the contrast-to-tissue-ratio (CTR) for each combination of contrast sequence, CA type, CA concentration, and MI. A circle with an area of ~12.5 mm² was placed manually in the phantom vessel lumen and in the surrounding tissue at the same depth, from which the mean intensity within that region of interest (ROI) was calculated (Figure 6.3 A). The mean intensity from each image frame (n=38) in the image sequence was exported to MATLAB and the CTR for each image frame was calculated as:

\[ CTR = I_{ROI_{Vessel}} - I_{ROI_{Phantom}} \] (6)

where I represents the mean intensity in decibels obtained from the phantom vessel lumen (ROI_{Vessel}) and the surrounding tissue phantom (ROI_{Phantom}).

![Figure 6.3 A) Illustration of the contrast-to-tissue ratio measurements. Circle 1 represents the region of interest (ROI) obtained from the phantom vessel lumen, and circle 2 represents the ROI obtained from the surrounding tissue-mimicking material. B) Illustration of the acoustic shadowing measurements. Circle 1 represents the ROI obtained from the upper part, and circle 2 represents the ROI obtained from the lower part of the phantom vessel lumen.](image-url)
6.5.2 Acoustic shadowing

Acoustic shadowing caused by CA absorption and scattering of the ultrasound was investigated in Study I according to the method proposed by Tiemann et al. [58]. This was tested for all CA dilutions, CA types, and contrast sequences at the MI at which the maximal CTR was obtained. Two identical ROIs with an area of ~3 mm² were placed manually in the upper and lower part of the phantom vessel lumen, see Figure 6.3B. A significant shadowing of a specific CA concentration was assumed if a significantly higher intensity was observed in the upper part of the vessel phantom lumen compared with the lower part.

6.5.3 Detection limit of contrast agent

To determine the detection limit of CAs for the high-frequency ultrasound system used in Study II, the image intensity within the phantom vessel lumen was analyzed. A circular ROI (15 mm²) was placed manually in the phantom vessel lumen. The mean intensity from each image frame (n=260) in the image sequence was then obtained for both the control fluid (ROI control) and for each CA dilution (ROI dilution) for both Plain PVA and SonoVue. By comparing the mean intensity within the ROI control with each ROI dilution, the lowest detectable concentration of the two CAs was obtained.

6.5.4 Blood elimination time

The blood elimination profile of each CA used in Study II was determined by investigating the change in image intensity within the rat vessel lumen over time after a bolus injection of a specific CA. An oblong ROI (area of 1.5 mm²) was placed manually in the center of the vessel lumen, and the mean intensity within the ROI from each image frame was exported to MATLAB for further processing. If the image view was shifted because the animal moved, the image sequence was excluded from further analysis. To minimize noise, a convolution filter with a normalized rectangular function of eight times the sample length was applied, after which the peak intensity for each CA injection was identified. Normalized curves were then approximated to an exponential decay curve (starting point=peak intensity) using the nonlinear last-squares method, from which the blood elimination time for each CA type was determined.

6.5.5 Endocardial border delineation

To assess the endocardial border delineation efficiency of the polymer-shelled CA, a comparative study was performed in a pig model using the commercially available CA SonoVue (Study III). The efficiency of endocardial border definition was evaluated visually by three readers with extensive experience in using contrast-enhanced echocardiography who were blinded to CA type and dose (Table 6.1). The uniformity of the LV opacification was investigated using semiautomatic software.

Before visual evaluation was initiated, the image sequences for each CA type and dose were divided into time intervals of 20 s starting from the first passage of CA into the left ventricle. From each time interval, the frame including the best filling of the left ventricle was selected. Thereafter, the optimal frames from the time intervals for each given CA type and dose were merged together into a chronological image series, starting with the time interval 0–20 s. From this image series, each reader individually identified the time for clinically useful contrast enhancement for each image series. Then, each reader selected the image with the best potential for endocardial border delineation from each image series. From that image, the endocardial border delineation capability was evaluated by dividing the left ventricle into six segments, see Figure 6.4. A nonvisible segment was graded as 0, weakly visible as 1, and well visible as 2.

The images with best potential for endocardial border delineation were used in the semiautomatic delineation of the left ventricle. The semiautomatic software was based on the level-set method developed in MATLAB [59, 60]. Because of the ambiguous structure between a contrast-enhanced atrium and contrast-enhanced ventricle, the semiautomatic software was modified for the current
application by allowing manual definition of the atrioventricular plane. Additionally, the number of iterations (2500) and the algorithm of Caselles et al. [59] were selected from initial tests. Before initiation of the delineation, the atrioventricular plane was defined manually. An elliptical region (with an area of about 1/4 of the total area of the left ventricle) was placed in the center of the left ventricle, after which the automated delineation was initiated. To evaluate the automatic delineation, the readers performed a manual delineation by selecting 20 border points, and then spline interpolation was used to create a reference border. The correlation between the two delineations was evaluated by calculating the Dice value (D) as:

\[ D(A, B) = \frac{2|A \cap B|}{|A| + |B|} \]  

where A is the LV cavity obtained from the semiautomated delineation, and B is the LV cavity obtained from the reference delineation. The union of A and B corresponded to the overlapped area of the two delineations (A and B). Hence, an ideal match corresponds to a Dice value of 1 (Figure 6.5).

6.5.6 Physiological data
In Study III, oxygen saturation, heart rate, and arterial pressure were sampled every minute during anesthesia using ECG, pulse oximetry, and invasive arterial blood pressure measurement, respectively. The effect of CA injection on these physiological variables was evaluated by comparing the values obtained before (n=3) and after (n=3) CA injection.

6.5.7 Myocardial wall motion
From the apical 2ch, 3ch, and 4ch views, the myocardial WMSI at rest was obtained for grayscale and contrast-enhanced images by visually assessment (Study IV). The patients (n=192) were allocated between four experienced readers, and the same reader evaluated both the grayscale and the contrast-enhanced images of one patient, at least two days apart. Before image analysis was initiated, all patient data were made anonymous. The left ventricle was divided into 18 segments, each of which was assigned a score depending on the myocardial thickening and movement pattern (Figure 6.4) [50]. A normally contracting segment was graded as 1, hypokinesis as 2, akinesis as 3 and dyskinesis as 4. The segment was scored as ‘NA’ if myocardial wall motion evaluation of that specific segment was not applicable. A patient’s WMSI was defined as the sum of all scores, divided by the number of graded segments [50].
To assess intraobserver variability, repeated analyses were performed on the images for five patients for each experienced reader (4 readers x 5 patients/reader), who were blinded to the previous results. The images for these five patients were also analyzed by another experienced reader, which allowed the determination of the interobserver variability for WMSI evaluation. In addition, the variability between readers with different experience levels was evaluated by having the inexperienced readers (n=2) perform repeated analysis on the reevaluated patients (n=20).

6.5.8 Left ventricular volume
In Study IV, EDV, ESV and EF were evaluated. As in the WMSI analysis, patients (n=192) were allocated between four experienced readers. The same reader evaluated both the grayscale and the contrast-enhanced images of one patient, with a minimum of two days between evaluations. Before image analysis, all patient data were made anonymous. The LV volumes and EF were calculated using Simpson’s biplane method [50]. A manual tracing of the endocardium was then performed at end systole and at end diastole from apical 2ch and 4ch views. End diastole was visually defined as the maximal cavity area in adjunction to mitral valve closing, and end systole was defined as the minimal cavity area in adjunction to mitral valve opening. According to guidelines, trabeculations and papillary muscles were excluded from the cavity during tracing [50].

To assess intraobserver variability, repeated analyses was performed on five patients for each experienced reader (4 readers x 5 patients/reader), who were blinded to the previous results. The images from these five patients were also analyzed by another experienced reader, which allowed the determination of the interobserver variability for EF measurements. The variability between readers with different experience level was evaluated by having inexperienced readers (n=2) perform repeated analysis on the reevaluated patients (n=20).

6.5.9 Left ventricular structural abnormalities
In Study IV, screening for LV structural abnormalities on the contrast-enhanced images compared with the grayscale images was performed by four experienced readers (48 patients/reader) who were blinded to patient information.

6.6 Statistical analysis
Data analyses were performed using the statistical software IBM SPSS Statistics19 (Armonk, NY, USA), MATLAB, and Excel (Microsoft Corporation, Redmond, WA, USA). A p-value of 0.05 was considered to be significant.

Continuous variables, such as image intensity, heart rate, oxygen saturation, blood pressure, body mass index, age, LV volumes and EF, are expressed as mean and standard deviation. Paired Student’s t tests and z tests were used to compare numerical groups (t test: acoustic shadowing (Study I), physiological data (Study III), LV volumes and EF (Study IV); z test: lowest detectable concentration (Study II)). The WMSI was determined by calculating the sum of all scores divided by the number of visualized segments [50].

Blood elimination time and endocardial segment score were classified as categorical data and were analyzed to compare categorical groups using the Mann–Whitney U test (for blood elimination time in Study II) or the Wilcoxon signed-rank test (for the endocardial segment score in Study II). In the Wilcoxon signed-rank test, identical segments from each pig were matched together during analysis.

The differences in Dice value distribution for the different CA types and concentrations (Study III), and for the WMSI and EF distribution between the grayscale and contrast-enhanced images (Study IV) were tested using McNemar’s test.
To assess the intra– and interobserver variability between different readers for the WMSI and EF (Study IV), intraclass correlation coefficients (ICCs) was calculated.

6.7 Summary of the methodology for each study

**Study I: Visualization of multimodal polymer-shelled contrast agents using ultrasound contrast sequences: an experimental study in a tissue mimicking flow phantom**

The acoustic response of three types of the polymer-shelled CA (Plain PVA, PVA Type A, and PVA Type B) was investigated in a phantom setup by calculating the CTR for various combinations of clinical ultrasound systems, contrast sequences, CA concentrations, and MI. The commercially available CA SonoVue was used as a reference. The optimal concentration of the different types of the polymer-shelled CA was evaluated further by studying the acoustic shadowing.

**Study II: Investigation of the elimination process of a multimodal polymer-shelled contrast agent in rats using ultrasound and transmission electron microscopy**

The blood elimination profile of three types of the polymer-shelled CA (Plain PVA, PVA Type A, and PVA Type B) was examined in a rat model by measuring the image intensity from ultrasound images of the common carotid artery over time after a bolus injection of CA. The commercially available CA SonoVue was used as a reference. The subcellular distribution of the polymer-shelled CAs was also investigated using TEM in a histological study.

**Study III: Endocardial border delineation capability of a novel polymer-shelled contrast agent**

To determine the diagnostic features of the polymer-shelled CA, the capability for endocardial border definition for contrast-enhanced ultrasound images was evaluated visually by experienced readers in a pig model. Apical 2ch views were obtained for different concentrations of the polymer-shelled CA. During image analysis, the left ventricle was divided in six segments and every segment was graded as 0= not visible, 1= weakly visible or 2= well visible. The duration of clinically useful contrast enhancement was evaluated visually. Semiautomatic segmentation software was used to investigate the uniformity of LV opacification. The results were evaluated against the responses using the commercially available CA SonoVue. In addition, the effect of the CA injection on physiological variables was studied.

**Study IV: The potential clinical value of contrast-enhanced echocardiography beyond current recommendations**

The clinical value of contrast-enhanced echocardiography beyond the current recommendations for CA use was investigated in a retrospective study. Patients with indications (≤2 contiguous segments not visualized on a grayscale image or suspicion of LV structural abnormalities) for CA use or lack of apical 2ch, 3ch, or 4ch images were excluded from the study. The diagnostic outcome was evaluated using the WMSI, EF classification, and detection of LV structural abnormalities. The inter- and intraobserver variability was also assessed.
7 Results

The main results from the four studies are presented in the subsections within this chapter. A more detailed presentation of the results can be found in the full versions of the papers in the appendixes.

Study I: Visualization of multimodal polymer-shelled contrast agents using ultrasound contrast sequences: an experimental study in a tissue mimicking flow phantom

For a specific CA type and contrast sequence, similar distribution patterns of the CTR were observed for the three CA concentrations tested (10^4, 10^5, and 10^6 MBs/ml). As shown in Figure 7.1, which includes the CTR distribution for 10^5 MBs/ml, the maximal CTR for each CA type was obtained for the PPI sequence. In general, the PPI and CPS sequences provided a higher CTR for the different types of the polymer-shelled CA, whereas PI and PM resulted in a higher CTR for SonoVue. A relatively low MI (0.2-0.6) was preferable for optimal visualization when using the PI and PM sequences, whereas a high MI (1.2) was optimal when using the PPI sequence. For the CPS sequence, an intermediate to high MI (0.6-1.0) gave the highest CTR. Acoustic shadowing was observed for all types of the polymer-shelled CA and contrast sequences for a concentration of 10^6 MBs/ml (Plain PVA and PVA Type A: p<0.001 for all contrast sequences; PVA Type B: p<0.001 for PI, PPI, and CPS, p=0.001 for PM), see Table 7.1. For concentrations <10^5 MBs/ml, significant acoustic shadowing was observed only for the PPI sequence (PVA Type A: p<0.001, PVA Type B: p<0.05).

![Figure 7.1](image_url) The maximal contrast-to-tissue-ratio (CTR) and its associated mechanical index (brackets) for each contrast agent (Plain PVA (light gray), PVA Type A (gray), PVA Type B (dark gray), and SonoVue (black)) for the different ultrasound contrast sequences (CPS=contrast pulse sequence PI=pulse inversion, PM=power modulation, PPI=power pulse inversion). A negative CTR indicates that the tissue response exceeded the response from the CA. The CA concentration was 10^5 microbubbles/ml.
Table 7.1 Presentation of the acoustic shadowing analysis, obtained at the MI (shown in parentheses) with maximal CTR for each combination of CA, ultrasound contrast sequence, and concentration (MBs/ml).

<table>
<thead>
<tr>
<th></th>
<th>Plain PVA</th>
<th>PVA Type A</th>
<th>PVA Type B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10^6</td>
<td>10^5</td>
<td>10^4</td>
</tr>
<tr>
<td>PI (5/10 MHz)</td>
<td>p&lt;0.001</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>(0.4)</td>
<td>(0.4)</td>
<td>(0.4)</td>
</tr>
<tr>
<td>PI (1.5/3.1 MHz)</td>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>(0.4)</td>
<td>(0.4)</td>
<td>(0.4)</td>
</tr>
<tr>
<td>PM (1.5/3.2 MHz)</td>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>(0.4)</td>
<td>(0.4)</td>
<td>(0.4)</td>
</tr>
<tr>
<td>PPI (1.3/2.6 MHz)</td>
<td>p&lt;0.01</td>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>(1.2)</td>
<td>(1.2)</td>
<td>(1.2)</td>
</tr>
<tr>
<td>CPS (4 MHz)</td>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>(1.0)</td>
<td>(0.8)</td>
<td>(0.6)</td>
</tr>
</tbody>
</table>

Significant effects are indicated by gray shading and p-values; non-significant effects are indicated by lack of shading and NS. CA=contrast agent, CPS=contrast pulse sequence, CTR=contrast-to-tissue ratio, MB=microbubble, MI=mechanical index, NS=nonsignificant, PI=pulse inversion, PM=power modulation, PPI=power pulse inversion, PVA=polyvinyl alcohol.

Study II: Investigation of the elimination process of a multimodal polymer-shelled contrast agent in rats using ultrasound and transmission electron microscopy

The lowest detectable CA concentration for the high-frequency ultrasound system was 10^4 MBs/ml for both Plain PVA and SonoVue. This was evident by the observation that a significantly higher mean intensity was recorded for CA dilutions >10^3 MBs/ml than for the control fluid.

A rapid decrease in the image intensity occurred after the peak enhancement for all CAs, indicating a fast elimination of the CAs in the early elimination phase, see Figure 7.2. However, a substantial difference in the pattern of the decrease in image intensity between the different CAs was observed. SonoVue and PVA Type A had a more rapid decrease in intensity in the early phase compared with Plain PVA and PVA Type B. By contrast, the blood half-life time (50% of the peak intensity) indicated that SonoVue had a significantly shorter detectable circulation time than the different types of polymer-shelled CAs (Plain PVA and PVA Type B: p<0.001; PVA Type A: p<0.05), see Figure 7.2 and Table 7.2. In addition, the blood elimination time (20% of peak intensity) for the three types of the polymer-shelled CA differed. Significant longer elimination times were observed for Plain PVA (p<0.01) and PVA Type B (p<0.001) than for PVA Type A (Table 7.2). No significant difference in blood elimination time was observed between Plain PVA and PVA Type B.

In the 144 tissue sections analyzed, all detected MBs (731) were found in the blood vessel, which indicated that no MBs were taken up by the parenchyma or endothelial cells. The MBs were localized inside, adherent to, or in the vicinity of macrophages (Figure 7.3). Several MBs could be phagocytized by one single macrophage (Figure 7.3 A) and both intact and collapsed MBs (Figure 7.3 C) were found for all analyzed time points (10 min, 40 min, and 24 h). However, a higher proportion of collapsed MBs were detected in tissue sections from the 24 h post-CA injection compared with those obtained 10 or 40 min after CA injection. Most MBs were found in the tissue sections analyzed 10 min (278 MBs) and 40 min (292 MBs) after CA injection. It was found that most of the MBs accumulated in the lungs during the first two time points and then shifted to the spleen and liver tissues after 24 h. Common to all time points was the fact that few MBs were observed in the kidney. None of these MBs passed through the filtration barrier between the blood and urine in the glomerulus.
Figure 7.2 Blood elimination profile for SonoVue (n=8) (solid), Plain PVA (n=7) (dash-dash), PVA Type A (n=8) (dash-dot), and PVA Type B (n=7) (dotted), $r^2$=coefficient of determination.

Table 7.2 Median blood elimination time to 50% and 20% of the peak intensity for each contrast agent (SonoVue, Plain PVA, PVA Type A, and PVA Type B).

<table>
<thead>
<tr>
<th>Blood elimination time (s)</th>
<th>Statistical significance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SonoVue</td>
</tr>
<tr>
<td></td>
<td>50%</td>
</tr>
<tr>
<td>SonoVue</td>
<td>7.5</td>
</tr>
<tr>
<td></td>
<td>(5-14)</td>
</tr>
<tr>
<td>Plain PVA</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td>(51-104)</td>
</tr>
<tr>
<td>PVA Type A</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>(13.3-24)</td>
</tr>
<tr>
<td>PVA Type B</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td>(54-88)</td>
</tr>
</tbody>
</table>

*p<0.05, **p<0.01, ***p<0.001, NS= nonsignificant, Interquartile ranges are shown in parentheses.

Figure 7.3 Transmission electron micrographs of localized microbubbles (MBs) in rat tissue. A) PVA Type B MBs observed in a lung macrophage 10 min after injection, B) Plain PVA MB inside a spleen macrophage 40 min after injection, C) Two MBs (PVA Type A), one intact (left) and one collapsed (right) in a lung macrophage 40 min after injection, D) One MB (Plain PVA) inside a rat kidney macrophage 10 min after injection. Scale bar represents 1 µm.
Study III: Endocardial border delineation capability of a novel polymer-shelled contrast agent

There was no significant difference in segment score distribution between SonoVue (1.5 ml) and the 5 ml injection of Plain PVA when considering all segments, see Table 7.3. This was opposite to what was observed for the two smaller injection volumes of Plain PVA, for which a significantly higher proportion of the segments were assigned a lower segment score compared with SonoVue (1.5 ml: p<0.05; 3 ml: p<0.001). Analysis of the differences in segment score on a regional level showed no significant differences in segment score between any of the injection volumes of Plain PVA and SonoVue for the apical segments. By contrast, significantly higher segment scores were obtained for SonoVue when studying the other regions (mid and basal segments) of the left ventricle (1.5 ml: p<0.001 for basal; 3 ml: p<0.05 for mid, p<0.001 for basal; 5 ml: p<0.05 for mid and basal segments), with one exception for the mid region when injecting 1.5 ml Plain PVA. The longest time of clinically useful contrast enhancement was observed for SonoVue and 5 ml Plain PVA. With this combination, LV opacification was sufficient for 20–40 s after the CA injection, whereas the two smaller injections of Plain PVA had only an acceptable LV opacification during the first 20 s post-CA injection.

The results from semiautomatic delineation confirmed that the highest dose (5 ml) of Plain PVA was needed to obtain comparable endocardial delineation capability as for SonoVue (1.5 ml). Figure 7.4 shows that similar Dice value distribution (<0.8; 0.8–0.9; >0.9) was obtained for these two injections. This indicates that an adequate LV opacification could be achieved when using these CAs because the semiautomatic delineation showed good agreement with the reference delineation. When comparing the Dice value distribution for SonoVue and 5 ml Plain PVA with the two smaller doses of Plain PVA (1.5 ml and 3 ml), a significantly higher percentage of the Dice values shifted toward the highest interval (>0.90) for SonoVue and 5 ml Plain PVA. The differences in homogeneity of the LV opacification between the three doses of Plain PVA are shown in Figure 7.5.

No significant change in oxygen saturation, heart rate, or mean arterial pressure for any dose of the polymer-shelled CA was observed. Table 7.4 shows the mean percentage differences before (mean 3 min) and after (mean 3min) CA injection for the different physiological variables.

Table 7.3 The segment score distribution for endocardial border delineation for each contrast agent (CA) and dose.

<table>
<thead>
<tr>
<th></th>
<th>All segments</th>
<th>Apical</th>
<th>Mid</th>
<th>Basal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 1 2</td>
<td>0 1 2</td>
<td>0 1</td>
<td>2 0 1 2</td>
</tr>
<tr>
<td>1.5 ml SonoVue</td>
<td>1 39 104</td>
<td>1 33 14</td>
<td>0 0</td>
<td>48 0 6</td>
</tr>
<tr>
<td>1.5 ml Plain PVA</td>
<td>4 49 91</td>
<td>1 26 21</td>
<td>0 3</td>
<td>45 3 20</td>
</tr>
<tr>
<td>p&lt;0.05</td>
<td>p&lt;0.001</td>
<td>NS</td>
<td>NS</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>3 ml Plain PVA</td>
<td>12 48 84</td>
<td>3 27 18</td>
<td>3 4</td>
<td>41 6 17</td>
</tr>
<tr>
<td>p&lt;0.001</td>
<td>p&lt;0.05</td>
<td>NS</td>
<td>p&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>5 ml Plain PVA</td>
<td>2 47 95</td>
<td>2 22 24</td>
<td>0 4</td>
<td>44 0 21</td>
</tr>
<tr>
<td>NS</td>
<td>NS</td>
<td>p&lt;0.05</td>
<td>p&lt;0.05</td>
<td></td>
</tr>
</tbody>
</table>

A significant distribution difference in segment scores between SonoVue and the different types of the polymer-shelled CA is indicated by its p-value and a nonsignificant difference with NS. 0= not visible, 1= weekly visible, 2= well visible.
Figure 7.4 The Dice value distribution for each contrast agent (SonoVue (black), 1.5 ml Plain PVA (dark gray), 3 ml Plain PVA (gray), and 5 ml Plain PVA (light gray)) over the Dice value ranges of <0.80, 0.80–0.90, and >0.90. In total, 24 delineations (3 readers x 8 pigs) were performed for each contrast agent and dose. A significant Dice value distribution between SonoVue and the different doses of the polymer-shelled CA is indicated by *.

![Dice value distribution graph](image)

Table 7.4 The mean percentage differences and standard deviations of oxygen saturation (SaO₂), heart rate, and mean arterial pressure 3 min after injection compared with 3 min before injection of a contrast agent (CA).

<table>
<thead>
<tr>
<th>Injected CA dose</th>
<th>SaO₂* (%)</th>
<th>Heart rate* (bpm)</th>
<th>Mean arterial pressure* (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5 ml SonoVue</td>
<td>0% ± 0</td>
<td>0% ± 1</td>
<td>5% ± 9</td>
</tr>
<tr>
<td>1.5 ml Plain PVA</td>
<td>0% ± 0</td>
<td>0% ± 2</td>
<td>-2% ± 2</td>
</tr>
<tr>
<td>3 ml Plain PVA</td>
<td>0% ± 0</td>
<td>-4% ± 8</td>
<td>-1% ± 4</td>
</tr>
<tr>
<td>5 ml Plain PVA</td>
<td>0% ± 0</td>
<td>-2% ± 3</td>
<td>2% ± 3</td>
</tr>
</tbody>
</table>

*Mean value before CA injection: SaO₂ =98%, heart rate=93 bpm, mean arterial pressure=78 mmHg.

Figure 7.5 Demonstration of the overlap between the areas delineated using the semiautomatic software (red line) and the reference delineation (yellow line) for 1.5 ml (A), 3 ml (B), and 5 ml (C) of Plain PVA. The corresponding Dice values were 0.61 (A), 0.82 (B), and 0.92 (C).

![Images A, B, C](image)
Study IV: The potential clinical value of contrast-enhanced echocardiography beyond current recommendations

In total, 321 patients were screened for this study. After the review process, 192 patients were included (body mass index=27±4 kg/m², systolic blood pressure=133±23 mmHg, diastolic blood pressure=73±11 mmHg). The indications for stress echocardiography for these patients were as follows: ischemic heart disease (n=171), follow-up after heart transplantation (n=9), ischemic heart disease and low-flow/low-gradient aortic stenosis (n=6), low-flow/low-gradient aortic stenosis (n=5), and arrhythmia (n=1).

The number of the segments that were not possible to analyze decreased from 4.9% (169 segments) to 0.4% (14 segments) when evaluating contrast-enhanced images instead of grayscale images. A significant difference (p<0.05) in the WMSI distribution was observed for patients with minor wall motion abnormalities (WMSI≤1.5) when comparing grayscale analysis with contrast-enhanced analysis. In the grayscale analysis, a significantly higher percentage of the patients were classified as normal (WMSI=1), whereas in the contrast-enhanced analysis, a significantly higher percentage of patients were classified with a wall motion abnormality (1<WMSI≤1.5) in any segment. When only considering patients with normal myocardial wall motion in every segment (i.e., patients with NA segments excluded) after grayscale analysis (n=72), 44 patients (59%) were reclassified as having abnormal wall motion. An increase in the wall motion score in ≥2 segments was the most common outcome (24 patients, 32%).

The EF classification was retained in most patients (139 patients, 72%) for both imaging modes. A slightly higher percentage of patients were classified as healthier (higher EF) when CA was present (30 patients (15%) compared with 23 patients (12%)). However, no significant difference in EF classification was observed between grayscale and contrast mode.

The intra- and interobserver variability for experienced readers decreased for the WMSI (ICC: 0.89–0.94 and 0.61–0.87, respectively) and EF (ICC: 0.90–0.98 and 0.80–0.95, respectively) when analyzing contrast-enhanced images instead of grayscale images. The variability between inexperienced and experienced readers decreased for both the WMSI (ICC: 0.58 to 0.81) and EF (ICC: 0.77 to 0.94) measurements after addition of CA.

More LV structural abnormalities, such as apical aneurysm (n=4), hypertrophy (n=1), apical thrombus (n=1), and increased trabecula formations (n=21), which indicates an enhanced risk of noncompaction, were detected with contrast analysis compared with grayscale analysis. Illustrations of the results can be seen in Appendix 4.
8 Discussion

The usability of the polymer-shelled CA, the clinical value of CA use beyond current recommendations during standard echocardiography, and the future of the next generation of ultrasound CAs are discussed in this section.

8.1 The novel polymer-shelled contrast agent

Study I showed that a high acoustic pressure was needed for optimal visualization of the polymer-shelled CA when applying the contrast sequences available in commercial ultrasound systems. The in vivo performance of the polymer-shelled CA was evaluated both in a rat model (Study II), in which the blood elimination time and subcellular distribution were determined, and in a pig model (Study III), in which the efficiency for endocardial border delineation was assessed. The results of these studies indicated that the polymer-shelled CA can be adapted in contrast-enhanced diagnostic imaging. Compared with the commercially available CA SonoVue, the polymer-shelled CA showed significantly longer blood circulation times. The visualization capability and the in vivo performance of the polymer-shelled CA are discussed in detail in the following sections of this chapter.

8.1.1 Visualization capability

The results of Study I and III suggest that the polymer-shelled CA can enhance contrast in ultrasound images when using the ultrasound contrast sequences available in clinical ultrasound systems. However, a high acoustic pressure combined with a relatively high CA concentration is needed to obtain the same backscattering efficiency as for the commercially available CA SonoVue. A common feature for the evaluated ultrasound contrast sequences is the detection of the nonlinear response from the MBs, which is generated during ultrasound exposure because of the nonlinear MB oscillation. Thus, it is essential for an ultrasound CA to be able to generate harmonics. The generation of harmonics is dependent on the viscoelastic properties of the MB shell, which in turn are determined by the shell thickness and shell material [33]. A thicker MB shell is less flexible and has considerably greater damping, which causes a reduction in MB oscillation and thereby a decrease in the nonlinear response. Compared with SonoVue, the polymer-shelled CA has a stiffer [37] and thicker shell (~700 nm for the polymer-shelled CA [19] vs ~4 nm for SonoVue [37]), implying that a higher MI (i.e., higher acoustic pressure) is needed to obtain a nonlinear response similar to that of SonoVue.

Although a high MI is needed for optimal visualization of the polymer-shelled CA, a high MI also increases the risk of MB destruction and a nonlinear response from tissue. However, MB destruction is limited even at high MI values when using the rather stable polymer-shelled CA. In addition, the nonlinear response from tissue can be limited by using an appropriate filtering technique because the nonlinear response from CAs has a wider bandwidth than the nonlinear response from the surrounding tissue [61].
Irrespective of the CA type tested in Study I, the highest CTR was observed when applying the PPI sequence. In the comparison of the maximal CTR for PPI with the maximal CTR obtained by the other contrast sequences tested, PPI was optimal at higher MI (MI≈1.2 vs MI≈0.8 for CPS, MI≈0.4 for PI and PM). Thus, the filtering technique in PPI must be designed so that the nonlinear response from tissue is minimized. Nevertheless, one limitation with the PPI technique could be the detection of single MBs in the microcirculation because of the increased probability for MB destruction at high acoustic pressures. This effect is however more pronounced for SonoVue than for the stiffer polymer-shelled CAs.

Because absorption of the ultrasound wave occurs constantly during wave propagation, it would be optimal to transmit high acoustic pressure pulses to enable the optimal image view of deeper regions. On the other hand, a high MI will result in significant MB destruction in the near-field regions when using the highly flexible and thin-shelled CA SonoVue. Therefore, it may be suggested that the more stable polymer-shelled CA would be superior to SonoVue in terms of visualization of both near- and far-field regions because the polymer-shelled CA tolerates higher acoustic pressure before it ruptures. However, this phenomenon was not observed in Study III, in which same efficiency for endocardial border delineation was observed for SonoVue and the polymer-shelled CA in the mid and basal segments of the heart. It has been shown that attenuation in a contrast media suspension increases with increased concentration and pressure [62]. Attenuation of the ultrasound pulse because of high CA concentration (injected dose 5 ml vs 1.5 ml) combined with the high acoustic pressure (MI=0.89–0.93 vs MI=0.39) may explain why the polymer-shelled CA had the same efficiency for endocardial border delineation as SonoVue.

To optimize the visualization of the polymer-shelled CA, it would be beneficial to have ultrasound contrast sequences optimized for stiff MBs. Efforts have been made within the 3MiCRON project to develop ultrasound contrast sequences that improve the visualization of the polymer-shelled CA. For example, a subtraction sequence with improved detection of the polymer-shelled CA was developed by our research group [63]. The subtraction sequence is based on the subtraction of a reference image (without CA) from a contrast-enhanced image (including CA). The two images are matched spatially using block matching with normalized cross-correlation and in time using the ECG. Nevertheless, this sequence does not provide real-time images because post-processing of the images is needed. The visualization is also limited to stationary or slowly moving organs because the subtraction sequence requires a reference image obtained at the same position as the contrast-enhanced image. Despite this, the subtraction sequence might be a useful tool for targeted imaging because the detection of a thin layer of the polymer-shelled CA was performed successfully in a phantom setup [63].

Another type of subtraction technique has also been developed within the 3MiCRON project by a group from SINTEF, Trondheim, Norway. Compared with the previously described subtraction sequence, this technique operates in real time. In brief, two identical pulses are transmitted along the same scan line, and the first pulse is received before transmission of the subsequent pulse. Because of the acoustic radiation force applied on the MBs upon ultrasound exposure, during which the absorbed energy causes a pressure gradient that displace the MBs in the direction of the ultrasound beam, the received echoes will differ. Thus, subtraction of the two echoes will produce only echoes from the MBs. As for the visualization studies published as part of this thesis, a relatively high MI (MI≈0.8) is needed to achieve an optimal visualization of the CA using this technique. Although the visualization capability of the MBs using ultrasound can be improved by new optimized ultrasound contrast sequences, there is still discussion about the clinical need for multimodal CAs. These issues are discussed in detail in Section 8.3 Future perspectives for ultrasound contrast agents.
8.1.2 In vivo performance

The lowest detectable in vitro concentration when using the high-frequency ultrasound system in Study II was $10^4$ MBs/ml. Balanced against the estimated CA blood concentration immediately after CA injection (total blood volume in the rat: $28\, ml\, [64]$, injected dose: $0.4\, ml$, CA concentration: $6.6 \times 10^8$ MBs/ml), up to one permille of the injected dose could be assumed to be detected when using the high-frequency system in the in vivo environment. The in vivo detection limit can differ from that measured in the in vitro environment because the MBs can be altered by macrophage internalization of the MBs or by protein complexes that surround the MBs in the blood. The latter is one possible explanation why the blood elimination profile of the different types of the polymer-shelled CA differed. Previous studies have shown that various coatings of iron oxide nanoparticles interact with different proteins in a biological solution and that these proteins form complexes that are referred to as the ‘protein corona’ [65]. The protein corona might affect the transportation across biological barriers [66], and consequently, the differences in protein corona complexes could be one reason why the blood elimination profile for PVA Type A, with SPIONs attached to the shell surface, was more rapid than the elimination profile obtained for Plain PVA and PVA Type B during the early elimination phase. Another possible explanation is the difference in shell stiffness. PVA Type B has been shown to be more rigid than PVA Type A, and therefore, it has a greater tendency to collapse [67]. By contrast, all types of the polymer-shelled CA showed significantly longer blood circulation times than did SonoVue. The prolonged blood circulation time seen for the different types of polymer-shelled CA is favorable for targeted CAs provided that the long circulation time increases the probability of attachment of MBs to the intended targets. Moreover, a long circulation time offers more time for image acquisition, enabling examination of organs in multiple scanning planes after a single bolus injection.

The data collected in Study II led to the conclusion that macrophages are responsible for the elimination of CAs. In this study, the MBs localized mainly inside, in the vicinity of, or adherent to macrophages in the tissue sections analyzed. Similar results have also been observed for other CAs with different MB shell compositions, such as lipid and albumin [68, 69], and in in vitro studies including the polymer-shelled CA (Grishenkov et al.: Ultrasound contrast agent loaded with nitric oxide as a theranostic microdevice, in press). The TEM analysis confirmed that the polymer-shelled CA was a pure blood pool agent because all of the identified MBs ($731\, MBs$) were located in the blood vessel. Intact MBs were observed in the blood $24\, h$ after CA injection, implying that free MBs can circulate for a long time in the blood. Additionally, a shift in MB accumulation from mainly in the lungs a short time after CA injection to the liver and spleen at $24\, h$ after CA injection has also been observed in other studies involving modified versions of the polymer-shelled CA [20, 21] (Barrefelt et al.: Multimodal imaging of fluorescence labelled microbubbles, submitted).

Because TEM analysis included only a modest area from each tissue type, it was not considered to be appropriate to perform quantitative measurements of the CA uptake in different tissues types over time. Nevertheless, the TEM analysis enabled an assessment of the subcellular interactions and analysis of the MB structures, which is an advantage compared with other indirect analytical methods that trace the release of encapsulated gas or detect radioactivity traces on the shell surface. These analytical methods are limited by not knowing how the encapsulated gas is released from the MBs or whether the radiolabeled molecules are still attached to MBs after interaction with biological material [70, 71]. To understand further the elimination process of polymer-shelled CAs, quantitative measurements of the biodistribution and clearance must be performed. In a collaboration with a group from the Department of Chemistry, School of Chemical Science and Engineering at KTH Royal Institute of Technology, we developed an analytical method based on capillary electrophoresis with ultraviolet detection (Josefsson et al.: Analysis of polyvinyl alcohol microbubbles in human blood plasma, submitted). In this study, we showed that this analytical method can detect both fragments of PVA and intact MBs of the polymer-shelled CA in human
blood plasma. When applying this method, it may therefore be possible to obtain a wider understanding of both the MB circulation time in blood and the elimination pathway by studying the PVA content in materials from excretion organs. Validation studies using biological fluids other than human blood plasma must be performed before this analytical method can be adapted for more comprehensive elimination studies. In addition, further concentration studies to provide standard values for a wide range of concentrations must be performed before quantitative measurements can be initiated.

An in vitro toxicity study that has included different types and concentrations of the polymer-shelled CA has been performed within the 3MiCRON project by a research group from INT, Milano, Italy. The cell viability of normal human fibroblasts subjected to different CA doses was evaluated by phase-contrast microscopy and fluorescent microscopy 3 and 7 days after CA exposure (unpublished data). None of the CA types tested in this thesis (Plain PVA, PVA Type A, and PVA Type B) showed significant effects on cell viability at a moderate dose (≤5 × 10⁶ MBs/ml). However, PVA Type A and PVA Type B showed slightly lower cell viability at high doses (>5 × 10⁶ MBs/ml) compared with Plain PVA, which had no apparent effect on cell viability.

In Study III, the highest dose (5 ml) of the polymer-shelled CA (Plain PVA) was needed to obtain the same efficiency for endocardial border delineation, time period of clinically sufficient contrast enhancement, and LV opacification as for SonoVue, when using the contrast sequences available in the present ultrasound systems. This indicates that the backscattering efficiency of SonoVue is superior to that of the polymer-shelled CA. As discussed in the visualization section (8.1.1 Visualization capability), the difference in shell composition is one possible explanation for this observed difference. No significant variations in the physiological variables were observed when comparing the physiological variables from before to after CA injection. This indicates that the anesthesia procedure was stable and that no untoward hemodynamic effects were caused by the CA injection.

Even though the visualization studies performed within this thesis indicate that the polymer-shelled MBs are too stiff to produce harmonics at low to medium MI values, there are still advantages of using MBs constructed with a polymeric shell. The relatively high proportion of reactive aldehyde groups on the shell surface enables the introduction of different substances into the shell surface, allowing for multimodal and targeted imaging. Specific examples are discussed in Section 8.3 Future perspectives for ultrasound contrast agents. It is important to remember that extensive studies are needed before this polymer-shelled CA can be adapted for clinical use. For example, the in vivo interactions, such as biodistribution and clearance, must be determined at a deeper level in both rodents and in nonrodents, and the adverse effects and the diagnostic features of the different modalities must be determined before initiation of clinical trials. To compete with commercially available CAs, it is essential that the polymer-shelled CAs can provide new features such as those that would allow their use in targeted and multimodal imaging. Otherwise, it will be difficult to attract pharmaceutical companies for large-scale production and to include the polymer-shelled CA in different clinical procedures.

8.2 The clinical value of broader contrast agent use
It has been proposed that 10–15% of all cardiac patients have indications for contrast-enhanced echocardiography [9, 72]. Despite this, a recent study concluded that only 3.8% of the echocardiographic examinations include CA during image acquisition [73]. This observation is surprising because several studies have demonstrated the clinical value of contrast-enhanced echocardiography in patients with suboptimal grayscale images [6, 74, 75].

The results of Study IV showed that the diagnosis from contrast-enhanced analysis differed significantly from that obtained from grayscale analysis in a patient group without indications for
CA use. This was related mainly to the improved detection of regional wall motion abnormalities, evidenced by the observation that a significantly higher percentage of the patients with a WMSI ≤ 1.5 were shifted toward the higher WMSI interval (1 < WMSI ≤ 1.5) after contrast-enhanced analysis. The shift in WMSI between the two imaging modes was not limited strictly to segments with poor image quality. For those segments that were rated with insufficient image quality during grayscale analysis (169 segments), the most common score shift after contrast-enhanced analysis was a change to normal wall motion (71% of the segments). When considering the patients with sufficient grayscale image quality and normal wall motion (WMSI = 1) in every segment, as many as 59% were classified as having regional wall motion and thickening abnormalities after contrast-enhanced analysis. The reclassification of previously healthy patients, together with the fact that no significant difference in diagnostics was found for patients with extensive wall motion abnormalities (i.e., WMSI > 1.5), suggests that CA use beyond current recommendations would be most beneficial for patients with small- to moderate-sized wall motion abnormalities. The clinical value of contrast-enhanced echocardiography was demonstrated further in the study of structural abnormalities, which found an increased sensitivity for LV structural abnormalities when analyzing contrast-enhanced images.

As described in literature [6], significantly larger LV volumes and EF were obtained for contrast-enhanced images compared with grayscale images. By contrast, no significant differences in EF classification (≥ 55%, 45–54%, 30–44% and < 30%) were observed between the two imaging modes. One possible explanation is that both EDV and ESV were underestimated in the grayscale analysis. As in previous studies [6, 9, 72], Study IV found improved reproducibility of WMSI and EF measurements when using contrast-enhanced images instead of greyscale images. The improved reproducibility was observed for repeated measurements both by experienced readers and between inexperienced and experienced readers. To improve the diagnostic accuracy of echocardiography further, automatic volume measurements from contrast-enhanced images instead of manual tracing could be adopted. Moreover, implementation of 3D echocardiography would minimize the geometrical assumptions and apical foreshortening that exists for 2D echocardiography.

In summary, this research shows that addition of a CA increases the reliability of the diagnosis and contributes to earlier detection of diseases. This suggests that it may be beneficial to implement a broader use of contrast-enhanced echocardiography instead of the selective approach used today. This may result in improved patient outcomes and increased cost-effectiveness. Nevertheless, such recommendation needs to be supported with further randomized multicenter studies to document the cost-effectiveness, risk assessment, and patient outcomes. At the same time, the major barriers to implementation of contrast use need to be overcome, such as the inadequate number of experienced specialists, and training and accreditation of sonographers to be able to perform contrast-enhanced echocardiography independently [73].

8.3 Future perspectives for ultrasound contrast agents
The original idea for ultrasound CAs was to develop a device that improves the contrast enhancement from relatively weak echogenic regions such as the vascular lumen. Today, ultrasound CAs are applied frequently in clinical practice, where they improve the diagnostic accuracy. However, new applications for the next generation of ultrasound CAs (i.e., third-generation CAs) such as multimodality, targeted imaging, and local drug-delivery may soon be developed and adopted. When available, these new ultrasound CAs will not be limited to diagnostic imaging only but will have theranostic capability; that is, the combination of therapeutic and diagnostic capability.

8.3.1 Multimodality imaging
At present, multimodality imaging with hybrid imaging systems, such as positron emission tomography (PET)/computed tomography (CT), single-photon emission computed tomography/CT,
and PET/MR, are used in clinical practice. These imaging modalities provide simultaneous information about anatomy and physiology. Compared with two separate imaging sessions for each modality, hybrid imaging allows for good alignment between images from the different modalities because the sequential imaging is performed without moving the patient. Additionally, temporal changes in physiology between image acquisitions of the two modalities are limited because the interval between image acquisitions is minimal. The multimodality imaging procedures may soon be expanded to other imaging combinations. Future uses of multimodality imaging may be to combine ultrasound with other imaging modalities. For example, research aiming to combine ultrasound with CT and MR imaging is currently being performed [76, 77]. Contrast-enhanced multimodality imaging may allow for a more comprehensive evaluation of suspected diseases or may help in difficult-to-image patients.

To enable contrast-enhanced imaging when using these hybrid imaging systems, a CA with multimodality potential is preferable. The polymer-shelled CA described in this thesis can meet these requirements because it can be visualized with several imaging modalities such as ultrasound and MR and emission imaging [20, 21, 54]. In addition, because of the CA’s considerable chemical versatility, it may also be possible to introduce substances (e.g., iodine) to allow contrast enhancement during transmission imaging. A problem that can arise when modifying an ultrasound CA is that the added particles, which enable multimodality imaging, may affect the elasticity profile of the MBs. Thus, generation of the nonlinear MB oscillation may be affected. It may be speculated that a decrease in the elasticity profile would be expected when particles are added into the shell. Consequently, the imaging performance for ultrasound could be impaired. However, the potentially decreased visualization performance for ultrasound may be acceptable, to some extent, because the information obtained from different image modalities may provide more information about a specific disease than what can be obtained with ultrasound alone.

Having a CA that can be applied for several modalities may decrease the time from suspected disease to diagnosis by making it possible to perform contrast-enhanced acquisition with different imaging modalities during the same examination. In addition, the hybrid imaging systems may provide a better basis for diagnosis by making available more comprehensive information. Even though hybrid imaging systems for several modalities and multimodal CAs are likely to be produced in the near future, there are still some practical issues concerning the feasibility and usability of multimodality imaging. For example, hybrid imaging must achieve imaging performance similar to that of each individual system. Moreover, large investment in new equipment and education of staff are needed. The clinical benefits of any hybrid imaging system must therefore be fully evaluated on the basis of the economic costs and patient well-being before they can be implemented in clinical practice. Because ultrasound by itself is portable and highly cost-effective, there will still be a need for traditional ultrasound systems in the future.

### 8.3.2 Targeted imaging

A number of diseases such as endothelial inflammation, angiogenesis, and atherosclerosis are characterized by overexpression of disease markers [78]. Decorating the MB shell surface with ligands (e.g., antibodies or peptides) that can bind specifically to a marker for a particular disease will allow for increased contrast enhancement of the diseased area, see Figure 8.1A. This will improve the ability to visualize a particular disease and diseased area. The binding affinity of the targeted MBs into a specific area can be enhanced further by acoustic radiation forces generated during ultrasound exposure, which will move the MBs to the vessel wall [79]. However, only a minor portion of the injected MBs will bind to the disease markers, implying that a sensitive imaging method is needed to enable detection of diseased areas.

At present, ongoing clinical trials are under way with a targeted ultrasound CA having an affinity for markers of angiogenesis [80, 81]. However, ultrasound targeted CAs can still be considered to be in
the development phase, and studies must demonstrate its potential. The detection of the ischemic myocardium [82-84], thrombus [85] and neovascularization of tumors [86] have been performed successfully in animal models. Additionally, the polymer-shelled CA described in this thesis has also shown targeted potential to inflamed tissue both during in vitro and in vivo studies (unpublished data). Targeted imaging is also being used for imaging modalities other than ultrasound imaging [87, 88]. Compared with MR imaging, ultrasound has a lower spatial resolution. On the other hand, ultrasound has advantages in that it can provide images in real time and is highly cost-effective. Compared with emission imaging, ultrasound operates without emitting ionizing radiation to the patient. Consequently, there are several advantages of having target-specific ultrasound CAs.

8.3.3 Local drug-delivery
The local uptake of drug by cells or tissue that is induced by ultrasound exposure is referred to as sonoporation. During sonoporation, the drug can be coadministered with the CA (nontargeted CA) or attached to, or incorporated into, the shell of the MBs (targeted CA) [89].

Figure 8.1 shows an illustration of targeted MBs that bind specifically to an intended target. The mechanism of sonoporation is not fully understood, but increased porosity and permeability of the cell membrane have been reported as possible explanations [89-91]. Shock waves and microjets generated after MB disruption at high pressure and stable MB oscillation at low to medium MI values have also been suggested as possible explanations for this phenomenon.

It would be beneficial to use a target-specific and drug-loaded CA to decrease a drug’s side effects and to direct a drug specifically toward the diseased tissue instead of affecting both healthy and diseased tissues. This may also reduce the volume of an injected drug needed. The therapeutic efficacy may increase because a higher proportion of the drug will be delivered into the diseased area. It may be beneficial to use a drug-loaded target-specific ultrasound CA when the diseased area can be located and its size can be determined. Moreover, the effects of treatment of a diseased area may be visualized and the targeting affinity may be improved by the acoustic radiation force that pushes the MBs toward the vessel wall. When using a drug-loaded CA, local release of the attached drug can be achieved by transmission of a high-pressure ultrasound pulse, which causes MB disruption, see Figure 8.1 B. The potential for local drug delivery of the polymer-shelled CA described in this thesis has been examined in several in vitro studies. For example, local release of an antitumor drug agent [92] and nitric oxide (Grishenkov et al.: Ultrasound contrast agent loaded with nitric oxide as a theranostic microdevice, in press), which can prevent clot formation [93],

![Figure 8.1](image_url)
has been performed successfully. Thus, the polymer-shelled CA may be a candidate for local drug delivery. In parallel with the research of the polymer-shelled CA, studies including other types of local drug-delivering CAs have also been reported [94-97].

Before the next generation of CAs can be implemented in clinical practice, improvements in chemical, technical, and practical issues are needed. The chemical problems include optimization of CAs with a high affinity for specific regions and a long blood circulation time, which would increase the probability of MB attachment to the diseased area. The polymer-shelled CA has been shown to have increased circulation time compared with standard ultrasound CAs (Study II). The technical problems involve improvements in ultrasound contrast sequences that allow the detection of single MBs and optimization of the ultrasound parameters to increase the acoustic radiation force and MB destruction. Finally, practical issues, such as training staff and investing in new equipment such as imaging systems and image analysis software, need to be solved before implementation of targeted imaging and local drug delivery in clinical practice can be realized.
9 Conclusions

The studies presented in this thesis demonstrated that the polymer-shelled CA has potential for contrast-enhanced diagnostic imaging and that there is a clinical value of increased CA use during standard echocardiography. The specific conclusions for each of the four papers included in the thesis are summarized below.

Study I  All types of the polymer-shelled CA tested (Plain PVA, PVA Type A and PVA Type B) have potential to enhance ultrasound images when using commercially available ultrasound systems with dedicated contrast sequences. The highest CTR was observed with PPI at a high MI. To minimize the influence of acoustic shadowing during image analysis, a concentration $<10^6$ MBs/ml is preferable for all contrast sequences and types of the polymer-shelled CA.

Study II  The blood circulation time for the three types of the polymer-shelled CA (Plain PVA, PVA Type A and PVA Type B) was prolonged compared with that of the commercially available CA SonoVue. When comparing the circulation time of the polymer-shelled CAs, a significantly longer blood circulation time was observed for Plain PVA and PVA Type B than for PVA Type A. Macrophages uptake of CA was observed for all tissue types and time points analyzed, suggesting that macrophages are responsible for the elimination of the polymer-shelled CA.

Study III  This study demonstrated that the novel polymer-shelled CA can be useful in contrast-enhanced diagnostic imaging for endocardial border delineation. The ability of the novel polymer-shelled CA to delineate endocardial border was comparable to SonoVue when injecting a higher dose. This was shown by similar values for overall segment scores from visual observations, time for clinically sufficient contrast enhancement, and the same ability for semiautomatic delineation of the left ventricle. Injection of CA did not significantly affect SaO2, heart rate, or arterial pressure.

Study IV  CA use beyond current recommendations for echocardiography improved the diagnostics in the studied patient group, as shown by the increased number of detected patients with regional wall motion and LV structural abnormalities. The reproducibility of the WMSI and EF measurements increased when analyzing contrast-enhanced images instead of grayscale images.
10 Future work

Three of the studies performed within this thesis demonstrated that the polymer-shelled CA has potential for contrast-enhanced diagnostic imaging. However, future work is needed to fully identify its potential in future applications. Some of the future studies needed are outlined below.

- Perform a more detailed elimination study in which the biodistribution and clearance of the polymer-shelled CA are determined in different animal models. Additionally, the effect on the physiological function must be further evaluated.

- Optimize ultrasound contrast sequences with respect to optimal visualization and local drug-delivery of the polymer-shelled CA.

- Continue to investigate the diagnostic features of the polymer-shelled CA for ultrasound, MR imaging and emission imaging.

- The multimodality, targeting and drug-delivery potential of the polymer-shelled CA must be further evaluated in vivo.

Study IV demonstrated the clinical value of CA use beyond current recommendations for standard echocardiography. Nevertheless, a randomized multicenter study that includes both high- and low-volume centers and that evaluates cost-effectiveness, assesses risks, and determines patient outcomes is needed before it can be concluded that increased CA use should be recommended during standard echocardiography.
References


