Structural studies of microbubbles and molecular chaperones using transmission electron microscopy

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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 Docent Philip Köck as the main supervisor, and Professor Hans Hebert as co-supervisor.

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Abstract

Ultrasound contrast agents (CAs) are typically used in clinic for perfusion studies (blood flow through a specific region) and border delineating (differentiate borders between tissue structures) during cardiac imaging. The CAs used during ultrasound imaging usually consist of gas filled microbubbles (MBs) (diameter 1-5 µm) that are injected intravenously into the circulatory system. This thesis partially involves a novel polymer-shelled ultrasound CA that consists of air filled MBs stabilized by a polyvinyl alcohol (PVA) shell. These MBs could be coupled with superparamagnetic iron oxide nanoparticles (SPIONs) in order to serve as a combined CA for ultrasound and magnetic resonance imaging. The first three papers (Paper A-C) in this thesis investigate the structural characteristic and the elimination process of the CA.

In Paper A, two types (PVA Type A and PVA Type B) of the novel CA were analyzed using transmission electron microscopy (TEM) images of thin sectioned MBs. The images demonstrated that the SPIONs were either attached to the PVA shell surface (PVA Type A) or embedded in the shell (PVA Type B). The average shell thickness of the MBs was determined in Paper B by introducing a model that calculated the shell thickness from TEM images of cross-sectioned MBs. The shell thickness of PVA Type A was determined to 651 nm, whereas the shell thickness of PVA Type B was calculated to 637 nm. In Paper C, a prolonged blood elimination time was obtained for PVA-shelled MBs compared to the lipid-shelled CA SonoVue used in clinic. In addition, TEM analyzed tissue sections showed that the PVA-shelled MBs were recognized by the macrophage system. However, structurally intact MBs were still found in the circulation 24 h post injection. These studies illustrate that the PVA-shelled MBs are stable and offer large chemical variability, which make them suitable as CA for multimodal imaging.

This thesis also involves studies (Paper D-E) of the molecular chaperones (Hsp21 and DNAJB6). The small heat shock protein Hsp21 effectively protects other proteins from unfolding and aggregation during stress. This chaperone ability requires oligomerization of the protein. In Paper D, cryo-electron microscopy together with complementary structural methods, obtained a structure model which showed that the Hsp21 dodecamer (12-mer) is kept together by paired C-terminal interactions.

The human protein DNAJB6 functions as a very efficient suppressor of polyglutamine (polyQ) and amyloid-β42 (Aβ42) aggregation. Aggregation of these peptides are associated with development of Huntington’s (polyQ) and Alzheimer’s (Aβ42) disease. In Paper E, a reconstructed map of this highly dynamic protein is presented, showing an oligomer with two-fold symmetry, indicating that the oligomers are assembled by two subunits.

Keywords: Transmission electron microscopy, Contrast agent, Microbubble, Polyvinyl alcohol, Single particle analysis, Heat shock protein, Molecular chaperone
Kontrastmedel för ultraljud används exempelvis för att studera perfusion (blodflöde genom ett specifikt område) och för utlinjering av endokardiet vid hjärtdiagnostik. Det består vanligtvis av gasfyllda mikrobubblor (MBs) (diameter 1-5 µm) som injiceras intravenöst. Den här avhandlingen behandlar delvis ett nytt kontrastmedel för ultraljud som innehåller luftfyllda MBs vars skal består av polyvinyl alkohol (PVA). Genom att binda partiklar av järnoxid till bubblorna kan dessa även fungera som kontrasmedel för magnetresonanstimografi. I de tre första artiklarna (artikel A-C) i den här avhandlingen studeras strukturen av dessa MBs samt hur de elimineras.


Det humana proteinet DNAJB6 dämpar effektivt aggregering av polyglutamin (polyQ) och amyloid-β42 (Aβ42). Aggregering av dessa peptider är kopplat till utvecklingen av sjukdomarna Huntington (polyQ) och Alzheimers (Aβ42). I artikel E gjordes en 3D rekonstruktion av proteinet. Rekonstruktionen visade att detta dynamiska protein bildar oligomerer med två-talig symmetri, vilket tyder på att de är sammansatta av två liknande subenheter.
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I also would like to thank Cecilia Emanuelsson for introducing me to molecular chaperones and for very nice visits in Lund. Thanks a lot for all your work during these projects. Gudrun Rutsdottir for your collaboration and great job on Hsp21 and DNAJB6. Wietske Lambert, thanks for your introduction to Hsp21. Cecilia Månsson for the collaboration on DNAJB6. Christopher Söderberg for the SAXS-data and modelling of Hsp21 and DNAJB6.

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Johan Härmark
Stockholm, May 2016
### Abbreviations

<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>2D</td>
<td>Two dimensional</td>
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<td>3D</td>
<td>Three dimensional</td>
</tr>
<tr>
<td>CA</td>
<td>Contrast agent</td>
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<tr>
<td>CCD</td>
<td>Charged-coupled device</td>
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<tr>
<td>CMOS</td>
<td>Complementary metal-oxide semiconductor</td>
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<tr>
<td>Cryo-EM</td>
<td>Cryo-electron microscopy</td>
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<tr>
<td>CTF</td>
<td>Contrast transfer function</td>
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<tr>
<td>CXMS</td>
<td>Crosslinking mass spectrometry</td>
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<tr>
<td>DDD</td>
<td>Direct detection device</td>
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<tr>
<td>EM</td>
<td>Electron microscopy</td>
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<tr>
<td>FSC</td>
<td>Fourier shell correlation</td>
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<td>GraFix</td>
<td>Gradient fixation</td>
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<td>Hsp21</td>
<td>Heat shock protein 21</td>
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<tr>
<td>MB</td>
<td>Microbubble</td>
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<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
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<tr>
<td>PVA</td>
<td>Polyvinyl alcohol</td>
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<tr>
<td>SAXS</td>
<td>Small angle x-ray scattering</td>
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<td>sHsp</td>
<td>Small heat shock protein</td>
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<tr>
<td>SPA</td>
<td>Single particle analysis</td>
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<td>SPION</td>
<td>Super paramagnetic iron oxide nanoparticle</td>
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<td>TEM</td>
<td>Transmission electron microscopy</td>
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1 Introduction

The transmission electron microscope (TEM) is an instrument that can be used for studying a wide range of micro/nanometer-scale particles, such as synthetic particles, cells, organelles, protein complexes and single protein molecules. The structure of these objects is decisive for their physical and biological function. In contrast to X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy, the TEM provides a direct image of the object, without having to convert diffraction patterns (X-ray) or NMR spectra to structural information. The finest structural details that can possibly be imaged are generally limited to the wavelength of the radiation used for illuminating the object. Since visible light has a much longer wavelength than electrons (~0.5 µm for visible light and ~2.5 pm for 200 kV electrons), the resolving power of a TEM is much higher compared to a conventional light microscope and TEM can provide structural details of objects down to atomic resolution.

Intravenously injected microbubbles (MBs) (1-5 µm) have made their way in to the clinic as a contrast enhancing tool for ultrasound imaging. Ultrasound contrast agents (CAs), consisting of MBs, are used in clinic to improve the signal from otherwise contrast weak areas, such as blood vessels. The blood flow through a particular area can then be determined, which is important information in cardiac and abdominal diagnostics. This thesis involves a novel polymer-shelled CA, that has been developed within the European FP7 project 3MiCRON (245572). This CA consists of MBs with a shell of crosslinked polyvinyl alcohol (PVA), that can be modified by coupling superparamagnetic iron oxide nanoparticles (SPIONs) to the shell, following two different production protocols. The introduction of SPIONs enables ultrasound and magnetic resonance imaging using the same CA. Thus, both functional and anatomical information could be acquired from one single CA injection. Besides this, the PVA shell offers large chemical flexibility for further modifications to support target specific imaging and to use these MBs as vehicles for local drug delivery.

In the field of biomedical microdevices, discovering the structure of the objects and their properties play a key role for optimizing the performance of the devices. In the case of the PVA-shelled MBs, TEM could provide important information for optimizing the production protocols, such as the distribution and relative size of the coupled SPIONs and the shell thickness and size of the MBs. These parameters are important in order to relate the structural differences to the function of the MBs.

Molecular chaperones are proteins that rescue other proteins that have been damaged during stress. The structure of these chaperones is decisive for understanding how these proteins interact with substrate proteins. Efficient chaperone activity often involves...
forming larger structures (oligomers) of the proteins. This thesis focuses on investigating the oligomeric structure of two molecular chaperones (Hsp21 and DNAJB6). Many chaperones are flexible by nature and exist in a wide range of conformations, with their own specific functions. The flexibility in combination with relatively large structures (250-500 kDa) prevents structural studies using conventional structural methods, such as X-ray crystallography and NMR spectroscopy. In contrast to X-ray crystallography, TEM and single particle analysis (SPA) does not require crystals and only small quantities of the protein are needed. Besides, the proteins are imaged in a close to their native state when imaged in cryo conditions. Since the first three-dimensional (3D) structure reconstructed from electron micrographs in 1968 [1], the method has advanced and was recently selected as ‘Method of the year 2015’ by *Nature Methods* [2]. The introduction of better detectors, microscopes and software are the main reasons for the latest development. In the foreseeable future, TEM will still be a widely used technique used for structural studies of large molecules and molecular complexes.

In this thesis I have focused on using TEM for structural studies of MBs and molecular chaperones to provide information for future development of MBs and deeper understanding on the oligomeric arrangement of two proteins involved in the cell’s quality control system for protein folding.
2 Aims

The aims of the first three papers (Paper A-C) in this thesis, were to investigate the structural characteristics and the elimination process of different types of a novel CA, that consists of air-filled PVA-shelled MBs, with multimodal imaging potential. The specific aims of Paper A-C were as follows.

Paper A To investigate the shell structure and distribution of SPIONs coupled to two types of PVA-shelled MBs (PVA Type A and PVA Type B) by using TEM.

Paper B To introduce a model for determining the average shell thickness of two types of PVA-shelled MBs (PVA Type A and PVA Type B) from TEM images of thin cross sectioned MBs.

Paper C To describe the elimination process of three types of PVA-shelled MBs (Plain PVA, PVA Type A and PVA Type B) in a rat model by measuring the ultrasound signal intensity over time and studying the subcellular localization of the MBs post injection using TEM.

The general aim of the work reported in Paper D and E was to investigate the oligomeric structure of two molecular chaperones. The specific aims and structures studied were as follows.

Paper D To investigate the oligomer arrangement in the small heat shock protein (sHsp) Hsp21 using single particle cryo-electron microscopy (cryo-EM) combined with small angle X-ray scattering (SAXS) and crosslinking mass spectrometry (CXMS).

Paper E To explore oligomers of the molecular chaperone DNAJB6 utilizing negative stain electron microscopy (EM) after gradient fixation (GraFix), combined with CXMS, SAXS and molecular modeling.
3 List of included papers

The thesis is based on the five papers listed below. Full versions of the papers are attached as appendixes at the end of the thesis. Reprints were made with permission from the respective publishers.


3.1 My contribution to the papers

Paper A I performed the structural characterization experiments of the MBs using TEM and took part in revision of the manuscript.

Paper B I took part in the design of the study, collected the data and wrote the manuscript.

Paper C I participated in the design of the study, collected and analyzed the TEM data and wrote the manuscript together with MKL.

Paper D I collected the cryo-EM data, performed the SPA, did the homology modelling and wrote the corresponding parts of the manuscript.
Paper E  I performed the GraFix purification, collected and analyzed the negative stain data and wrote the corresponding parts of the manuscript.
4 Other scientific contributions

4.1 Peer-reviewed papers


4.2 Conference contributions


Härmark J, Koeck PJB, Hebert H: Structural Characterization of Multifunctional Microbubbles by TEM, Scandem, Annual Meeting of the Nordic Microscopy Society, Copenhagen, Denmark, 2013.

Härmark J, Margheritelli S, Oddo L, Paradossi G and Hebert H: Magnetic nanoparticles coupled to microbubbles enable multimodal imaging, CLINAM, Basel, Switzerland, 2012.

5 Background

5.1 Electron microscopy
In electron microscopes (EMs) a beam of accelerated electrons is used to illuminate the sample. In contrast to light, electrons have a much shorter wavelength (~0.5 µm for visible light and ~2.5 pm for 200 kV electrons). However, due to limitations, such as imperfect lenses, the resolution in conventional EMs is typically limited to about 1 Å. The two common types of electron microscopes are scanning electron microscopes (SEM) and TEM. In SEM the electron beam is focused and scanned over the specimen while detecting the electrons that scatter back from the specimen. Thus, the collected image represents the surface of the specimen. In contrast, using a TEM the electron beam is spread before illuminating the sample and the electrons that pass through the sample are detected. Hence, the TEM produces images representing internal structures of the sample. In this thesis, structural studies were only performed using TEM.

5.1.1 The transmission electron microscope
The main components of the TEM include the column, electron gun, lens systems and detector. The following section will describe these components in detail.

The TEM column is only a few mm wide, shielded by lead and kept at high vacuum. The sample is mounted into the column, either from the side or the top of the column. In most TEM, the electron gun, the unit that emits the electrons, is placed at the top of the column. Electrons with enough energy escape a filament, which is held at a very high negative voltage (e.g. ~200 kV). The filament is usually composed of a tungsten wire or a lanthanum hexaboride (LaB₆) crystal with a very sharp tip. The electrons can also be emitted in a more controlled manner using a field emission gun (FEG). The electrons are then pulled out of the filament in the FEG by applying an electric field close to the tip. Compared to LaB₆ filaments, the electron beam produced by the FEG have both higher spatial and temporal coherence. The electrons escaping the tip are accelerated down the column by an accelerator stack with graded potential. The lowest plate of the stack is grounded (at 0 V).

The lens systems of the TEM have two main functions, focusing the electron beam and magnifying the image produced by the microscope. The lenses are stacked in the column and are grouped into different categories. The condenser lens system is positioned after the accelerator stack and this system focuses and directs the electrons onto the sample. The objective lens system focuses and magnifies the image of the sample. Next, the projector lens system further magnifies the image by a set of lenses. Each lens system consists of deflectors, a lenses, stigmators and apertures. The deflectors can bend the incoming electrons towards the optical axis by shifting and tilting the beam. The lenses are coils of metal wires, applied with a current, that produce a magnetic field that bends the electron path towards the optical axis. The EM lenses are astigmatic, which means that they have
different focal lengths in two planes orthogonal to each other. The stigmators can compensate for this effect by adding field strength in a particular direction. The aperture is basically a disc with a hole that blocks radiation far from the optical axis.

The image generated by the TEM can be shown on a fluorescent screen in the microscope. Besides, the image can also be recorded on a detector that is placed at the bottom of the column. Photographic film, charge-coupled devices (CCDs) or direct detection devices (DDDs) are typical detectors used to record the images. Photographic film can generate high quality images covering a large field of view. However, the films need to be developed and digitized, a process which is very time consuming. Thus, the use of film has declined after the introduction of CCDs and DDDs. The CCD contains a scintillator layer that converts the electrons to light (photons). The photons are then directed and registered by pixels in the CCD array via fiber optics. Due to multiple scattering events, many photons can be generated by the same electron, which adds noise to the images. In DDDs the electrons are converted directly to charge by a complementary metal-oxide semiconductor (CMOS) detector. The CMOS detector is commonly back-thinned, which means that multiple scattering events produced by the same electron are less likely and the amount of noise is reduced. In addition, CMOS detectors have a fast read-out of the pixels, which enables collecting images at very high frame rates. This feature can be used to collect ‘movie-mode’ exposures, i.e. a set of image frames recorded during one single exposure.

5.1.2 Image formation

Just like any other moving object, electrons can be described as both particles and waves. This wave-particle duality is fundamental to describing how an image is formed in the TEM. The electrons that pass through the sample are either unscattered, inelastically scattered (with reduced energy) or elastically scattered (deflected with the same energy). The elastically scattered electrons are deflected by attraction from the nucleus of the sample atoms. Inelastically scattered electrons cause events that are damaging the sample. For vitrified biological samples (see section 5.2.3) these events limit the possible dose used when collecting images of these objects. The aperture of the objective lens system blocks scattering too far away from the optical axis. Thus, denser areas of the sample will appear darker in the image as fewer electrons passing through these areas will hit the detector than in the vicinity. This is called amplitude contrast, since the amplitude of the electron wave appears to be reduced.

In the TEM there is an interference of un-, inelastically- and elastically-scattered waves that are brought together to form the image. The elastically scattered waves interfere either constructively or destructively with the unscattered beam. For proteins (weak scatterers) the image formed by the microscope is composed of the object multiplied by the contrast transfer function (CTF) of the microscope. The CTF can be calculated as follows.

$$CTF = \sin \left( \frac{\pi}{2} C_s \lambda^3 k^4 - \pi \Delta z \lambda k^2 \right)$$  \hspace{1cm} (5.1)

where $C_s$ is the spherical aberration of the objective lens, $\lambda$ the electron wavelength, $k$ the spatial frequency and $\Delta z$ the defocus [3]. The effect of the CTF for two images with different defocus values is shown in Figure 5-1. At the higher defocus value, the CTF curve oscillates more rapidly and the contrast contributions from lower spatial frequencies are
Figure 5-1 Comparing the contrast transfer function (CTF) for three different example images. The solid line represents an image recorded close to focus. The dotted line represents an image recorded at a higher defocus value compared to the image plotted with a solid line. The CTF represented by the dotted line is shifted to the left, towards lower spatial frequencies, and has more rapid oscillations than the image corresponding to the solid line. The image represented by the dashed line is collected at the same defocus value as the dotted line image, but dampened to illustrate the effect of the envelope function.

enhanced. The TEM can be described as a band pass filter since some spatial frequencies are fully transferred, while others are dampened or cancelled. Due to partial spatial coherence of the electron gun, i.e. electrons escaping the filament at slightly different positions, some components will be detected with an additional shift in the image. This effect will reduce the contrast (blur the image), particularly for high frequency (high angle scattering) components. In addition, electrons with partial temporal coherence (different energy) will be focused at different heights and this will also reduce the contrast. Both these effects can be plotted as the envelope function, that will estimate how much the contrast is dampened as a function of the spatial frequency. In Figure 5-1, an envelope function is added to the CTF curve for the image with a higher defocus value. As shown, the dampening typically reduces the contribution from higher spatial frequencies. Besides, the envelope function is more generous in images obtained closer to focus. When CTF correcting the images, the phases corresponding to negative contrast are flipped (multiplied by -1).

5.2 Sample preparation
In biology, TEM is an important imaging technique for studying the subcellular organization in tissues. In addition, viruses and macromolecules can be imaged by TEM either as individual particles or in complex. To view biological specimens in the TEM, the sample must be prepared so that structural information is preserved after it has been positioned in the high vacuum of the microscope. Apart from not collapsing when exposed to the high vacuum, the sample also needs to be protected from radiation damage caused by the electron beam. For cell and tissue samples, this can be achieved by chemically stabilizing the sample followed by dehydration and embedding the sample in a plastic resin (plastic embedding). Biological samples are mainly composed of atoms with low atomic numbers, which produce low contrast images. Hence, these samples are often stained with heavy metal salt (such as uranyl or osmium) containing solutions to increase the contrast during TEM imaging. Sample preparation of viruses and macromolecules is mainly performed either by staining the specimen (negative staining) or by quickly freezing it in
liquid ethane (vitrification). The following sections cover the sample preparation techniques used for the TEM studies in this thesis.

5.2.1 Plastic embedding
Preparation of tissue and cell samples for TEM imaging usually starts by immersing the entire sample in a fixative solution, such as a mixture of formaldehyde and glutaraldehyde. The solution penetrates and fixates cellular structures by crosslinking proteins in the tissue. This primary fixation is often followed by adding osmium tetroxide as a secondary fixative. Osmium tetroxide stabilizes lipid membranes and proteins. In addition, the heavy osmium atoms contribute to contrast, which enhances the contrast of cellular membranes [4]. Tissue samples and cells are too thick to be imaged directly in the TEM. Thus, the samples need to be cut in thin sections before imaging. To enable cutting, the fixed sample is first dehydrated in a solvent, e.g. acetone, ethanol or a combination of both. After dehydration the sample is embedded in a resin, for example Epon, and polymerized. The solid polymerized plastic block is then cut by a diamond knife in approximately 50 nm thin sections. The sections are transferred onto metal grids coated with a thin plastic (often Formvar) film and supported by a layer of continuous carbon. Before TEM imaging, additional staining of the sections is often done with uranyl salts to enhance the contrast.

5.2.2 Negative staining
The negative staining method, first described for studying virus particles [5], can be used for imaging biological structures in the TEM. The sample is added to metal grids supported by a thin layer of continuous carbon. After incubating the sample on the grid, the excess sample solution is blotted away. The sample is then stained with a heavy metal salt (such as uranyl acetate, phosphotungstic acid or ammonium molybdate) containing solution. A thin layer of stain solution is left to let dry on the grid. The heavy metal atoms now cover all areas not occupied by the biological sample. Thus, the sample is embedded in the stain solution that stabilizes the structure and protects the sample from radiation damage when exposed to the electron beam. Another consequence of the staining is the increased contrast obtained by the heavy metal atoms. As an effect, the biological sample will have lower contrast than the surrounding background, i.e. the sample is visible due to ‘negative’ contrast. Since the generated contrast mainly represents external parts of the sample, the resolution of negative stained samples is limited [6]. Other limitations include partial sample deformation upon drying [7] and introduction of possible staining artifacts.

5.2.3 Vitrification
The vitrification method for structural studies of biological samples was first described about 40 years ago [8]. Since then, the method has been further developed and established in the field of structural biology [9], [10]. By using this method, the sample is embedded in a thin layer of vitrified (amorphous) ice in a fully hydrated (‘close-to-native’) state. Initially, the sample is applied to a metal grid coated with a layer of carbon. The carbon layer is usually perforated with micrometer-sized holes. After incubating the sample, the excess liquid is blotted away with filter paper. A thin layer of sample is now left on the grid that cover the holes of the perforated carbon film. The grid is then quickly plunged into liquid ethane. The ethane is kept cooled by liquid nitrogen, enabling very fast cooling of the sample. The cooling process prevents ice crystal formation that would damage the sample and instead the sample is embedded in a thin layer of vitrified ice. The vitrification process
is often performed using semi-automated instruments, such as a Vitrobot [11]. After freezing, the grid is transferred to liquid nitrogen for storage until further analysis. Vitrification enable studies of the sample in the TEM, without additional staining and the contrast is related to the biological sample itself. However, the contrast is lower compared to stained samples. During imaging the sample need to be kept at low (cryo) temperature to avoid crystal formation. Thus, a specially designed specimen holder is used that is constantly cooled by liquid nitrogen. Imaging at cryo temperatures also reduces the radiation damage produced by the electron beam [12]. Still, the electron doses need to be kept low to minimize the radiation damage. Thus, this is another reason why the contrast is low in images of vitrified samples. Despite low image contrast, cryo-EM methods have been developed (see section 5.6) that enable structural studies of macromolecules at atomic resolution [13], [14].

5.3 Ultrasound contrast agents

CAs can be introduced during ultrasound image acquisition to increase the image intensity from specific regions. Increased image intensity improves the diagnostic outcomes as it enables microcirculation detection, definition of borders between different tissue structures and increases the reproducibility of image analysis [15]–[17]. One of the most commonly used clinical applications for ultrasound CAs is cardiac imaging. CAs are also utilized for abdominal diagnostics, such as kidney and liver imaging [18], [19].

Ultrasound CAs consist of micrometer sized (1-5 µm) gas filled MBs that are injected intravenously into the circulatory system via either a bolus or continuous injection. The use of CAs during image acquisition can be considered as a safe procedure, with only minor side effects reported, such as headache and flushing [20], [21]. However, the Food and Drug Administration (FDA) recommends electrocardiogram and oxygen saturation monitoring up to 30 minutes after CA injection in patients with defected heart and lung function [22].

The use of ultrasound CAs for enhanced contrast imaging was first described in 1968 [23]. This first generation of CA, consisting of free gas MBs, had a lifetime of a few seconds after injection. Additionally, the large size of these free gas MBs only enabled imaging prior to the pulmonary circulation. Thus, to improve the clinical usability for ultrasound CAs, stabilized MBs with narrowed size distribution have been developed. Examples of commercially available CAs, consisting of shell stabilized gas-filled MBs, are listed in Table 5-1. One of the most frequently used CA in Europe is SonoVue (Bracco Imaging, Italy). SonoVue consist of thin flexible MBs stabilized with a phospholipid shell that encapsulates the low soluble gas sulfur hexafluoride [24], [25].

Table 5-1 Examples of ultrasound contrast agents approved for diagnostic ultrasound [26].

<table>
<thead>
<tr>
<th>Contrast agent</th>
<th>Gas</th>
<th>Shell material</th>
</tr>
</thead>
<tbody>
<tr>
<td>SonoVue*</td>
<td>Sulfur hexafluoride</td>
<td>Phospholipids</td>
</tr>
<tr>
<td>Definity</td>
<td>Octafluoropropane</td>
<td>Phospholipids</td>
</tr>
<tr>
<td>Optison</td>
<td>Octafluoropropane</td>
<td>Albumins</td>
</tr>
<tr>
<td>Levovist</td>
<td>Air</td>
<td>Lipids</td>
</tr>
<tr>
<td>Sonazoid</td>
<td>Perfluorocarbon</td>
<td>Lipids</td>
</tr>
</tbody>
</table>

*=Lumason in USA
By introducing magnetic and ionization particles on the shell surface of the MBs, multimodal imaging can be achieved. Multimodal imaging has great potential as a diagnostic tool, as both anatomical (magnetic resonance imaging (MRI)) and functional (ultrasound and emission imaging) images can be analyzed either separately or by fused images. Additionally, the MBs can be employed as a target specific agent to assist local drug delivery. This can be accomplished by introducing antibodies on the shell surface and loading of drugs either embedded inside or connected to the shell surface of the MBs.

5.3.1 Theory of ultrasound contrast agents

An ultrasound image is constructed by reflected echoes that arise when the transmitted ultrasound wave passes boundaries with different acoustic impedance (i.e. resistance to the acoustic pressure wave). A high difference in acoustic impedance between two materials will produce a strong reflection. Due to the high acoustic impedance between blood and MBs, a significant echo will be generated when the ultrasound wave propagates into this region. In addition, the MBs can also act as an active reflector when exposed to ultrasound as it starts to oscillate when exposed to an ultrasound wave that consists of alternating pressure waves. The MB oscillation generates an echo that can be detected by the ultrasound system. Depending on the frequency and pressure of the ultrasound wave, in combination with the properties of the MBs, the magnitude of the MB oscillation will differ [27]–[29]. In general, a flexible shell in combination with a high acoustic pressure wave will generate significantly higher oscillations compared to a stiffer shell at the same acoustic pressure [28], [30].

Compared to tissues, MBs can oscillate nonlinearly at high acoustic pressures (~100 kPa-1 MPa), as they expand more at negative pressures than the compress at positive pressures. This nonlinearity of the MBs can be utilized when implementing contrast specific ultrasound sequences. Common for all contrast specific sequences are transmission of multiple ultrasound waves with alternating phase and/or amplitude. By adding the response from subsequent echoes, the linear response from the tissue will be cancelled out, while the nonlinear response from the MBs will be enhanced. Hence, the image will mainly represent an echo from the MBs, see Figure 5-2.

![Figure 5-2](image)

**Figure 5-2** Example of a pulse scheme for contrast specific ultrasound sequences. After addition of two inverted ultrasound pulses, only the nonlinear signal from microbubbles oscillations remain.
5.3.2 A novel polymer-shelled contrast agent
This thesis involves a novel polymer-shelled ultrasound CA, developed within the European research project 3MiCRON (project ID, 245572). This novel CA consists of air filled MBs (3-4 µm in diameter), which are stabilized by a PVA shell [31]. One aim of the 3MiCRON project was to modify these MBs so that they could be used as CA for multimodal imaging. For example, by coupling iron oxide nanoparticles to the PVA shell, these MBs can also be used as CA for MRI (Paper A). The first part of this thesis (Paper A-C) will focus on studying the structural characteristics and investigating the elimination process of different types of the polymer-shelled CA.

5.4 Small heat shock proteins
The sHsps are molecular chaperones that exist in almost all organisms and are particularly abundant in plants [32], [33]. The sHsps play an important role to prevent protein aggregation during heat induced stress [34]. The sHsps interact with partially denatured target proteins to overcome irreversible protein aggregation [35]. Thus, sHsps act as fast and dynamic rescue systems in the cell [36]. In humans, the most well-known sHsps are the α-crystallins (αA- and αB-crystallin), which are major structural components in the eye lens [37]. The chaperone utility was the first known function of α-crystallin and other sHsps [38], [39]. Due to the chaperone ability, to act as the protein quality control unit in cells, sHsps have been described as “Nature’s molecular sponges” [40]. In addition, the sHsps might not only be limited to function as chaperones, but also involved in a number of other cellular functions that require protein interactions [41]. A number of human diseases, including Alzheimer’s, Parkinson’s and multiple sclerosis, are caused by an accumulation of protein aggregates (amyloids) in neurons, a process which might be prevented by sHsps [42]. Additionally, sHsps have been associated with development of cancer, fibrosis, cardiac diseases and cataract [43], [44]. In order to function as a rescue system during stress conditions, the structural flexibility and variability of sHsps is large. Hence, the heterogeneity of sHsps has limited the ability to investigate these proteins using conventional structural characterization methods. However, crystal structure studies show that sHsps form large oligomers assembled by dimeric building blocks [45], [46]. The oligomerization of sHsps will be discussed in the following section.

5.4.1 Structure of small heat shock proteins
The α-crystallin domain, named after the human sHsps αA- and αB-crystallin, constitutes the central structural domain in all members of the sHsps superfamily [47]. This conserved region consists of 80-100 amino acids, that form an immunoglobulin domain comprises up to nine β-strands [48]. The N-terminal arm is the most variable region in sHsps, both in number and type of residues present in this part of the sequence. The third domain in sHsps is the C-terminal tail. This region also shows large sequence variability, except for a short conserved part at the end of the sequence. This part, containing a I/V/L-X-I/V/L motif, is essential for oligomerization of sHsps [48]. The domain architecture in three sHsps, human αB-crystallin (UniProt ID, P02511), Hsp16.9 from T. aestivum (UniProt ID, Q41560) and Hsp21 from A. thaliana (UniProt ID, P31170) is illustrated in Figure 5-3.

Owing to heterogeneous terminal domains, high-resolution structures solved for full-length sHsps remain quite rare. However, in almost all solved structures, the sHsps are built up by dimeric building blocks, consisting of two interacting α-crystallin domains from
Figure 5-3 Domain architecture in three small heat shock proteins. The proteins consist of the variable N-terminal arm (dark gray), the conserved α-crystallin domain (gray) and the C-terminal tail with a conserved I/V/L-X-I/V/L motif. The domain sequence alignment is shown for human αB-crystallin (UniProt ID, P02511), Hsp16.9 from *T. aestivum* (UniProt ID, Q41560), Hsp21 from *A. thaliana* (UniProt ID, P31170) and was adapted from [48], [49].

Figure 5-4 Dimeric arrangement of wheat Hsp16.9. A loop extends from the β-strands in the α-crystallin domain in one monomer and interacts with a β-strand in the neighboring monomer. The figure was prepared with Chimera [50] after retrieving atomic coordinates of chain A (white) and chain B (black) from the crystal structure of Hsp16.9 (PDB ID, 1GME). The N-terminal region was only resolved for chain A in the crystal structure [46].

different monomers. Two different arrangements of the dimeric building blocks exist in the structures solved. In mammalian sHsps the dimer formation occurs via interactions between elongated β-strands (numbered β-6 and β-7) in neighboring monomers [51]. In contrast, crystal structures of plant and archaeal sHsps show dimer formation via interactions in the loop forming region and the β-strand α-crystallin domain in neighboring monomers [45], [46]. This dimeric arrangement is illustrated in Figure 5-4, using the crystal structure of wheat Hsp16.9 (PDB ID, 1GME) [46].

In most cases, the dimeric building blocks form larger oligomeric structures. Several interactions are identified that keep the dimers in the oligomers together. The most well-known interaction involves the conserved I/V/L-X-I/V/L motif in the C-terminal tail. The motif interacts with a hydrophobic groove formed by β-strands in the α-crystallin β-sandwich of a neighboring dimer. This interaction was first described in the crystal structure of Hsp16.5 from *M. Jannaschii* [45]. In wheat Hsp16.9, a dodecamer (12-mer) is
formed by a double hexameric disc structure, each assembled from a trimer of dimers [46]. The hexameric assembly is supported by the C-terminal tail interaction in neighboring dimers, see Figure 5-5. As the N-terminal regions are partially or fully unresolved in most crystal structures, the involvement of the N-terminal in the oligomer formation is not fully understood. However, as illustrated for wheat Hsp16.9 in Figure 5-5, the resolved N-terminal regions are often found in the center of the oligomer [46]. In Hsp16.9, the hexameric discs are suggested to hold together by both C-terminal tail interactions and N-terminal arm connections in between the two discs, see Figure 5-5. Apart from stabilizing the oligomers, the N-terminal arms are involved in substrate binding [52]. Thus, the N-terminals are important to support the chaperone activity of the sHsps [53].

5.4.2 The chloroplast localized small heat shock protein, Hsp21
One of the proteins studied in this thesis is the sHsp Hsp21 from the flowering plant Arabidopsis thaliana. Hsp21 is localized in the plant chloroplasts and the protein evolved when the land-plants developed [54]. The N-terminal domain is extended compared to other sHsps (See Figure 5-3) and contains a methionine-rich region, which is conserved among chloroplast-localized sHsps. This region forms an amphipathic α-helix-motif [55], that has been developed during evolution as response to a selection pressure on higher land plants [56]. Hsp21 can protect plants during heat stress, which involves rearrangements of the oligomeric structure of the protein [57]. The only crystal structure solved for a plant sHsp is the previously discussed cytosolic homolog from wheat Hsp16.9 [46]. As seen in Figure 5-3, Hsp16.9 is smaller than Hsp21. The main difference is the N-terminal region, which is 41 amino acids longer in Hsp21 than in Hsp16.9. It has been shown that Hsp21 forms dodecameric oligomers [58]. Single particle negative stain EM suggested the dodecamer to be organized in the similar double disc arrangement as shown for Hsp16.9 [59]. Cryo-EM was used in this thesis to further investigate the oligomer arrangement in Hsp21.

Figure 5-5 The oligomeric arrangement in wheat Hsp16.9. The dodecamer (PDB ID, 1GME) is arranged as a double hexameric disc structure, where each disc consists of a trimer of dimers. (Left) 3-fold axis view showing two dimers in the upper disc (blue), the remaining dimer in the upper disc illustrated in white (Chain A) and black (Chain B) and the three dimers in the lower disc (yellow). (Right) 2-fold axis view of the dodecamer (rotated 90° around the 2-fold axis). The C-terminal tail dimer-dimer interactions, within and between the discs and the N-terminal arm connection are highlighted for one of the dimers.
5.5 DNAJ proteins

DNAJ proteins, also known as Hsp40 proteins, are molecular chaperones that recruit and transport unfolded proteins to the larger Hsp70 proteins that perform refolding of the substrate proteins. The DNAJ protein is also responsible for supporting the substrate refolding by initiating ATP hydrolysis of the Hsp70 proteins. The DNAJ protein family is the largest chaperone family and contains members which are involved in a large number of biological processes [60]. The DNAJ family constitutes 49 human homologues and is divided in 3 classes (A, B and C), which all contain a conserved J-domain that can interact with Hsp70. The members that belong to class A and B have the J-domain in the N-termini, that contain many glycine and phenylalanine residues. The DNAJB protein class contains 13 homologues that vary in size and function. The most well studied DNAJ protein is the DNAJB1, which forms dimers via interacting α-helices in the C-terminal [61]. The space between the connecting monomers of the dimer is suggested to work as a binding site for unfolded substrate proteins [61].

5.5.1 The molecular chaperone DNAJB6

The DNAJB6 has been reported to work as a suppressor of polyglutamine (polyQ) aggregation [62]. The accumulation of polyQ peptides is associated with development of Huntington’s disease. DNAJB6 has been demonstrated to work as an effective suppressor even at low protein to peptide ratios [63]. Besides this, DNAJB6 can also inhibit the aggregation of another peptide, amyloid-β42 (Aβ42), which forms amyloid fibrils that are associated with Alzheimer’s disease [64]. Also this inhibition works at very low protein to peptide ratios and the activity is significantly larger than for any other known chaperones [65]. It has been reported that the inhibition is maintained by revoking the primary nucleation process, which is necessary for developing the amyloid fibrils. In DNAJB6 proteins, a conserved domain contains many serine (S) and threonine (T) residues (the S/T-rich region), which is specific and only exist in DNAJB6 and its closest homologue, DNAJB8. Gradual substitution of S/T to alanine in this region results in lower chaperone activity and it is suggested that the hydroxyl groups in this region are necessary for efficient fibril suppression [66].

DNAJB6 form a large variety of oligomers [63] and the flexibility and conformational diversity of oligomers have prevented structural studies using conventional methods, such as X-ray crystallography. Thus, the importance of oligomerization of DNAJB6 for efficient chaperone activity is not yet understood. The S/T-rich domain in DNAJB6 expands through both the middle and the C-terminal domain and is required for suppressing the aggregation of huntingtin exon-1 [62]. The S/T-rich region flexibility and the conformational diversity of DNAJB6 oligomers are two specific features of the protein which are thought to be essential for the function of this chaperone. Negative stain EM after gradient fixation was used in this thesis to possibly get a glimpse of the dynamic DNAJB6 oligomers.

5.6 Single particle analysis

SPA has been used to reconstruct the molecular chaperones (Hsp21 and DNAJB6) presented in papers D-E. The method is a combination of image processing techniques that use projection images of biological objects, typically proteins or viruses, obtained by TEM,
to generate a 3D reconstruction of the object. In general, the sample is either vitrified or negatively stained, which will leave the protein particles in random orientation on the EM grid. The idea is to select a large number of particles and to group and average particles together that show similar orientations in order to then compute a 3D reconstruction of the object. The image processing steps involved in the SPA will be discussed in the following section.

5.6.1 The reconstruction process

The whole reconstruction process is based on the projection theorem, which says that the Fourier transform of a projected image matches one central slice through the Fourier transform of the 3D object [67]. Thus, the amplitudes and phases in the Fourier transform of the projected image will be the same as in one central slice through the Fourier transform of the object. The main task of SPA is to record lots of projection images and to determine the orientation of these to calculate a 3D reconstruction of the object.

The first step involves identifying (or boxing) the particles in the TEM images. The selection can either be done manually or automatically by cross-correlation using a set of reference particles. Since the images typically show particles with relatively low contrast (particularly for small proteins), this task might be one of the most time consuming steps during the whole process. CTF correction of the image can either be done before or after boxing the particles. In a homogeneous sample, each particle represents one view of the same object. Two-dimensional (2D) classes showing particles in similar views can be constructed by aligning the particles, allowing in plane rotations and translations, and then averaging particles that look the same into one class average. One way classification can be performed is by iteratively placing the particles in a predefined number of clusters based on their similarity [67]. 2D class averaging can be used to identify variations in the particle population, caused by partially degraded particles, clustering of particles or conformational changes. Particles that belong to class averages showing clustered particles or other features not originating from the particles can be removed from the data set. In this way the data set can be purified computationally before computing the final 3D model.

The next step is to generate an initial 3D model that will be used as a seed for the reconstruction process. The initial model can be generated in many ways. One method is based on finding the common lines, i.e. finding the intersections between the Fourier transform of projection images where the amplitudes and phases of their Fourier transforms are almost similar [68]. Some good representative 2D classes are selected and their Fourier transforms calculated. The Fourier transform of each 2D class average is then oriented using the common line method and the best fit will help orienting the class average correctly in the Fourier transform of the object. After fitting all the slices and interpolating to fill the space between them, the inverse Fourier transform of the volume will generate the initial model. Other ways of generating initial models involve collecting tilt pair images and using the predetermined tilt angles to compute the models [69], [70].

The initial model is then projected in many different directions and these model projections are compared and matched with all individual particle images. Thus, the particle images are associated with the best matching model projection. All the particle images that belong to the same model projection are then averaged to form a new set of 2D class averages. These classes are then used to compute a new ‘refined’ 3D model of the
Figure 5-6 Summary of a typical single particle reconstruction process. The refinement loop is iterated until the class averages and the model converge.

Object. This process is iterated until both the class averages and model converges. For each iteration, the resolution of the model typically increases as the model converge. The resolution of the final map can be estimated by comparing structural features of two models generated from two separate halves of the total data set [71]. The reconstruction process is summarized in Figure 5-6.
6 Materials and methodology

This chapter presents the materials and methods that were used in Paper A-E. A more detailed description of the procedures can be found in the full versions of the papers in the appendixes.

6.1 Contrast agents

In this thesis, three different types of a novel CA, consisting of gas filled MBs stabilized with a shell of PVA, has been investigated. One type consists of pure PVA-shelled MBs (Plain PVA) that can be visualized with ultrasound, whereas the other two types consist of MBs with magnetic nanoparticles linked to the shell (PVA Type A and PVA Type B). These shell modified MBs can serve as a combined CA for ultrasound and MRI [72]. For the ultrasound imaging studies in Paper C, the commercially available ultrasound CA SonoVue (Bracco Imaging, Italy) [25], [73] was used as a reference when evaluating the ultrasound imaging performance of the novel CA. An overview of the CAs used in Paper A-C is shown in Table 6-1. The synthesis of the three PVA-shelled MBs (Plain PVA, PVA Type A and PVA Type B) investigated in Paper A-C are described in the following section.

6.1.1 Synthesis of Plain PVA microbubbles

Synthesis of Plain PVA MBs has been described elsewhere [31], [74]. In summary, 2% (w/v) PVA was dissolved in Milli-Q water. Sodium metaperiodate was added to the solution in order to selectively split the PVA chains. Splitting occurred at sequences of the PVA chains that contain two neighboring hydroxyl groups facing the opposite side (1,2-diols) of the PVA backbone (approx. 2 % of the total sequences) [75]. The products of this splitting reaction were PVA chains with aldehydes as terminal groups. A cross-linking reaction between the mixture of these shorter chains and longer PVA chains (1,3-diols) was carried out during high shear stirring, using an Ultra-Turrax T-25 (IKA, Germany) equipped with a Teflon coated tip, at 8000 rpm for 2 h. During this high shear stirring reaction a foam, consisting of air-filled PVA MBs, was produced at the air-water interface. The floating MB solution was washed repetitively in Milli-Q water using a separation funnel, until no debris was observed at the bottom of the funnel.

Table 6-1 Properties of the contrast agents used in Paper A-C.

<table>
<thead>
<tr>
<th>Type of CA</th>
<th>Shell Material</th>
<th>Gas</th>
<th>Paper</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plain PVA</td>
<td>PVA</td>
<td>Air</td>
<td>C</td>
</tr>
<tr>
<td>PVA Type A</td>
<td>PVA + SPION</td>
<td>Air</td>
<td>A-C</td>
</tr>
<tr>
<td>PVA Type B</td>
<td>PVA + SPION</td>
<td>Air</td>
<td>A-C</td>
</tr>
<tr>
<td>SonoVue</td>
<td>Phospholipids</td>
<td>Sulfur hexafluoride</td>
<td>C</td>
</tr>
</tbody>
</table>
6.1.2 Synthesis of PVA Type A microbubbles

For the synthesis of PVA Type A MBs, SPIONs were covalently attached to the PVA shell following a five step process. First, Plain PVA MBs were produced according to the protocol described in section 6.1.1. Next, a silanization processes that introduced amino groups on the SPIONs surface was performed [72]. Then, the SPIONs were sonicated for 90 min in an ultrasonic bath (Ultrasonic cleaner CP104, CEIA, Italy). Immediately after sonication, SPIONs at a concentration of 20 mg/ml, were added to a volume corresponding to 10 mg of Plain PVA MBs. Reductive amination was used for coupling amino groups on SPIONs with aldehyde groups on Plain PVA MBs. The coupling reaction was initiated by adding NaBH₃CN and continued by gently shaking the solution for five days. Finally, in order to stabilize the MBs oxidized chitosan molecules were coupled to hydroxyl groups of the PVA shell. A more detailed description of the synthesis of these MBs, herein entitled PVA Type A, can be found in Paper A.

6.1.3 Synthesis of PVA Type B microbubbles

Synthesis of PVA Type B MBs was performed by adding SPIONs during the production of Plain PVA MBs. In short, unmodified SPIONs at a concentration of 5 mg/ml were sonicated 90 min in an ultrasonic bath (Ultrasonic cleaner CP104, CEIA, Italy). Subsequently, sonicated SPIONs at a concentration of 20 mg/ml, were added during the Plain PVA MB cross-linking reaction described in section 6.1.1. As shown in previous studies, iron oxide nanoparticles could interact with PVA molecules [76], [77]. Consequently, by adding SPIONs during the PVA cross-linking reaction, the SPIONs were embedded in the MB shell. A schematic overview of shell structure of the three different types of PVA MBs is shown in Figure 6-1.

![Figure 6-1](image.png)

**Figure 6-1** Schematic overview of the shell structure of (A) Plain PVA, (B) PVA Type A with superparamagnetic iron oxide nanoparticles (SPIONs) covalently attached to the PVA shell and (C) PVA Type B with SPIONs embedded inside the PVA shell. The SPIONs are illustrated as black dots.
6.2 Sample preparation and imaging of PVA-shelled microbubbles using transmission electron microscopy

This section describes the sample preparation procedures and imaging conditions for the structural characterization studies performed in Paper A and B.

6.2.1 Morphology and distribution of SPIONs
The distribution and morphology of SPIONs, chemically coupled to (PVA Type A) or physically embedded inside (PVA Type B) the MB shell, were analyzed by TEM. 3 µl of the sample solution were applied to glow-discharged copper grids, that were coated with a thin layer of continuous carbon. Subsequently, after 1 min adsorption to the carbon film, the excess liquid was blotted away with filter paper. Thereafter, the grids were immediately washed in 2 drops of Milli-Q water, blotted again and dried at room temperature for 10-15 min. Imaging was performed using a JEM-2100F (JEOL, Japan) electron microscope, operated at an accelerating voltage of 200 kV. Images were recorded at high defocus (~20 µm defocus) on a 4k × 4k CCD camera (TVIPS, Germany) at a magnification of ×8,000-10,000.

6.2.2 Shell structure characterization
The preparation and imaging protocol described above generated projection images of entire MBs. In other words, the projection images reflected the complete volume parallel to the electron beam. Hence, by using only this method, it was not possible to determine whether the observed SPIONs were attached to or embedded inside the PVA shell. Therefore, in order to determine the position of the SPIONs more precisely, a preparation method involving thin sectioning of the MBs was developed. First, in order to trap the floating MBs, an aliquot of the MB solution was added to Eppendorf tubes containing preheated 10 % (w/v) gelatin (Merck, Germany) solution. Next, the tubes were placed in a stand at room temperature to solidify the gelatin. Magnets were placed at the bottom of the tubes during this process in order to trap the MBs in the gelatin. The gelatin was further stabilized by adding 3 % (w/v) paraformaldehyde in 0.1 M phosphate buffer and the tubes were placed in a refrigerator overnight. Then, the gelatin pellets containing the MBs were cut into smaller blocks. The fixed MBs were then dehydrated in ethanol followed by

Figure 6-2 A theoretical section through the epoxy resin (gray). As illustrated, slicing at varying distances from the MB center will affect the observed shell thickness (T₁ > T₂). [78]
acetone and embedded in epoxy resin LX 112 (Ladd, USA) as described [79]. Finally, ~50 nm thin sections were cut using a Leica Ultracut UCT (Leica, Austria) and placed on formvar coated copper grids stabilized with a thin layer of continuous carbon film. The sections were imaged using Tecnai 10 (FEI, The Netherlands) and JEM-2100F (JEOL, Japan) EMs.

6.2.3 Shell thickness determination
In order to determine the shell thickness of both PVA Type A and PVA Type B MBs, thin sections suitable for TEM imaging were prepared as described in section 6.2.2. The sections were imaged on a Philips/FEI CM120 EM (FEI, The Netherlands) at an acceleration voltage of 120 kV. A total of 100 images, that each contained one MB per image of both PVA Type A and PVA Type B MBs, were recorded at a magnification of ×3,000. A model for calculating the average shell thickness of the MBs from these TEM images was introduced that is discussed in detail in the following section. In order to use the model, images of MBs showing solid discs, sliced far from the MB center, were also included in the data.

6.3 Model for determining the shell thickness of PVA-shelled microbubbles
The apparent shell thickness and radius seen in the TEM images of thin sectioned MBs depends on the position of the cut. Thus, even for MBs with identical radius and shell thickness, sections that were cut at an increasing distance from the MB center will produce larger shell thickness and smaller radius compared to the values at the MB equatorial center, see Figure 6-2. Hence, a model was introduced that corrects for this random slicing effect. The following section presents the model that is discussed in detail in Paper B.

6.3.1 The model
The model introduced for determining the shell thickness of PVA Type A and PVA Type B MBs, see Figure 6-3, was adapted from models utilized for determining the shell thickness of microcapsules and yeast cells [80], [81]. In those models, the observed outer radius, shell thickness and calculated slicing angle, were integrated within a range of slicing angles where the apparent inner radius is different from 0. Thus, those models excluded the objects imaged at very high slicing angles that only cut though their shell material and in order to use these models, the limits for the slicing angle first need to be determined numerically. Instead of using this approach, we introduce a model for determining the MB shell thickness directly from the TEM images by independently calculating the mean outer and inner radius from the data and then taking the difference of these as the average MB shell thickness.

Following the parameters defined in Figure 6-3, the projection z onto the y-axis at slicing angle \( \varphi \) can be described as

\[
z = R \sin \varphi \quad \rightarrow \quad \varphi = \sin^{-1} \frac{z}{R}
\]

(6.1)
The projection $z$ is limited to heights ranging from $-R$ to $+R$, while cuts along $z$ should be of equal probability. Hence, a probability function for cuts along $z$ can be expressed as

$$f(z) = \begin{cases} \frac{1}{2R}, & \text{for } |z| \leq R, \\ 0, & \text{else} \end{cases} \quad \int_{-R}^{+R} f(z) \, dz = \frac{1}{2R} \cdot [z]_{-R}^{+R} = 1 \quad (6.2)$$

The observed outer radius ($R_o$) is given by geometry

$$R_o(z) = R \cos(\varphi(z)) \quad (6.3)$$

The mean value of the observed outer radius ($\bar{R}_o$) can now be calculated from the following expectation value, where $f(R)$ is the probability distribution of the outer radius

$$\bar{R}_o = \int_0^\infty f(R) \int_{-R}^R R \cos \varphi f(z) \, dz \, dR \quad (6.4)$$

Following the calculations given in detail in Paper B, equation (6.4) can be described as

$$\bar{R}_o = \frac{R}{2} \int_{-\pi/2}^{\pi/2} \cos^2 \varphi \, d\varphi = \frac{\pi}{4} \cdot \bar{R} \quad (6.5)$$

As seen in equation (6.5), the mean outer radius ($\bar{R}$) can be calculated directly from the mean value of the observed outer radius ($\bar{R}_o$).

From the parameters illustrated in Figure 6-3, the projection along $z$ can also be written as

$$z = r \sin \theta \quad \rightarrow \quad \theta = \sin^{-1} \frac{z}{r} \quad (6.6)$$

The projection $z$ now ranges from $-r$ to $+r$. Hence, by utilizing similar methodology as for determining the outer radius, the mean inner radius ($\bar{r}$) can be calculated from the mean value of the observed inner radius ($\bar{r}_o$) using equation (6.7).
\[
\bar{r}_o = \int_0^\infty f(r) \int_{-\infty}^\infty r \cos \theta f(z) \, dz \, dr = \frac{\pi}{4} \cdot \bar{r} \tag{6.7}
\]

Consequently, the average shell thickness (\(\bar{T}\)) can be calculated from the TEM images using equation (6.8).

\[
\bar{T} = \bar{R} - \bar{r} = \frac{4}{\pi} (\bar{R}_o - \bar{r}_o) \tag{6.8}
\]

The inner radius does not exist for sections that were cut only through the MB shell, e.g. for \(r < z \leq R\). Thus, the data sets for calculating \(\bar{R}\) and \(\bar{r}\) will have different size.

6.3.2 Simulation
The model described above was validated by simulating the sectioning computationally, using MBs with known shell thickness and radius. For each selected MB radii (1500, 2000 and 2500 nm) a number of corresponding MB shell thicknesses (200, 400, 600, 800 and 1000 nm) were selected. The selected MB radii and shell thicknesses were within the range of the observed values from the TEM sections. For each selected MB dimension (n=15), a total of 1000 sections were generated, slicing the MBs at random projection heights \(z\), within the range of -R to +R, using Microsoft Excel (2007). The observed outer and inner radius were calculated by basic geometry, see Figure 6-3. The mean shell thicknesses of the simulated MBs were calculated utilizing equation (6.8). The simulation for each selected MB dimension was iterated 10 times.

6.4 Investigating the elimination process of PVA-shelled microbubbles
In this section, a summary of the methods used for investigating the elimination profile of Plain PVA, PVA Type A and PVA Type B are presented. Detailed descriptions of the methods are reported in Paper C.

6.4.1 Study subjects
The study protocol for the in-vivo investigation (N372/10) was approved by the regional ethical committee for animal experiments in Solna, Sweden. The study comprised a total of 25 rats. The ultrasound imaging and TEM histology parts included 16 and 9 rats, respectively.

6.4.2 Ultrasound imaging
Two repeated bolus injections, each containing 0.4 ml of the CAs listed in Table 6-1, were manually injected through the right jugular vein of the rats. The CAs were administrated at a flow rate of approximately 0.03 ml/s, followed by a flush of saline. The second dose was injected 30 min after the initial dose. Ultrasound image sequences (1.5-2 min) of the common carotid artery were recorded 10 min post CA injection. However, the recordings were aborted if the signal from the CA was no longer visually detected in the vessel lumen. Details on the ultrasound imaging system and setups are reported in Paper C. After completed recordings, the ultrasound image sequences were exported to a workstation, Vevo LAB (Fujifilm, VisualSonics, Canada), for offline analysis. The mean intensity value, from an area positioned at the center of the vessel lumen (see Figure 6-4), was extracted for each frame of the sequences and exported to MATLAB (MathWorks, USA) for further
processing. The maximum intensity (peak intensity) for each CA injection was used as starting point for estimating the blood elimination time. The mean intensity values, normalized against the peak intensity, were approximated to exponential decay curves using the nonlinear least square method. The median blood elimination time for each CA was then determined from the decay curves.

6.4.3 Histology analysis by transmission electron microscopy

Histology analysis using TEM was performed to study the subcellular localization of the MBs post injection. The animals were given a 0.4 ml bolus injection of the three types of PVA-shelled MBs listed in Table 6-1, in similar fashion as described above. Three rats were euthanized at different time points (10 min, 40 min and 24 h) post injection of each MB type. Tissue samples were collected from liver, spleen, kidney and lung at each time point. Immediately after collection, the tissue samples were cut in 1 mm thin slices and immersed into a fixation buffer (2 % glutaraldehyde, 1 % paraformaldehyde in 0.1 M phosphate buffer) and placed in a refrigerator until further preparation. Tissue sections of approximately 1 mm² that contained the glomerulus, the filtration barrier between the blood and urine, were selectively chosen for the kidney. For the more homogenously composed liver, spleen and lung tissues, regions (1 mm²) containing parenchyma and endothelial cells, were randomly selected. After fixation, the tissue samples were transferred to a wash buffer (0.1 M phosphate buffer) and placed in a refrigerator overnight. Next, the tissue samples were fixed and contrasted with an Osmium buffer (2% OsO₄, in 0.1 M phosphate buffer). The tissue samples were then dehydrated in ethanol followed by acetone and embedded in epoxy resin, LX 112 (Ladd, USA) [79]. Prior to TEM imaging, semithin sections (~0.5 µm) of the kidney were cut using a Leica Ultracut UCT (Leica, Austria) and imaged in a light microscope to identify the areas containing the glomerulus. Finally, tissue sections for TEM imaging (~50 nm thick) were cut and placed on formvar coated copper grids, coated with a thin layer of continuous carbon. In total, 144 tissue sections were included in the study, corresponding to 4 sections from each rat (n=9) and tissue (n=4). Sections were imaged using a Philips/FEI CM120 (FEI, The Netherlands) electron microscope, operated at an acceleration voltage of 80 kV. Areas containing MBs were recorded onto Kodak SO-163 films and digitized using an Epson Perfection V600 photo scanner (Epson, USA).
6.5 Sample preparation and imaging of molecular chaperones using transmission electron microscopy

6.5.1 Cryo-EM preparation and imaging of Hsp21

3 µl of Hsp21 solution (5 µg/ml) were applied onto 400 mesh glow-discharged Quantifoil R2/4 grids (Quantifoil Micro Tools GmbH, Germany), coated with a continuous carbon film. After adding of Hsp21, the grids were incubated for 30 s, blotted for 3 s and then plunge-frozen in liquid ethane using an FEI Vitrobot (temp. = 18 °C, relative humidity = 100 %). Frozen grids were stored in liquid nitrogen until analyze. The grids were imaged in a JEOL JEM2100F EM, operating at 200 kV. The grids were maintained at approximately -180 °C during the entire data collection processes. Images were recorded, aiming at 2-5 µm under-focus, on a DE-20 DDD (Direct Electron, USA). Images were collected at a magnification of ×50,000, resulting in a sampling distance of 1.24 Å/pixel. Each exposure was acquired during 2 s using a frame rate of 20 frames/s (dose rate approx. 1.4 e⁻/Å²/frame), giving an accumulated dose of approximately 60 e⁻/Å². The data set consisted of a total of 152 images.

6.5.2 GraFix purification of DNAJB6 oligomers

For EM, DNAJB6 oligomers were purified and stabilized following the GraFix procedure [83]. A 4 ml linear 5-30% (w/w) sucrose and 0-0.2 % (w/w) glutaraldehyde gradient (in 20 mM NaPO₄, 150 mM Nacl, pH 8.0) was prepared using a gradient master (Biocomp, Canada). 200 µl of DNAJB6 solution (0.4 mg/ml) were added on top of the gradient and run for 16 h at 4 ºC with a speed of 30,000 rpm in a Beckman SW 55 rotor. Fractions of approx. 250 µl were collected from the bottom of the tube using a fraction collector (Gilson, USA). The collected fractions were analyzed by blue native-PAGE on precast 4-16 % Bis-Tris Gels (Life Technologies, Sweden) and scanned with an Epson Perfection V600 photo scanner.

6.5.3 Negative staining EM preparation and imaging of DNAJB6

5 µl of sample from fractions 1-6 collected after the GraFix procedure were applied to glow-discharged continuous carbon-coated 400 mesh copper grids. The grids were subsequently blotted with filter paper, washed with two drops of Milli-Q water and negatively stained with 2 % (w/v) uranyl acetate. Fraction five obtained homogenous particles at relatively high concentration (~ 100 particles/image) and was selected for SPA. The sample was imaged using a JEM2100F EM (JEOL, Japan) operated at 200 kV. Images were recorded on a DE-20 DDD (Direct Electron, USA) at a magnification of ×30,000 and 0.9-1.7 µm defocus. The selected magnification resulted in a pixel size of 2.08 Å at the specimen level. Images were recorded using a frame rate of 20 frames/s and 2 s exposure time. The accumulated dose for the whole exposure was approximately 20 e⁻/Å². A total of 40 images were recorded.

6.6 Single particle analysis of molecular chaperones

6.6.1 Image processing of Hsp21

At first, each frame-set was corrected for sample drift using the DE_process_frames-2.7.1.py script [84]. The initial five frames were discarded due to large amount of drift. For
the remaining frames (6-40), full frame alignment was performed. The alignment procedure was iterated four times and the final drift corrected images were imported to EMAN2 (version 2.12) for initial quality control [85]. 12 images were discarded due to contaminations or too high defocus, reducing the dataset to 140 images. Reference particles for template-based particle picking were selected manually using XMIPP [86], which generated 1,008 particles from a subset of 21 images. The particle coordinate files were imported to RELION and used for 2D classification [87]. The predominant class averages (n=8) were used as templates for automatically selecting 33,456 particles from the full (140 images) dataset in RELION [88]. Particles in ice contaminated areas were manually deselected. The coordinates of the automatically picked particles were then imported to EMAN2 [85] for further processing. 2D class averages were generated to discard false positives in the dataset of the automatically picked particles and to select references for building an initial model. The 2D classification was iterated for another round to discard more false positives, resulting in a dataset containing 18,407 particles, which was selected for 3D refinement. In a previous study, using negative stain EM, it was demonstrated that Hsp21 oligomer has D3 symmetry [59]. In addition, reference free classification of Hsp21 particles from the cryo-EM data showed mainly class averages with 2- and 3-fold symmetry. Hence, the 3D refinements were performed in D3 symmetry. An initial round of 3D refinement was performed using a downscaled (2.48 Å/pixel) dataset, aiming at low resolution (targetres = 25 Å). The final map of the first 3D refinement was used as starting model for a second 3D refinement, using the full-sized (1.24 Å/pixel) dataset as input, aiming at medium resolution (targetres = 15 Å). Finally, a third 3D refinement, using the final map of the second refinement as starting model and the full-sized dataset, aiming at high resolution (targetres = 8 Å) was generated. The resolution reported for the final map of the third 3D refinement, generated using the gold-standard Fourier shell correlation (FSC) procedure [71], was calculated at FSC = 0.143 [89].

6.6.2 Image processing of DNAJB6

The frame-sets from each exposure were drift corrected using the DE_process_frames-2.7.1.py script [84]. The first five frames were excluded from the data due to large amount of drift. The remaining frames (6-40) were processed using full frame alignment. The alignment procedure was iterated four times and the resulting drift corrected images were imported to EMAN2 (version 2.12) for further processing [85]. First, defocus, particle separation and contrast were evaluated with e2evalimage.py. Two images were discarded due to mistargeted defocus. 4,344 particles were selected semi-automatically from the remaining 38 images using the swarm mode particle boxing procedure in e2boxer.py. False positives from the picking procedure, representing clusters of aggregated particles or staining artifacts, were discarded. For each image, CTF parameters were estimated using 160 × 160 pixels boxed out, particle containing, regions using e2ctf.py. 2D classification was performed on 3,717 phase-flipped particles with e2refine2d.py. 2D class averages representing particles in different orientations were selected and used as input for initial model generation using e2initialmodel.py, assuming asymmetric particles (sym = C1). The model with best matching 2D class averages and 3D model projections was selected as starting model for 3D refinement. First, a 3D refinement was performed with downscaled (4.16 Å/pixel) data, aiming at low resolution (targetres = 25 Å), using e2refine_easy.py. A second round of 3D refinement was performed, using the full-size (2.08 Å/pixel) data, with the final map from the first 3D refinement as seeding model, targeting medium resolution.
The presence of two-fold symmetry was tested by imposing this symmetry element along a chosen axis of the asymmetric model. Subsequently, another 3D map was produced by applying C2 symmetry during the reconstruction process.

6.7 Modelling of Hsp21

6.7.1 Homology modeling
The crystal structure of Hsp16.9 was used as template for generating a homology model of Hsp21. The primary sequence of Hsp21 was extracted from (UniProt ID) P31170 by removing the chloroplast transit peptide [59]. Sequence alignment of Hsp21 and Hsp16.9 (UniProt ID, Q41560) was performed with Clustal Omega [90]. 10 different Hsp21 homology models, using chain A and B in Hsp16.9 (PDB ID, 1GME) as templates, were generated using the software MODELLER [91]. The extended sequence in Hsp21, representing the prolonged N-terminal region (41 amino acids) and the unresolved region (42 amino acids) in Hsp16.9 chain B were deleted from the produced Hsp21 models. The best matching models were used for construction of Hsp21 dimers. Model building of the Hsp21 dimer was performed using the Match-Maker extension in Chimera [92].

6.7.2 Docking the Hsp21 model into the density map
The two hexameric discs in wheat Hsp16.9, retrieved from the crystal structure assembly (PDB ID, 1GME.pdb1), acted as templates for producing two hexameric discs of Hsp21, using the homology modelled Hsp21 dimers as building blocks. The Hsp21 hexameric discs were individually docked into the cryo-EM density map. First, rough manual fitting was performed, followed by utilizing the Fit in Map command in Chimera [50].

6.7.3 Difference map calculation
Dimers retrieved from the Hsp16.9 crystal structure (PDB ID, 1GME) was fitted to the Hsp21 cryo-EM map using the same method as described for the Hsp21 dimers above. A 10 Å map from the rearranged Hsp16.9 model was generated by sfall in the CCP4-package [93]. After resampling and scaling a difference map was determined by vop in Chimera [50], which subtracted the Hsp16.9 map from the Hsp21 map. Hence, positive contours should correspond to density arising from the additional residues present in Hsp21.


7 Results

The main results from Paper A-E will be presented in separate sections within this chapter. A more detailed presentation of the results can be found in the full versions of the papers in the appendixes.

7.1 Paper A

In this study, two techniques for coupling SPIONs to PVA-shelled MBs were evaluated. In PVA Type A, the SPIONs were covalently attached to the PVA shell after MB formation. For PVA Type B MBs, SPIONs were added during the PVA shell formation and thus theoretically embedded inside the PVA shell. The following sections will focus on the structural characterization of these two types of MBs using TEM. In addition, the main results from the study acquired by other techniques will also be reported.

7.1.1 SPIONs distribution

The distribution of the SPIONs coupled to MBs was investigated using TEM. Images showing the projected volume of entire MBs were acquired at high acceleration voltage (200 kV). The dense SPIONs are clearly visible in the images and represented as dark particles and clusters. The SPIONs tend to aggregate and form large clusters in PVA Type A MBs (Figure 7-1 A), whereas the SPIONs embedded in PVA Type B MBs were homogeneously distributed (Figure 7-1 B). In addition, small parts of the shell material were observed as separated from the PVA Type A MBs. The iron content in PVA Type A and PVA Type B MBs were 29 % (w/w) and 15 % (w/w), respectively. The higher iron amount for PVA Type A, estimated by thermogravimetric analysis (TGA) combined with differential thermal analysis (DTA), was most probably caused by the large heterogeneous SPION clustering seen in the TEM images.

![Figure 7-1](https://via.placeholder.com/150)

**Figure 7-1** Transmission electron microscopy images of two types of superparamagnetic iron oxide nanoparticles (SPIONs) coupled microbubbles (MBs). (A) PVA Type A with SPIONs covalently attached to the MB shell and (B) PVA Type B with SPIONs embedded inside the MB shell. Scale bars represent 500 nm. [72]
7.1.2 Shell structure

The TEM images shown in Figure 7-1 represent projections through the full volume of the MBs. To determine the position of the attached SPIONs more precisely, i.e. to distinguish if the SPIONs were attached to the surface or embedded inside the PVA shell, further analysis was necessary. Thus, thin cross sections of MBs were prepared and imaged using TEM. Two images displaying the location of the SPIONs are shown in Figure 7-2. As seen for PVA Type A (Figure 7-2 A), the SPIONs were attached to the PVA shell surface. For PVA Type B, the SPIONs were smaller and embedded inside the PVA shell, see Figure 7-2 B. Consequently, as illustrated in Figure 7-2, the TEM images confirmed the theoretical design of these MBs. As shown in the TGA and DTA analysis, the iron content coupled to PVA Type A MBs was also higher compared to the amount of SPIONs in PVA Type B MBs.

7.1.3 Imaging properties

In addition to the structural studies, the acoustical properties of these two MB types were evaluated based on the ultrasound backscatter enhancement. The backscatter enhancement was studied for different concentrations of MBs. For PVA Type A, the maximum backscatter enhancement was achieved at a concentration of \( \sim 4.4 \times 10^5 \) MBs/ml, while for PVA Type B a comparable level required a concentration of \( \sim 2.0 \times 10^6 \) MBs/ml. Hence, attaching SPIONs after the PVA shell formation (PVA Type A) produced a more efficient ultrasound CA.

To work as a CA for combined ultrasound and MR imaging, both acoustic and magnetization properties needed to be evaluated for these MB types. Magnetization studies showed that PVA Type B produced \( \sim 43\% \) higher net magnetization than PVA Type A. These results suggest that PVA Type B should be a more efficient CA for MRI. Consequently, despite the fact that the iron content was higher in PVA Type A than in PVA Type B, embedding the SPIONs in the PVA shell produced a more efficient MRI CA.

![Figure 7-2](image)

**Figure 7-2** Schematic illustration (upper) and transmission electron microscopy images of thin sectioned microbubbles (lower). (A) PVA Type A with superparamagnetic iron oxide nanoparticles (SPIONs) (dark dots) covalently bound to the shell surface and (B) PVA Type B with SPIONs embedded inside the shell. Scale bars represent 500 nm.
7.2 Paper B

In this study a model was introduced for calculating the average shell thickness of PVA Type A and PVA Type B MBs from TEM images of thin cross sectioned MBs (see Figure 7-2). The average shell thickness was calculated from the outer and inner radius after correcting for the random slicing effect illustrated in Figure 6-2.

7.2.1 Microbubble shell thickness determined by the model

The mean values of the inner ($r_o$) and outer ($R_o$) MB radius observed in the TEM images, the mean outer and inner radii ($\bar{r}$ and $\bar{R}$) corrected by the model and the average shell thickness ($\bar{T}$) calculated from the model are presented in Table 7-1. The average shell thickness of PVA Type A MBs, with SPIONs covalently attached to the PVA shell, was 651 nm. For PVA Type B MBs, with SPIONs embedded inside the PVA shell, the average shell thickness was 637 nm. Additionally, the mean diameter of PVA Type A MBs (3.8 µm) was larger compared to PVA Type B MBs (3.6 µm). These numbers for the diameters are equivalent to the ones determined by confocal laser scanning microscopy (3.8 µm ± 0.6 µm) reported in Paper A.

7.2.2 Simulation of microbubbles with known dimensions

Studies using other techniques and models for calculating the shell thickness of PVA-shelled MBs, have reported a wide range (200-900 nm) of values [31], [94]–[97]. The values reported for the average shell thickness of PVA Type A (651 nm) and PVA Type B (637 nm), using our model, was within this range. To validate our model, simulated sectioning of MBs with known shell thickness and radius were generated. The average shell thicknesses and the standard deviation for the MBs are reported in Table 7-2. The values calculated by the model correlates with the known values of the MBs.

<table>
<thead>
<tr>
<th>Microbubbles</th>
<th>$\bar{r}$ [nm]</th>
<th>$\bar{R}$ [nm]</th>
<th>$\bar{r}$ [nm]</th>
<th>$\bar{R}$ [nm]</th>
<th>$\bar{T}$ [nm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>PVA Type A</td>
<td>967</td>
<td>1478</td>
<td>1231</td>
<td>1882</td>
<td>651</td>
</tr>
<tr>
<td>PVA Type B</td>
<td>920</td>
<td>1421</td>
<td>1171</td>
<td>1809</td>
<td>637</td>
</tr>
</tbody>
</table>

Notes: the mean outer ($\bar{R}$) and inner ($\bar{r}$) radius were calculated from the observed mean outer ($\bar{R}_o$) ($n=100$ for both PVA Type A and PVA Type B) and inner ($\bar{r}_o$) ($n=80$ for PVA Type A and $n=82$ for PVA Type B) radius of the microbubbles in the transmission electron microscopy images.

Table 7-1 Average shell thickness $\bar{T}$ of PVA Type A and PVA Type B microbubbles determined by the model.

<table>
<thead>
<tr>
<th>Shell thickness [nm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radius [nm] 200 400 600 800 1000</td>
</tr>
<tr>
<td>1500</td>
</tr>
<tr>
<td>2000</td>
</tr>
<tr>
<td>2500</td>
</tr>
</tbody>
</table>

Table 7-2 The average shell thickness and standard deviation calculated from the model by iteratively ($n = 10$) cutting random slices ($n = 1000$) of simulated microbubbles with known shell thickness and radius.
7.3 Paper C

In this paper, the elimination profiles of three PVA-shelled MBs (Plain PVA, PVA Type A and PVA Type B) were investigated by measuring the ultrasound signal intensity over time. In addition, tissue sections were imaged using TEM to examine the subcellular localization of the MBs post injection.

7.3.1 Blood elimination profile

Decay curves were calculated from the mean intensity in a selected region to estimate the elimination times for each MB type. In addition to the three PVA-shelled MBs, the commercially available ultrasound CA SonoVue was used as a reference in this part of the study. After registering the peak intensity, all CAs showed a rapid decrease in the signal enhancement. However, a difference in the blood half-life times, measured at 50 % of the peak intensity, was observed. SonoVue had a shorter detectable half-life time compared with the investigated PVA-shelled MBs (Figure 7-3). In addition, Plain PVA and PVA Type B had longer detectable half-life times compared with PVA Type A. The blood elimination times, estimated at 20 % of the peak intensity, shows similar tendency as SonoVue had shorter blood elimination time than the three PVA-shelled MBs (Figure 7-3). As for the blood half-life times, Plain PVA and PVA Type B showed longer blood elimination times than PVA Type A at 20 % of the peak intensity. A summary of the median blood elimination times is reported in Table 7-3.

![Figure 7-3](image)

**Figure 7-3** Box plots displaying the blood elimination times at 50 % (A) and 20 % (B) of the ultrasound peak intensity are shown for each contrast agent (CA). The median blood elimination time for all CA injections (SonoVue (n = 8), Plain PVA (n = 7), PVA Type A (n = 8) and PVA Type B (n = 7)) are highlighted in the boxes. [82]

<table>
<thead>
<tr>
<th>Peak intensity</th>
<th>Blood elimination time [s]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SonoVue</td>
</tr>
<tr>
<td>50 %</td>
<td>7.5 (5-14)</td>
</tr>
<tr>
<td>20 %</td>
<td>19.5 (14-30)</td>
</tr>
</tbody>
</table>

**Table 7-3** Median blood elimination times calculated at 50 % and 20 % of the ultrasound peak intensity for the different contrast agents.
7.3.2 Histology

In the analyzed tissue sections, MBs were easy to localize even at low magnifications and due to the specific MB characteristics, such as the large air-filled central cavity and the PVA shell structure, they were clearly differentiated from other cellular structures. A total of 134 tissue sections were imaged by TEM. All detected MBs (n = 731) in these sections were found in the circulatory system, either located in vicinity of, or adherent to macrophages, see Figure 7-4. Both intact and partially collapsed MBs could be detected (Figure 7-4 A). Uptake by parenchyma or endothelial cells was not observed in any of the investigated organs. Owing to the large variety of cells accumulated in the spleen, it was more difficult to determine the location of the MBs in tissue samples from the spleen compared with the other organs. However, as illustrated in Figure 7-4 B, lysosomes were found in close proximity to the MBs, indicating an uptake of MBs by macrophages also in the spleen. As seen in Figure 7-4 C, more than one MB could be phagocytized by one single macrophage.

MBs of all types were found in tissue sections at every analyzed time points (10 min, 40 min and 24 h). However, most MBs were observed in tissue sections collected 10 and 40 min post injection, see Table 7-4. Only a few MBs were found in the tissue sections collected from the kidney (Figure 7-4 D). None of these MBs had passed through the filtration barrier between the blood and urine in the glomerulus. MBs detected in tissue sections collected 24 h post injection still had SPIONs coupled to the PVA shell (Figure 7-4 E). Most of the MBs were found in the lungs (Figure 7-4 F) during the first two investigated time points. A shift in accumulation towards the spleen and liver was noticed in the sections collected 24 h post injection.

Figure 7-4 Tissue sections containing localized microbubbles (MBs) imaged using transmission electron microscopy. (A) Two PVA Type A MBs, one intact (left) and one collapsed (right), internalized in a rat lung macrophage 40 min post injection; (B) Plain PVA MB surrounded by lysosomes in a rat spleen macrophage 40 min post injection; (C) PVA Type B MBs observed in a rat lung macrophage 10 min post injection; (D) Plain PVA MB found inside a rat kidney macrophage 10 min post injection; (E) Structurally intact PVA Type A MB observed in a rat spleen macrophage 24 h post injection; (F) PVA Type B MB found inside a rat lung macrophage 40 min post injection. Scale bars represent 1 µm.
Table 7-4 The number of microbubbles (MBs) observed in tissue sections collected at different times post injection. [82]

<table>
<thead>
<tr>
<th>Time after injection</th>
<th>Analyzed tissue sections</th>
<th>Number of localized MBs</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 min</td>
<td>42*</td>
<td>278</td>
</tr>
<tr>
<td>40 min</td>
<td>46*</td>
<td>292</td>
</tr>
<tr>
<td>24 h</td>
<td>46*</td>
<td>161</td>
</tr>
</tbody>
</table>

* 6 grids were damaged during preparation and therefore excluded.
\* 2 grids were damaged during preparation and therefore excluded.

7.4 Paper D

In this paper, cryo-EM and homology modelling combined with SAXS and CXMS were utilized to investigate the oligomer arrangement in Hsp21. The following section presents the results from cryo-EM and homology modelling. Additional illustrations of the results can be seen in Paper D.

7.4.1 Cryo-electron microscopy of Hsp21

The subunit arrangement of Hsp21 was analyzed using SPA. Hsp21 particles (oligomers) were observed in cryo-EM images (Figure 7-5), and had an approximate diameter of 100 Å. The particles were found in high concentration on the grid (~200 particles per image). Despite the high concentration, the particles were well-separated, thus the images were selected for SPA. A previous study using negative stain EM, has demonstrated that the 12 Hsp21 monomers are arranged in D3 symmetry [59]. In addition, reference-free classification of boxed out Hsp21 particles from the cryo-EM data shows mainly class averages with 2- and 3-fold symmetry (Fig S2 Paper D). Thus, the single-particle analysis was performed in D3-symmetry.

![Figure 7-5 Image showing Hsp21 oligomers obtained by cryo-electron microscopy. The sample was vitrified and imaged in a JEOL JEM2100F electron microscope. The image was recorded on a DE-20 direct detection device. The total exposure time was 2 s, using a frame rate of 20 frames per s. The final image was drift corrected by aligning all the acquired frames.](image)
The reconstructed Hsp21 map (Figure 7-6), obtained from 18,407 boxed out particles, shows that the oligomers are ~100 Å wide (3-fold axis view (Figure 7-6 A)) and ~90 Å high (2-fold axis view (Figure 7-6 B)). The resolution of the reconstructed map was 10.0 Å, which was calculated using gold-standard Fourier shell correlation.

7.4.2 Modeling of Hsp21
Hsp21 dimers were generated by homology modeling, using the sequence homolog from wheat Hsp16.9 (PDB, 1GME) as template. The hexameric disc arrangement (trimer of dimers) in Hsp16.9 [46] was then used as reference for building two Hsp21 discs. Each disc was subsequently fitted individually into the cryo-EM map (Figure 7-7). In order to fit the density, a relative rotation of the hexameric discs by ~30° was required compared to the Hsp16.9 model (Figure 7-8 A and B). In addition, a separation of the two discs by ~35 Å was needed to fit the Hsp21 density map (Figure 7-8 C and D). These two changes required to fit the Hsp21 model to the density map can be described as a stretching in combination with a rotation of the two discs. In other words, the remodeling can be described as a motion along an imaginary screw centered at the 3-fold axis. As a result of this motion, the C-terminal tails from dimers in opposite discs are brought closer together (Figure 7-8 C). As reported for Hsp16.9, the remaining C-terminal tail in each Hsp21 dimer is probably stabilizing the hexameric disc structure [46]. A possible ion pair (K177/D180), located in connecting C-terminal tails in Hsp21, could be stabilizing the double discs in the oligomer (Fig. 4B Paper D). A difference map was calculated between the Hsp21 cryo-EM map and a 10 Å resolution map generated from a remodeled version of Hsp16.9. The map showed extra density extending from the center of the 3-fold axis to the α-crystallin domains in the dimers (Fig 5 Paper D). It is possible that this extra density corresponds to the truncated N-termini of the fitted Hsp21 model.
Figure 7-7 The homology model of Hsp21 fitted into the density map. The views are along the 3-fold axis (A) and one side of the 2-fold axis (B). The view in (B) can be seen as a rotation along the 2-fold axis by 90°. The Hsp21 density map is shown with partially transparent mesh surface representation at contour level 1.11. The two hexameric discs of the Hsp21 model (shown in blue and green for illustrational purpose) were individually fitted into the cryo-EM map using the Fit in Map feature in Chimera [50]. Scale bars represent 10 Å.

Figure 7-8 Comparison of the Hsp21 model with the crystal structure of Hsp16.9. The 3-fold axis view of Hsp21 (A) and Hsp16.9 (B), with the lower discs (green) aligned illustrates a relative rotation by approximately 30° of the upper disc (blue) in Hsp21 compared to Hsp16.9. In addition, one side view of the 2-fold axis demonstrates a separation (~35 Å) of the two discs in Hsp21 (C) compared to Hsp16.9 (D). The changes made to fit the Hsp21 model to the cryo-EM map can be described as an imaginary screw movement along the 3-fold axis.
In this study, gradient fixation of DNAJB6 followed by negative stain EM was performed to obtain structural information on the arrangement of DNAJB6 oligomers. Complementary structural methods were utilized to acquire information about DNAJB6 monomer interactions and binding partners and are reported in Paper E.

7.5.1 Image processing of DNAJB6

The conformation of DNAJB6 oligomers has previously shown to be very polydisperse [63]. In order to limit the number of oligomer conformations, the sample was purified using GraFix [83]. Fractions collected after GraFix (Figure 7-9) show distinct bands when analyzed on Native PAGE. The first six fractions were selected for studies using negative stain EM. One of the fractions (Fraction number 5 in Figure 7-9) showed homogenous particles at a high concentration on the grid. Negative stain EM images showed slightly elongated particles. 2D classification, generated from a set of 3,717 boxed out particles, showed mainly elongated class averages, with the lengths of 160 Å and 120 Å for the major and minor axis, respectively (Fig. 2 Paper E). The asymmetric 3D map (Fig. 3 a and b Paper E) showed a pseudo two-fold symmetry. A map with imposed two-fold symmetry was generated that could be compared to the reconstructed map with C2-symmetry (Figure 7-10). A high degree of similarity between these maps was seen, which suggests that the analyzed DNAJB6 oligomer is symmetrical, at least at this resolution level. The DNAJB6 map with C2-symmetry was reconstructed at a resolution level of 20 Å (Fig. 4 Paper E).

Figure 7-9 DNAJB6 oligomers imaged by negative staining electron microscopy (EM). Insert shows Native PAGE analysis of GraFix purified (10-30 % sucrose gradient, 30 000 rpm, 16 h, 4° C) DNAJB6. The sample was fractionated, starting from the bottom of the tube. The well named 'Inp' is the DNAJB6 sample before GraFix. The EM image shows negatively stained (2% uranyl acetate) DNAJB6 oligomers collected from fraction 5.
Figure 7-10 Density maps of the DNAJB6 oligomer. (A, B) The map reconstructed with C2 symmetry, viewed from two opposite sides of the two-fold axis. (C, D) the asymmetric map, with imposed two-fold symmetry, shown in mesh overlay of the map obtained using C2 symmetry. The surfaces of the C2 and asymmetric maps were rendered in Chimera [50] at contour level 1.23 and 2.69, respectively. Scale bars represent 10 Å.
8 Discussion

8.1 PVA-shelled microbubbles

8.1.1 Structural characterization

Two techniques for coupling SPIONs to PVA-shelled MBs were evaluated in Paper A. In PVA Type A the SPIONs were aggregated and observed as large clusters attached to the MBs, while the SPIONs were more homogeneously distributed in PVA Type B. Thus, the higher iron content measured in PVA Type A (29%) compared to PVA Type B (15%) was probably caused by the fact the SPIONs were more aggregated in these MBs. Despite the higher iron content in PVA Type A, the net magnetization produced by PVA Type B was 43% higher than for PVA Type A. Thus, PVA Type B would be a more efficient MRI CA than PVA Type A. However, these PVA-shelled MBs were designed to work as CA for both MRI and ultrasound imaging and the results from experiments measuring the ultrasound backscatter enhancement concluded that PVA Type A was a more efficient ultrasound CA. These results illustrate an example of compromises that need to be satisfied when introducing new modifications to these multimodal imaging microdevices. The modifications need to be optimized for each imaging modality, while not adversely affecting the other imaging modalities. In order to optimize the contrast enhancement during ultrasound and MR imaging, it is important to know how the modifications change the structure of the MBs. Thus, detailed images, such as those obtained by TEM, are important for optimizing the function of the MBs based on the acquired structural information. As reported above, the position and the degree of aggregation of coupled SPIONs has proved to be important for both the ultrasound and MR imaging performance. A possible explanation to the increased backscattering intensity of PVA Type A could be a difference in the shell stiffness. PVA Type A MBs have been shown to have a much softer shell compared to PVA Type B MBs. It was demonstrated that the chemical coupling of SPIONs in PVA Type A resulted in lost crosslinks within the PVA shell, while the embedding of SPIONs in PVA Type B had the opposite effect and instead stabilized the shell and generate more rigid MBs [94].

The average shell thickness of PVA Type A and B was calculated using the model described in Paper B. The calculations reported a slightly thicker shell for PVA Type A (651 nm) compared to PVA Type B (637 nm). PVA Type A was also larger, with a diameter of ~3.8 µm compared to ~3.6 µm for PVA Type B. Thus, the thickness of the shell of these MBs is ~17% of the particle diameter, which is similar to values reported for Plain PVA MBs [98]. However, other studies using different techniques and models for measuring the shell thickness of PVA-shelled MBs have reported values ranging from 200-900 nm [31], [94]–[97]. Thus, to validate the model used in Paper B, MBs with known dimensions were simulated. Within one standard deviation of the mean, the thickness values obtained by
the model varied in between 10-30 nm from the known value. Hence, the larger thickness reported for PVA Type A (14 nm thicker than PVA Type B) could originate from variations in the model used for calculating the mean. The SPIONs vary in size and have a diameter of 8-12 nm [99]. As the SPIONs were attached as larger clusters on PVA Type A MBs, this could potentially lead to an increased shell thickness. Despite this, comparable shell thicknesses could be explained by the fact that, due to the heavy loading of SPIONs and the softening of the PVA network might lead to a small compression of the shell in PVA Type A.

A few collapsed MBs, mainly PVA Type A, were observed in the sections imaged by TEM in Paper B. Even though the thickness actually could be observed for these MBs, they were not included in the data since the model is only valid for spherical objects. Besides, it is not known if the shell thickness will be consistent after collapsing. It is possible that the lost crosslinks in the PVA network of PVA Type A, which produce softer MBs, also generate less stable MBs with a higher tendency to collapse.

To determine the average shell thickness of MBs, the model presented in Paper B requires sampling at varying slicing angles. At very high slicing angles, as when the projection z is close to -R or +R, the sectioned MBs are difficult to find due to their small size. Since these are excluded from the data the average outer radius is overestimated, which also overestimates the average shell thickness. Another limitation of using the model is that no information on the variation of the MB shell thickness is given by the model. However, by knowing the average shell thickness, optimized conditions for enhanced contrast during ultrasound imaging can be determined more precisely.

The model used for determining the MB shell thickness could potentially be applied to calculate the shell thickness for any spherical particles within the micro-nanometer range, such as other types of MBs, cell-walls and microcapsules. For new drug candidates, structural characterization of the particles is essential before regulatory approval. Thus, this model can be used for determining the shell thickness of such particles.

8.1.2 The elimination process
To investigate the elimination of PVA-shelled MBs, tissue sections were collected at different time points after MB injection and imaged using TEM. None of the 731 localized MBs were found outside the circulatory system, but were instead mainly found in vicinity of, adherent to or internalized in macrophages. Thus, the MBs were recognized by the macrophage system that initiated the elimination of these particles. It was also observed that one single macrophage could incorporate several MBs. It has been reported in other studies that the macrophage system was responsible for elimination of lipid-shelled [100] and albumin [101] MBs. The histology using TEM enable direct detection of MBs at a subcellular level, which is an advantage compared to secondary detection methods. Secondary methods used for investigating CA elimination and distribution include radiolabeling [102] and measuring the amount of gas released from MBs [103]. Although these methods provide quantified data it is not sure how long the radiolabeled molecules are attached to the MBs after CA injection or if the gas release also means that the MBs are eliminated. Thus, no information on both the actual MB structure and localization can be obtained using these secondary methods. As seen in the tissue sections imaged by TEM, intact MBs were still registered in the blood circulation system 24 h after MB injection.
This has also been reported in another study using PVA-shelled MBs, which measured the contribution from the coupled SPIONs by MRI \[104\]. In the TEM histology analysis, a small shift in accumulation of MBs from lungs towards the spleen and liver was noticed when comparing the sections collected 10 min, 40 min and 24 h post injection. This shift has also been observed when investigating the biodistribution of SPIONs coupled PVA-shelled MBs, which was produced utilizing a layer-by-layer coupling technique for attaching the SPIONs \[105\].

For the TEM histology analysis, only a few animals and limited volumes from each tissue were imaged. The limitations are set by the method, such as restrictions in the area and thickness possible to image in one TEM section, and practical reasons, as the time spent at the microscope imaging all sections. Although a total of 134 tissue sections were imaged, no quantitative measurements of the distribution per organ or MB type could be made using this technique. However, the possibility to observe how these PVA-shelled MBs interact with biological tissues on a subcellular level is very important for designing stable structures suitable for longer image acquisition times. A capillary electrophoresis method for quantitative analysis of both intact and fragmented PVA-shelled MBs in blood plasma has recently been developed, which can be used to further investigate the elimination of these MBs \[106\].

The ultrasound investigation showed that all the three PVA-shelled MBs had longer blood circulation times compared to the phospholipid-shelled CA (SonoVue) used in clinic. Besides, the blood circulation time for Plain PVA and PVA Type B was prolonged compared to PVA Type A. As the PVA network is the outermost layer in Plain PVA and PVA Type B, while SPIONs is the outer layer in PVA Type A, the outer surface facing the circulatory system differ for these MBs. Proteins present in the circulatory system attach to the injected particles, creating a so called protein corona that encapsulates the particles. The protein corona is suggested to determine the circulation time of injected particles as it might decide the recognition time needed to activate the macrophage system \[107\]. Thus, differences in the structure and material of the particle’s outer layer facing the circulatory system can change the elimination profile of the particles and the SPIONs coating of PVA Type A MBs can be responsible for the shorter blood circulation time \[108\]. In addition, once internalized by macrophages, the ultrasound signal enhancement from the MBs might possibly be decreased. As described previously, PVA Type A also have a higher tendency to collapse compared to PVA Type B. This would influence the acquired ultrasound signal intensity sequences as a lower percentage of intact MBs are present in the circulatory system post injection. The blood elimination times were measured based on the mean ultrasound signal intensity for a selected region. The signal intensity from structurally intact MBs can be suppressed or completely cancelled by the macrophage encapsulation or the protein corona formation, thus the true blood elimination time of the MBs might differ from the time calculated using the ultrasound signal intensity decay.

8.1.3 Potential diagnostic and therapeutic use of contrast agents
Prolonged blood circulation time offers longer image acquisition, which enables collecting more data from one single injection of MBs. In addition, SPIONs coupled PVA-shelled MBs can offer enhanced contrast when imaging contrast weak regions, such as blood vessels. These CAs can combine both anatomical (MR) and physiological (ultrasound) information.
when used for different imaging modalities. In comparison to MR, ultrasound has lower spatial resolution, but instead enable real time measurements using less expensive and movable instruments. PVA-shelled MBs have also been modified to support combined MR and emission/computed tomography (SPECT/CT) imaging \[104\] and fluorescence/ultrasound imaging \[109\]. Thus, the chemical versatility of the PVA polymers offer possibilities to modify the shell to support a large number of imaging modalities. As seen for the SPIONs modified MBs, the acoustic performance might be impaired when introducing additional modification. However, these changes can be acceptable if more information is gained from the extra modality.

It has been demonstrated that MBs coupled with antibodies directed to disease markers can find target areas, such as inflamed tissues \[110\]. A prolonged blood circulation time is essential for imaging particular target sites, since a longer circulation time increases the probability of finding the intended target site. Still only a few MBs will possibly reach the target sites, hence development of more sensitive imaging methods is needed to enable single MB detection at target sites. In addition to inflamed tissues, ultrasound CAs have successfully been utilized to detect and diagnose cardiac ischemia \[111\], thrombosis \[112\] and angiogenesis \[113\]. It has also been demonstrated that MRI CAs can be used for imaging and diagnosis of tumors by targeting inflammatory markers \[114\]. Thus, a large number of diseases can be detected using injectable CAs.

Besides being used as a diagnostic tool for enhanced image contrast, MBs can also work as potential drug delivery vehicles. The drugs can either be embedded in or attached to the shell of the MBs. This technique enables imaging of target sites before and after drug treatment, hence the effect of the released drug can be monitored and evaluated directly after administration. When the MBs have reached the target site, the MBs can potentially be destroyed by transmission of high pressure ultrasound pulses, which releases the drugs. MBs can also be injected together with a drug, which can increase the uptake by targeted cells. This effect, called sonoporation, is initiated by ultrasound exposure of the MBs. The ultrasound exposed MBs is assumed to change the permeability of the cell membrane of targeted cells, which will make the drug treatment more efficient \[115\]. PVA-shelled MBs have successfully been loaded with doxorubicin for local delivery at cancer cells \[116\] and nitric oxide for treatment of cardiac ischemia \[117\].

In summary, PVA-shelled MBs offer large chemical variability and can be used as CA for multimodal imaging and as a local drug delivery system. The PVA-shelled MBs are stable enough to offer longer circulation times compared to a phospholipid-shelled CA, but they can still be destroyed by high pressure ultrasound pulses to support local drug release.

8.2 The small heat shock protein Hsp21

8.2.1 Oligomeric structure of Hsp21

Cryo-EM in combination with homology modelling, SAXS and CXMS were used to describe the structure and function of the sHsp Hsp21. The structural model of the Hsp21 dodecamer was docked into the cryo-EM density map, which was obtained at 10.0 Å resolution. The model was created by individually fitting two homology modelled hexameric discs into the cryo-EM map. The hexameric discs were produced using the
dimeric arrangement in the crystal structure of Hsp16.9 as template. In contrast to the Hsp16.9 crystal structure, the Hsp21 model shows a relative rotation of the two discs by approximately 30° and a separation of the discs by about 35 Å (Figure 7-8). These two changes can also be seen as a screw motion along the 3-fold axis. This motion separates the discs and the interaction observed in Hsp16.9 between the C-terminal tail and a binding site in the α-crystallin domain, a connection that helps keeping the discs together, is not conserved in the Hsp21 model. However, the screw motion brings the C-terminal tails located in opposite discs closer together. The corresponding sequence in these neighboring C-terminal tails comprise the conserved sHsps I/V/L-X-I/V/L motif, which in Hsp21 is extended to I/V-X-I/V-X-I/V. In addition, a potential ion pair (K177/D180) located in close connection to the extended motif was identified in the Hsp21 model. This ion pair is conserved among chloroplast-localized sHsps homologues, but does not exist in Hsp16.9 and other homologues (Fig S7 Paper D). These paired C-terminal tail interactions, which include the extended motif and the potential ion pair, are involved in the Hsp21 dodecamer formation. In addition, a mutation (V substituted to A) in the potential binding site of the α-crystallin domain in Hsp21, corresponding to residue 109, did not affect the dodecameric arrangement, while a mutation (Hsp21V181A) in the C-terminal tail motif (residue V181 replaced by A) only produced hexamers and dimers. Hence, the C-terminal tail region, including the conserved I/V-X-I/V-X-I/V motif, is responsible for holding the hexameric discs in the Hsp21 dodecamer together.

In contrast to Hsp16.9, the N-terminal arm region is about 40 amino acids longer in Hsp21 and only six of the twelve N-terminal arms were resolved in the crystal structure (PDB ID, 1GME). Hence, the sequence corresponding to these regions were truncated when constructing the homology model of Hsp21 and should appear as extra density in the cryo-EM map. The differential map shows six regions with additional density on the outside of the dodecamer and six regions extending from the α-crystallin domain towards the inside of the structure (Fig 5 Paper D). The simulations based on the SAXS data shows that the N-terminal arms are flexible and dynamic (Fig 9 Paper D). This flexibility has also been shown in simulations of the N-terminal arms of Hsp16.9 [118]. The N-terminal arms in Hsp21 consists of a conserved methionine-rich region, which has been demonstrated to be important for substrate binding and maintaining the chaperone activity [57], [119].

8.2.2 The importance of oligomerization for chaperone activity
The mutated variant Hsp21V181A was chosen for investigating the importance of oligomerization for the chaperone activity of Hsp21. As mentioned above, this Hsp21 variant only contains hexamers and dimers. In theory the hexamers and dimers of Hsp21V181A should have more easily accessed N-terminal arms available for substrate binding. However, the chaperone activity of Hsp21V181A was significantly reduced compared to Hsp21 (Fig. 8 Paper D). Such dramatic effects can possibly be explained by the fact that the dodecameric conformation is essential for Hsp21 to support efficient substrate interaction. The dodecameric arrangement offers flexibility to this dynamic structure, which is suggested to support rapid binding as well as quick release of substrate proteins. Thus, the dodecameric arrangement in Hsp21 might work as a support for weak and flexible interactions, which are essential for full chaperone activity.
8.3 The molecular chaperone DNAJB6

8.3.1 Towards a structural model of DNAJB6 oligomer

The human DNAJB6 has been shown to effectively suppress fibril formation of peptides involved in development of Huntington’s (polyQ) and Alzheimer’s (Aβ42) diseases. In contrast to other less effective fibril suppressors of the DNAJB6 family, DNAJB6 contains a conserved region, rich in serine (S) and threonine (T) residues, and forms oligomers. The DNAJB6 oligomers has been reported to be flexible and analysis of negative stained oligomers in the EM showed heterogeneous particles [63]. Thus, in order to enable structural analysis of DNAJB6, the sample was pre-treated by GraFix. The collected fractions after GraFix were subsequently evaluated by negative stain EM. One of the fractions showed homogenous particles, which was used for SPA. The density maps were generated with and without applied symmetry. The asymmetric map matched the density of the map reconstructed with applied C2 symmetry, suggesting that the DNAJB6 oligomers have two-fold symmetry. Hence, the oligomers probably consist of two subunit building blocks. The subunit building blocks are probably dynamic due to the large flexibility demonstrated for this sample and the reconstructed map may represent one of many possible conformations of the DNAJB6 oligomers. The limited resolution reported for the negative stain EM map makes it difficult to perform reliable docking of the monomers. However, based on the density map it might be possible to fit 16-20 monomers in the oligomer.

The crosslinking mass spectrometry suggests that there is a possible interaction site for the Aβ42 peptide in the C-terminal domain of DNAJB6. In addition, simulations of the DNAJB6 monomer depicted a very dynamic loop region around residues N199 and G200, that might be involved in the formation of the DNAJB6 oligomers. Still more structural information is needed to fully understand the function of this important human molecular chaperone.
9 Conclusions

The conclusions for each paper included in the thesis are as follows:

Paper A TEM images of thin sectioned MBs confirmed that SPIONs could be coupled to the MBs, which enable dual modality (ultrasound and MRI) contrast enhanced imaging. The SPIONs were attached as large clusters to the PVA shell surface in PVA Type A, whereas the SPIONs were smaller and embedded inside the shell in PVA Type B.

Paper B The average shell thickness of the PVA-shelled MBs could be determined from TEM images of cross-sectioned MBs using our mathematical model. The model was validated using simulated slices of MBs with known shell thickness and radius. The values obtained from the model correlated with the known values. The average shell thickness of PVA Type A was 651 nm, whereas the average shell thickness of PVA Type B was 637 nm.

Paper C The elimination profiles of the three CAs (Plain PVA, PVA Type A and PVA Type B), investigated by measuring the ultrasound signal intensity, indicated that the blood elimination time for these three CAs were prolonged compared with the commercially available CA SonoVue. In addition, longer blood elimination times were observed for Plain PVA and PVA Type B than for PVA Type A. The MBs observed in the TEM analyzed tissue sections were exclusively located inside the circulatory system, either found in vicinity of, or internalized by macrophages.

Paper D A structure model of the Hsp21 dodecamer was obtained by combining cryo-EM, homology modelling, SAXS and CXMS. Compared to the crystal structure of its homolog Hsp16.9, the hexameric discs are rotated by about 30° and separated by ~35 Å in the Hsp21 model. Besides, the discs are interacting with each other via the C-terminal tails. A mutation in the C-terminal tail (Hsp21V181A) effected both the oligomerization and chaperone activity of Hsp21. The N-terminal arms are dynamic and involved in substrate binding.

Paper E The oligomerization of DNAJB6 was investigated by SPA of negatively stained oligomers after gradient fixation. The reconstructed maps showed DNAJB6 oligomers with pseudo two-fold symmetry, indicating that the oligomers are assembled by two subunit building blocks.
10 Future work

10.1 PVA-shelled microbubbles
The structural studies and the in vivo investigation of PVA-shelled MBs performed within this thesis have demonstrated that the MBs offer enhanced contrast when used as CA for different imaging modalities. In addition, the evaluated MBs have prolonged circulation time compared to the lipid-shelled CA SonoVue, which supports longer image acquisition times. However, the following studies are needed to further develop these MBs.

- Modify the PVA-shell surface to increase the time needed for recognition of the MBs by the macrophages.
- Optimize the coupling of SPIONs and produce more stable MB, that has a higher net magnetization without compromising the acoustical properties.
- Develop ultrasound contrast sequences that enable single MB detection in real time to support targeting and local drug delivery.
- A complete investigation describing the biodistribution and elimination pathways of the PVA-shelled MBs.

10.2 The small heat shock protein Hsp21
A structure model of the Hsp21 dodecamer was obtained based on a 10.0 Å cryo-EM map. Some additional studies are suggested to further investigate the structure and function of the Hsp21 oligomers.

- Collect more cryo-EM data to obtain a density map at higher resolution, that might allow fitting of secondary structures directly to the EM-map.
- Investigate conformational changes induced by substrate interactions to better understand the dynamics of the Hsp21 oligomers.

10.3 The molecular chaperone DNAJB6
The study presented in this thesis provides just a glimpse of the structure and function of DNAJB6 oligomers. Further studies are needed to understand these flexible structures.

- The analyzed sample might represent one of several possible conformations and optimization of the GraFix protocol is needed to study the others.
- A higher resolution map, possibly acquired by cryo-EM, can provide more information on the oligomeric arrangement.
11 References


