3MiCRON

PROJECT FINAL REPORT

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1 Final publishable summary report

1.1 Executive summary

Multi-modality imaging is an important research field for the medical imaging sector. Multimodality imaging aims to combine the strong points of two or more imaging modalities, thereby improving the diagnostic capabilities. For example, contrast enhanced ultrasound (CEUS) imaging is the only truly bedside applicable diagnostic modality at present and it is also a very powerful bedside decision-making tool in cardiac care. The latest trend in CEUS is so-called molecular imaging, which is a non-invasive technique to visualise and monitor in real-time very fine changes at the molecular level, as they may occur as a result of disease. Nowadays, the key modalities for molecular imaging are Magnetic Resonance Imaging (MRI), single photon emission computed tomography (SPECT) and positron emission tomography (PET). Increasing interest is being shown in the introduction of specific contrast enhanced targeted ultrasound techniques to molecular imaging. In that respect, ultrasound has unique features, i.e. high temporal resolution, low-cost and availability.

The 3MICRON project investigated the potential of *in vitro* and *in vivo* multimodal medical imaging with contrast enhanced ultrasound (CEUS) and various combinations of MRI, PET and SPECT, using polymer microballoons (MBs) as imaging contrast enhancement agent. The micro-balloons were the basic ultrasound contrast enhancement agent, and they were modified by incorporating superparamagnetic iron oxide particles (SPIONs) for MRI or ^{99m}Tc for SPECT imaging, and by attaching ligands for targeting the microballoons at inflamed tissues.

3MiCRON gathered multidisciplinary competences from ten European laboratories with expert knowledge in fields ranging from nanotechnology, pharmaceutics and chemistry to pre-clinical and clinical applications of different imaging modalities and therapy of atherosclerosis and cancer.

During the project multimodal MBs with enhanced biodegradability and desired properties were synthesized and characterized. A quality control procedure including characterization of structural, magnetic, mechanical and interaction properties of MBs was developed, which allowed optimization of the MB production process and performance with respect to the imaging modalities investigated in the project. The manufacturing of the 3MiCRON MBs was up-scaled in accordance with the ISO 9001 standard enabling a delivery of MBs with reproducible properties, such as shell thickness, diameter, and magnetic content. The 3MiCRON MBs were visualized *in vitro* and *in vivo* with several clinical available imaging modalities, such as ultrasound, MRI, SPECT, PET, fluorescent visualization. Several desopsonin proteins, such as serum albumin, have been identified as major protein corona binders indicating good biocompatibility of 3MiCRON MBs. The macrophages were responsible for the elimination of 3MiCRON MBs. The biodistribution of MBs in different organs was determined by contrast enhancement evaluation using different imaging modalities. The biodistribution was shown to be dependent on the attached ligand type, indicating promising steps towards multimodal molecular imaging. The blood half-life time of 3MiCRON MBs was longer than for commercially available contrast agents, indicating potential use in tissue specific targeted imaging. Initial results demonstrate that 3MiCRON MBs have diagnostic potential to be applied in cardiovascular diseases for quantification of myocardial perfusion and endocardial border

delineation. In addition, increased uptake of targeted MBs was shown in inflammation models demonstrating the possibility of molecular imaging of inflammation, which is pathophysiological mechanism of development of various diseases from atherosclerosis through rheumatoid arthritis and cancer.

The 3MiCRON project resulted in 14 peer reviewed publications, 3 patent applications and around 70 presentations and posters at conferences and workshops, as well as 6 PhD theses. 3MiCRON also organised several specialised high level training workshops.

1.2 Project context and objectives

1.2.1 3MiCRON partners

Participant legal name (acronym)	Country	Organisation type
Kungliga Tekniska högskolan (KTH)	SE	University
Università di Roma Tor Vergata (UNITV)	IT	University
University College Dublin, Dublin (UCD)	IE	University
Karolinska Institutet (KI)	SE	University
Fondazione IRCCS Istituto Nazionale dei Tumouri (INT)	IT	Research organization
Surflay Nanotec GmbH (Surflay)	DE	SME
Universitaet Bayreuth (UBT)	DE	University
Esaote S.p.A. (Esaote)	IT	Multinational industry
Sintef (Sintef)	NO	Research organization
Deutsches Krebsforschungszentrum Heidelberg (DKFZ)	DE	University

1.2.2 Background

Contrast enhanced ultrasound (CEUS) is the only truly bedside applicable diagnostic modality at present. It is probably also the most powerful bedside decision-making tool in modern cardiac care for assessment of myocardial perfusion. By using CEUS it is possible to distinguish low-risk patients from high risk patients in the emergency room. CEUS has proven superior to today's clinical practice using ECG and risk scoring. Moreover CEUS, being the modality with a very high negative predictive value (i.e. the test rarely misclassifies a sick person as being healthy), gives the possibility to exclude acute coronary artery disease and safely send patients home directly from the emergency room. This is potentially one of the most powerful strategies for cutting hospitalization costs without jeopardizing patient safety in modern cardiac care. Today the majority of low-risk patients are admitted with chest pain to coronary care units for a cost of around 2,500-3,000 EUR/day. The estimated savings from this strategy for a university hospital-sized emergency room is between 2 and 7 MEUR per year.

In 3MiCRON, a new multimodal contrast agent with target specific potential consisting of microballoons (also known as microbubbles, MBs) with a diameter of 3 µm has been developed and tested. The protocol for improved segmentation and blood differentiation using 3MiCRON MBs was established and results were compared with current commercially available contrast of this type,

SonoVue. Important applications of CEUS are to detect, diagnose, and monitor progression of angiogenesis in atherosclerotic plaques or tumours. Angiogenesis is a physiological process involving the growth of new blood vessels from already existing vessels, which is a fundamental step in the transition towards vulnerable or actively growing plaques in the case of atherosclerosis, or the transition from benign to malignant in the case of tumours. Much of the blood volume resides in the microcirculation, with capillaries playing a particularly important role in pathophysiology and drug delivery. Since characterization of the flow in the capillaries is essential for understanding such diseases as atherosclerosis, diabetes and cancer, both *in vitro* and *in vivo* investigations carried out in this project were performed not only on big vessels but rather at the microcapillary level.

The new medical and technological approach of CEUS concerns *theranostics*, i.e. the combining of therapy with diagnostics. Therapeutic systems are no longer a stand-alone approach against disease. On the contrary, they are more and more integrated into diagnostic techniques such as ultrasound, fluoroscopy, nuclear medicine, computed tomography (CT) and magnetic resonance imaging (MRI). With the help of these techniques, in combination with 3MiCRON MBs, local and specific drug delivery becomes possible.

Within the frame of the 3MiCRON project the surface of the polymer MB was decorated with pharmacological agents. In addition to the therapeutic payload, MBs can be coated with ligands. Specific ligands can thereby bind to the specific receptors of the cell, enabling targeted imaging, and helping to differentiate atherosclerosis or tumour from normal tissue. The cutting-edge result of these integrated functions is the administration of a much lower drug dosage, thus avoiding the side effects of pharmaceutical overloading, a well-known drawback of chemotherapy.

The latest trend in CEUS is so-called molecular imaging, which is a non-invasive imaging technique to visualize and monitor in real time very fine changes at the molecular level. Nowadays, the key modalities for molecular imaging are MRI, single photon emission computed tomography (SPECT) and positron emission tomography (PET). However, increasing interest is being shown in the introduction of specific contrast enhanced targeted ultrasound techniques to molecular imaging. In that aspect, ultrasound has unique features, i.e. high temporal resolution, low-cost and availability.

Even though the application areas of CEUS are growing every year, there are several fundamental problems when performing clinical tests using this technique. Not taking into account the expertise and experience of the physician who acquires and interprets the image, those fundamental problems are caused by the physical properties including inhomogeneity and instability of the commercially available contrast agents typically based on lipidic shells. In practice, this restricts the use of the technique to dedicated laboratories. Moreover, the self-resonance of currently available lipidic MBs gives intense non-linear reflections that introduce shadow artefacts and distortion of the returning wave. This obscures the deeper structures and leads to an overestimation of the true tissue velocities. Thus, there is a need for a more stable, tissue specific, drug loaded contrast agent that supports simultaneous imaging with several modalities, for example, ultrasound, MRI and nuclear medicine.

1.2.3 Overall objectives

The overall objectives of the project are listed below.

1) Synthesis and characterization of MBs with different properties (thermoresponsivity, response to magnetic fields, controlled size, binding capability toward ^{99m}Tc). Development of MBs with enhanced biodegradability.
2) Characterization of the structural, magnetic, mechanical and interaction properties of MBs in order to provide feedback for the production of MB, quality control for upscaling of the production, and clarification of correlations between more complex properties of MBs like imaging performance and pharmacological properties and structural/physical properties. These correlations should allow optimization of the production process of MBs and their performance.
3) Preparation of larger homogeneous batches of MBs under ISO 9001 and magnetic MBs with defined properties (wall thickness, diameter, magnetite content).
4) Management of clinical and research orientated imaging modalities (ultrasound, MRI, SPECT, PET, fluorescent visualization) with reference to the new contrast agent.
5) Assessment of the long-term biocompatibility of the MBs, using proteomics approaches, such as screening of the binding partners via protein arrays. Recovery of the MBs from exposed cells, tissues and organs and determination of the adsorbed biomolecules to determine biomarkers of compatibility, fate and distribution.
6) Assessment of the biodistribution and clearance of new MBs *in vivo*. In particular the following specific objectives: the distribution of MBs in the different organs; the biological half-life of MBs; evaluation of the biological elimination pathways; evaluation of the contrast enhancement of the MBs using ultrasound, MRI and nuclear medicine.
7) Visualization of disease in animal models *in vivo*. In particular the visualization and quantification of attachment of targeted MBs.

The overall scientific structure of the project is presented in Figure 1 and it can be summarized as follows; two work packages (WP3 and WP5) were responsible for the development and optimization of the MB production. MBs were continuously sent for characterization of structural, mechanical, acoustic and magnetic properties in WP4. *In vitro* imaging in tissue-mimicking phantoms using clinical equipment operating in research mode was carried out within WP6. Questions related to biocompatibility were addressed within WP7. The key role of these testing WPs was to perform screening and to provide instant feedback to the manufacturing of the MBs in order to modify/optimize the synthesis/ production process. As a final step, promising MBs were sent to the pre-clinical WPs. In particular, WP8 evaluated the possibility to visualize multimodal MBs in various animal models, WP9 investigated the possibility to detect inflammation, and WP10 assessed the therapeutic effect and visualized/quantified perfusion of tissues. Moreover, three WPs (WP1, WP2 and WP11) followed the progress of the work, steered the overall direction of the research and promoted the results of the project to the key stakeholders.

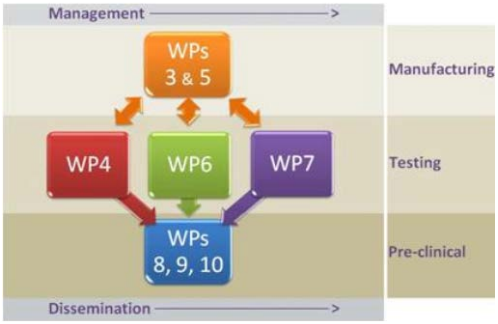

Figure 1. The scientific WP structure of 3MiCRON

1.3 Description of the main S&T results/foreground

This chapter summarizes the major scientific activities carried out during the 36 months of the project and highlights the major results achieved in the eight scientific research WPs.

1.3.1 WP3: Synthesis of a new generation of polymeric MB

Objectives – WP3

The activity of WP3 was focused on the fabrication of a polymer shelled MB with enhanced functionality as a device for multimodal imaging. Poly(vinyl alcohol) (PVA) MBs were the platform used in this WP as derived from the background know-how of UNITV concerning their synthesis. The MB is a microparticles characterized by a gas core and a cross-linked PVA shell, with an average diameter of 3 µm and a limited size distribution. Starting from this platform, several coatings were conjugated on the surface of the PVA MB (Plain MB) in order to support different types of imaging, namely ultrasound, MRI and SPECT. Moreover this next-generation MB was designed for targeting inflamed cells and, for this purpose, specific molecules which recognize markers of inflammation were conjugated on the MB surface, as necessitated by WP objectives. Three partners UNITV, Surflay and UBT as designers of the new MBs, proceeded with three strategies: a) direct decoration of Plain MBs with new molecules, enhancing the functionalities of the device without losing their ultrasound properties (ultrasound scattering efficiency, cavitation at low mechanical index, MI); b) use of layer-by-layer (LbL) technology for the functionalization of the Plain MBs; and c) design of new polypyrrole-based MBs by electrochemical methods. The tasks of WP3 are listed below.

Task 3.1 Fabrication of untargeted Plain MBs with size distribution assessment for *in vivo* tests

Task 3.2 Fabrication of MBs with the following features:

a) Biodegradable polymeric MBs and study of the biodegradation pathways *in vitro*
b) Polymeric MBs responsive to temperature and study of the structural changes with the temperature
c) New polymer MBs formed by application of ultrasound of high intensity
d) Gas loaded MBs (therapeutic/diagnostic gasses, i.e. nitric oxide, xenon for MRI)
e) LbL MBs
f) Magnetic MBs
g) Magnetic LbL MBs

Task 3.3 Surface engineered LbL and polymeric MBs:

a) Fluorescently marked MBs
b) Polymeric MBs with enhanced bio-adhesiveness and targeting properties
c) MBs binding ^{99m}Tc

Results – WP3

Task 3.1 All partners participating to this WP were instructed by UNITV to the fabrication of Plain MBs. The Plain MB samples were delivered to all partners for the assessment *in vitro* and *in vivo*.

Task 3.2 UNITV designed biodegradable polymer vesicles with a cross-linked biopolymer shell containing a hydrophobic liquid as a perfluorocarbon (PFC), Figure 2a. These vesicles are transformed into MBs by acoustic droplet vaporization (ADV) of the hydrophobic core by ultrasounds irradiation, Figure 2b. This process, together with the enzyme catalysed degradation, Figure 2c, is the main novelty with respect to the PVA shelled MB behaviour. We have evidenced that these vesicles can also be temperature responsive by using N-isopropyl acryl amide (NiPAAm) as co-monomer in the polymer shell formation (point b of Task 3.2). The outcome of this research activity resulted in filing an Italian patent (RM2012A000290, 2012) and a paper published (*Chem. Comm.,* 2013). This strategy can be applied to polymers with different structures and elasticity in order to fine-tune the acoustic properties of the shells. Moreover, in the vesicle state the liquid, hydrophobic core can be used as a pool with superior capability of loading drugs that can be vaporized upon ultrasound application and delivered to address organs/tissues. With regard to point (c) of Task 3.2, UBT proceeded with the use of high-power ultrasound during for the electrochemical formation of polypyrrole MBs with precise control of shell growth and diameters. This we consider to be a promising option for the design of alternative microdevices with imaging and drug delivery functions.

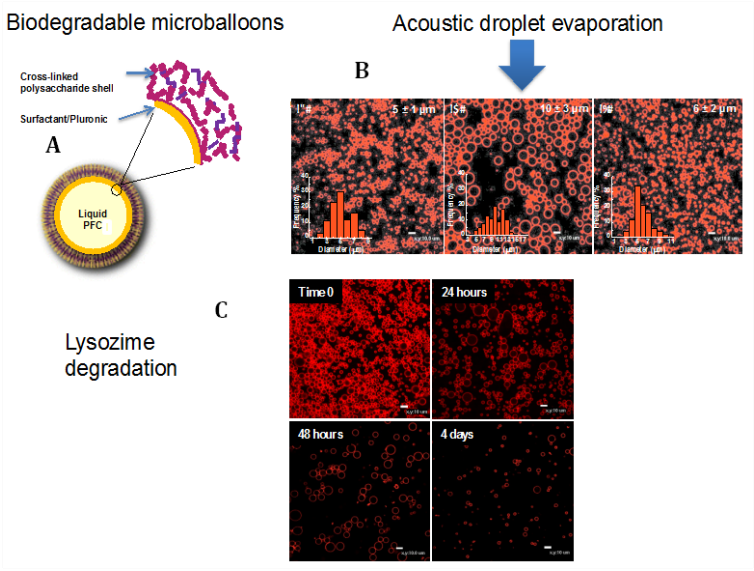

Figure 2. (A): Scheme of the vesicle containing a liquid PFC core stabilized by a lipid monolayer and a cross-linked biopolymer based shell. (B): A confocal micrograph sequence of vesicles (left) before ultrasound irradiation, (center) immediately after ultrasound irradiation (ADV), (right) after return to the initial state. (C) Vesicle biodegradation by lysozyme.

For item (e) of Task 3.2, Surflay has developed an LbL technology for coating Plain MBs with layers of oppositely charged polyelectrolytes, i.e. poly(styrene) sulphonate (PSS)/polyallylamine (PAH). For enabling the LbL coating, an introduction of positive charges to the PVA surface via functionalization with amino-guanidine was necessary. To obtain MBs supporting MRI, superparamagnetic iron oxide nanoparticles (SPIONs) were attached to MBs shell using (i) the LbL technology sandwiched the SPION between PAH layers (Type C), or (ii) the direct embedding of SPIONs in the PVA shell (Type B) and (iii) the SPIONs chemical attachment onto the external surface of the shell (Type A), Figure 3. Transmission Electron Microscopy (TEM) micrography of the different types of MB is reported in Figure 4a-c. The results of the study are described in the paper in *Biomacromolecules*, 2012 (see Section 2.2).

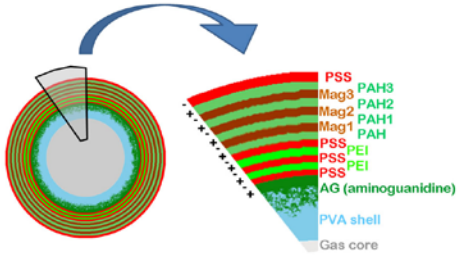

Figure 3. Type C MBs obtained with LbL technology

Task 3.3 MBs with surface engineered were obtained. Labelling with several fluorescent probes (a, Task 3.3) was accomplished as an additional imaging modality. The dyes anchored to MBs were e.g: Fluorescein isothiocyanate (FITC), rhodamine B isothiocyanate (RBITC), cyanine 3 (Cy3), AlexaFluor 488, the near Infrared (NIR) dye VivoTag 680XL. NIR fluorescence imaging is a powerful diagnostic approach in transdermal, tracking in preclinical models, flow cytometry. As for targeting, UNITV and Surflay have worked out a coating with antibodies (anti P-selectin, anti-ICAM-1, anti-VCAM-1) for the respective antigens, through biotin – streptavidin bridging bond, see Figure 5.

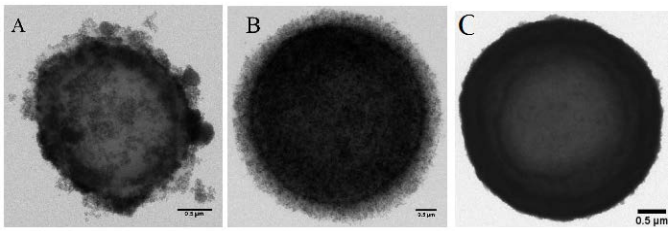

Figure 4. Electron micrographs of A) chemically coupled Type A, B) physically entrapped Type B and C) LbL immobilized Type C, SPION/MBs

Flow cytometry methodology was used for studying the interaction with inflamed endothelial cells (MyEnd) and macrophages (RAW 264.7) (see also WP9). As for item (c) of task 3.3, i.e. MBs binding ^{99m}Tc, UNITV and Surflay have worked on the functionalization of MB shell with bifunctional macrocyclic ligands derived from DOTA (UNITV) and NOTA (Surflay), able to chelate radiometals.

Conclusion – WP3

To summarize the activity carried out in WP3, we demonstrated that PVA shelled MBs (Plain MBs) can be equipped with different functionalities for imaging purposes (ultrasound, MRI, PET, SPECT, fluorescence microscopy), which can be added in a singular way or in combination.

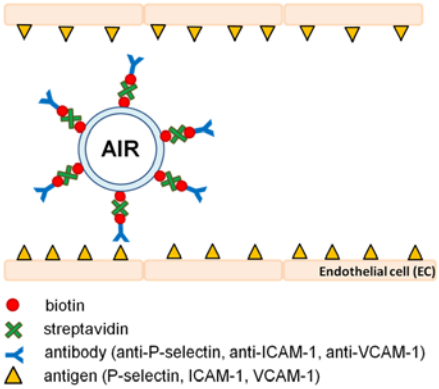

Figure 5. MB biotin – streptavidin bridging to antibody

The surface of the MBs was functionalised by stable coating of defined synthetic or natural polyelectrolytes (e.g. heparine, albumine, hyaluronic acid or just poly(methacrylate) or poly(allylamine) for coupling sites. Moreover introduction of antibodies or enzymes was achieved by direct covalent coupling to the surface or by coupling via the Streptavidin-Biotin link. In addition covalent linkage of polyethylene glycol moieties was proposed for using the stealth effect of this material. Last but not least the new technique to increase storage time by freeze-drying the MBs was developed.

1.3.2 WP4: Structural/physical characterization of MBs

Objectives – WP4

The activities in WP4 aimed on the physical/structural characterization of multimodality contrast agents. The focus of WP4 was on the characterization of structural, magnetic, mechanical and interaction properties of the 3MiCRON MBs. The obtained results served in close collaboration with WP3, WP5 and WP6 for the optimization of the MBs' manufacturing process, for the development of quality assurance criteria during upscaling, and for the prediction of the MBs' imaging performance. The work was divided into the following tasks:

Task 4.1 Structural characterization

Task 4.2 Characterization of mechanical and surface properties

Task 4.3 Investigation of ultrasound properties

Task 4.4 Investigation of magnetic properties

Results – WP4

Plain MBs were the platform for the development of new hybrid probes, which were supplemented with e.g. SPIONs or specific surface modifications to become active in MRI, SPECT or NIR:

Task 4.1 First, WP4 identified the critical parameters that are relevant for the MRI and ultrasound imaging performance of the investigated multimodality contrast agents: shell thickness, shell morphology, particle radius, polydispersity, distribution and density of magnetic nanoparticles. WP4 showed that changes in the MBs' manufacturing process strongly affect these structural parameters. Major structural changes were observed for the integration of SPIONs into the MBs where physical or chemical strategies were used. Because these structural changes showed to be

relevant for ultrasound and MRI, the magnetic MBs were subdivided in three different design types as shown in Figure 6.

The following characterization techniques proved suitable for the quantification of structural differences between the produced magnetic MBs:

- TEM was successfully used to image ultrathin sections of MBs to localize the SPIONs and their distribution in the MBs.
- Atomic force microscopy (AFM) and TEM proved suitable for shell thickness determination.
- Differential scanning calorimetry was successfully employed for the quantification of the PVA network structure (cross-linking density) constituting the polymeric shell.

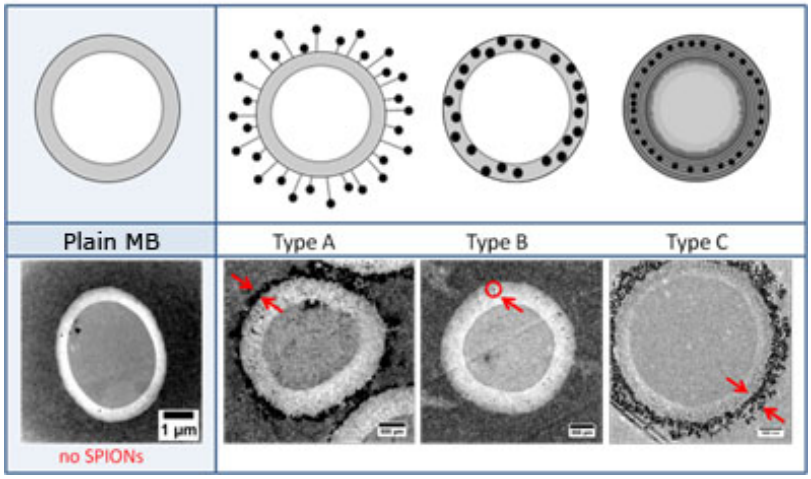

Figure 6. Different structural designs of MBs produced in the 3MiCRON project. (Top) schematic of the structure of US/MRI hybrid probes and (bottom) Ultrathin sections of MBs imaged with TEM. Red arrows indicate SPIONs.

Tasks 4.2 and 4.3 Regarding the MBs' acoustic response, the mechanical properties of the shell were of major interest. Therefore, we investigated the MBs' mechanical response in the low and high frequency regimes. Low frequency mechanics were tested by quasi-static deformation experiments using AFM with a maximal deformation frequency of about 2 Hz. Thus, we were able to quantify shell stiffness and the shell's elastic modulus on the single particle level. From these force deformation experiments additional information on adhesion properties and critical burst forces were gained. High frequency mechanics were tested in acoustic tests with a maximum deformation frequency of about 16 MHz. Thus, backscatter intensity, attenuation coefficient and the pressure threshold for the fracturing of MBs were determined. Moreover, modelling and simulations were successfully used to reconstruct the shear modulus and shear viscosity of the shell material. The results from structural characterization were used as input parameters for the theoretical models. The obtained results made clear that the manufacturing process affects the PVA network structure constituting the PVA shell and thus has an impact on the shell's mechanical properties. In brief, Type A showed a softer shell compared to the Plain MBs, while Type C were much stiffer than Type B and the reference sample (Figure 7 and *Soft Matter*, 2012). For ultrasound contrast agents the soft-shelled MBs showed a higher echogenicity than the stiff-shelled MBs. This

finding was further supported by ultrasound tests performed in tissue mimicking phantoms reported in WP6.

To enable further modalities such as SPECT, NIR and the targeting of inflammation, additional molecules were introduced by surface modifications of the MB shell. In summary, these modifications showed a minor effect on the structural/mechanical parameters of MBs. However, the number/concentration of functional molecules on the MBs surface is crucial to ensure an adequate stability and activity of the hybrid MBs. With qualitative and quantitative experiments the successful surface modifications were proofed and quantified. An example for carried out surface modifications were antibody-modified MBs, where the biotin concentration on the MB surface was determined by using a HABA biotin binding assay (see WP3). Another example is SPECT active MBs with DOTA or NOTA on the shell surface and corresponding binding sites for ^{99m}Tc.

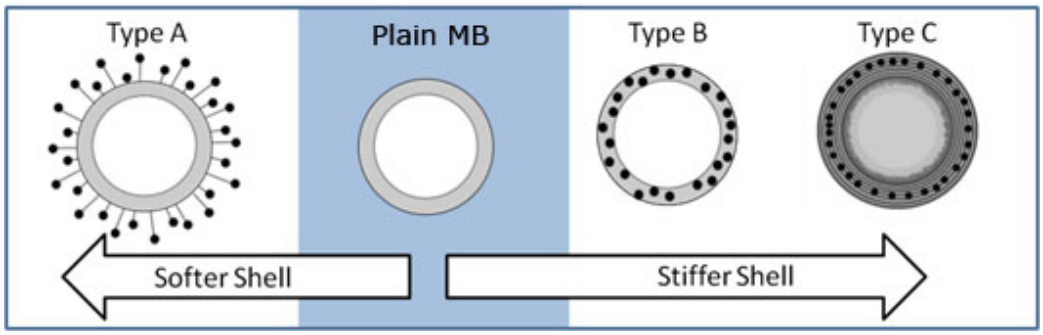

Figure 7. Overview of shell structure and shell mechanics investigated in the low and high frequency deformation regime

Task 4.4 Magnetic properties of MBs were investigated with regard to the content of SPIONs in the synthesized MBs using thermogravimetry analysis and the corresponding magnetization by a home-built magnetic balance. Thus, it was possible to study the impact of the SPIONs content on the MBs performance in MRI and ultrasound imaging.

Conclusion – WP4

The characterization techniques developed within this project are of fundamental interest for a rational design of multimodal contrast agents. Towards a prediction of the imaging performance of MBs, the performed work provides a strategic approach to link structural parameters such as shell structure, shell thickness, and shell mechanics with low and high mechanics and thus allows bridging the gap between synthesis and application.

1.3.3 WP5: Optimization and upscaling of MB preparation

Objectives – WP5

In WP5 the controlled preparation of MBs and magnetic MBs with desired properties was developed in an applicable batch size. The construction of new devices with technology enabling large scale and controllable MB production was performed. The tasks of WP5 are listed below.

Task 5.1 Optimization of the MB preparation

Task 5.2 Upscaling of MB preparation

Task 5.3 LbL coating of the MBs with fluorescence, Iodo-compounds or particles, chelating agents for PET-materials

Task 5.4 Preparation of magnetic MBs with defined magnetism was developed

Task 5.5 Optimization and upscaling of reproducible surface modification of the multitasking MBs

Results – WP5

Tasks 5.1 All surface modifications by Surflay were based on the LbL technology. As the initial MB surface is uncharged and also chemically relatively neutral, all attempts of applying the LbL-coating technology to the bubbles failed. Therefore a pre-coating step was developed which involved a "priming" by aminoguanidine (AG) aminoguanidine and the introduction of a positive surface charge. This modification did neither change the biocompatibility nor the ultrasound response. The pre-coating is followed by the primary coating with stable LbL-films: alternating negative and positive layers highly stabilize the MB and make them a good initial body for further modification. However, the stiffness of the shell increased and therewith the ultrasound response decreased slightly compared to non-coated MBs. Schematic representation of the LbL-coating procedure is given in Figure 8.

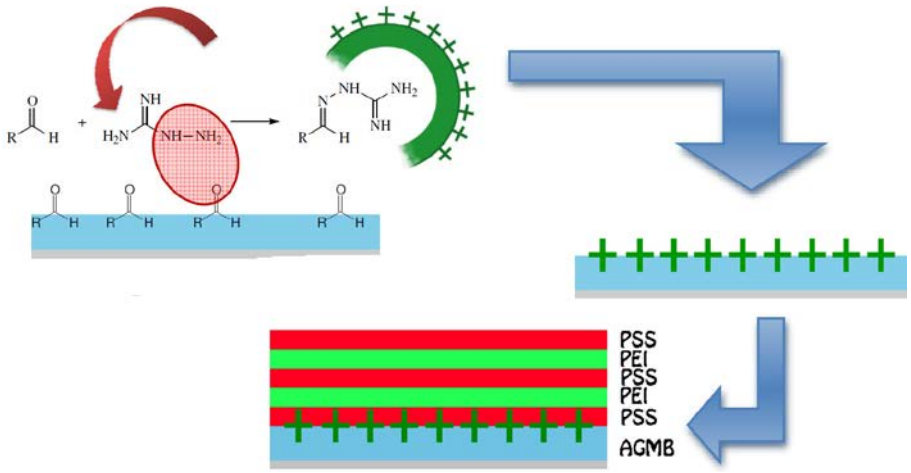

Figure 8. Top: priming the surface by aminoguanidine (hydrazine chemistry), bottom: adding the first 5 layers for stabilizing purposes and for additional charge.

The so encapsulated MBs were further derivatized. We developed a modular system, enabling the production of any desired modality within each MB. As the Plain MBs are already ultrasound compatible, each further function ensures multimodality, which had been set as the project goal. We produced multimodality MBs in good quality (no aggregation, no leaching, homogeneous functionality) and in quantities which are sufficient for the investigation by the consortium partners. Table 1 highlights the coupling method to introduce substances that support multimodal approach in the project.

Table 1. Modalities and methods

Substance	Manner	Method	Modality
Priming	coupling	hydrazine chemistry	-
Primary LbL encapsulation	LbL	LbL	-
SPION	LbL	alternating SPION and PAH layers	MRI
NOTA	coupling	NOTA-SCN reacted with PAH	SPECT/ PET
NIR dye	coupling / LbL	Dye-SCN reacted with the amine groups of the outmost layer / LbL coating using fluorescent dye labeled PAH	IVIS
fluorescein/rhodamine	coupling / LbL	Dye reacted with PAH / LbL using labeled polymers	-
Streptavidin	complexation	Complexation onto a biotin-covalently linked to PAH top layer	Targeting

Task 5.2 Parameters for an upscaled process for industrial MB preparation were optimized. These parameters were used for a stepwise upscaling in the batch size, allowing finally the preparation of above 2 x 10^{11} MBs within one batch with constant yield of 1.2 % in respect to PVA and absolutely repeatable MB-quality in terms of size, aggregation and stability. Several special devices as stirring, ice bath cooling and additional air supply into the solution were introduced in order to achieve this high yield. The development of a continuous process was not successful and further development was not possible within the scope of the project. The optimized batch process is nevertheless stable and reproducible. The excessive and time consuming washing procedure could be reduced from 3 days per batch to below 1 hour by using a continuously working centrifuge system (cream separator), allowing the potential handling of large quantities.

Figure 9. Measurement of the magnetic properties by a magnetic balance for SPIONs, magnetic MBs and magnetic capsules. left: whole setup; right: capsules during the attraction process.

Task 5.3 LbL coated MBs were functionalized with corresponding label agents or nanoparticles in order to address further modalities. For MRI the deposition of SPION nanoparticles of 10 nm in diameter within polycation layers was developed and optimized. For SPECT imaging the chelating agent NOTA was coupled to the PAH and could assembled in one or more layers in the LbL-film. Several different fluorescent dyes were covalently linked with PAH and assembled in layers independently of the other functions.

Task 5.4 A fundamental requirement for a reproducible SPION-MBs production is a reliable process for the SPION suspension itself. Beside the manufacturing and purification the analytics of the

SPION (concentration by UV-VIS, size distribution by Dynamic Light Scattering, Zeta-potential by electrophoretic measurements) were developed. Furthermore, the magnetic properties of MBs Type C, for which SPION were introduced using LbL technology, were characterized by a home-made magnetic balance (Figure 9). The magnetism of MBs Type C can be controlled by varying the number of layers. At about the fourth SPION layer the MBs become so heavy that they do not float any more, which is the upper limit for increasing the magnetism, as the sedimenting MBs cannot be separated from the sedimenting debris. However, the coating and washing liquors can always be removed by withholding the MBs by a magnet. The final step is the sedimentation of the magnetic debris.

Task 5.5 By the end of the project we were able to produce the following: a Plain MB, an LbL-coated MB, a magnetic MB, a multimodal MB; at 1-2×10^{11} scale (2L) in batches or according to the needs of the consortium partners. Regarding the documentation, analytics, process control and reproducibility, production was performed under ISO9001 conditions.

Conclusion – WP5

The preparation of Plain MBs was upscaled in accordance with the ISO9001 standard in the project, enabling future delivery of MBs with reproducible properties. A possible way to apply the LbL-coating to the inert MBs was developed. By the LbL-technology a modular system was established allowing the simultaneous functionalization for several modalities together or in desired combinations. Furthermore, these MBs were further coated by Streptavidine, thus enabling targeting via biotinylated antibodies. The production of these MBs could be upscaled to 2 l batches of 1.2% MB volume, which was sufficient to fulfil all partner requests for the experiments. By the trimodal LbL-MBs the proof of multimodality concept was achieved.

1.3.4 WP6: Multimodal imaging approaches

Objectives – WP6

The objectives of WP6 were the setting up of all the imaging modalities considered in the project, namely ultrasound, MRI and SPECT according to the advanced contrast agent under investigation. The accomplishment of imaging systems-related tasks was strongly based on the results obtained from synthesis, physico-chemical characterization and optimization/upscaling of MBs carried out in WP3, WP4 and WP5 respectively. Moreover, the knowledge acquired within this WP was crucial to address the *in vivo* testing. The main tasks of WP6 are summarised below.

Task 6.1 The design and the development of multimodalities phantoms in order to address the *in vitro* testing and minimize as much as possible the animals use

Task 6.2 Ultrasound modality tuning to enhance the feature of the new contrast agent by identifying the most suitable signal processing algorithms, scanner frequency and imaging mode

Task 6.3 MRI modality tuning concerns the design of sequences and the set-up / optimization of coil in order to maximize the signal-to-noise ratio (SNR) according to new contrast agent

Task 6.4 The Nuclear Imaging (SPECT) activity will be focused on the tuning of isotope imaging according to the contrast agent features as well as of targeted disease

Task 6.5 The design and development of an analysis and visualization software tool for contrast agent perfusion dynamics in order to support both diagnosis and therapeutic follow-up

Results – WP6

The combination of imaging modalities to get the most relevant properties from each modality is a great interest for the future clinics. One of the main objectives of the 3MiCRON project has been to test how the multimodal PVA MBs perform in ultrasound, MRI and SPECT imaging modalities.

Task 6.1 WP6 was designed to develop the signal processing strategies for ultrasound imaging, the sequences for MRI and fitting of coils, and to identify *ad hoc* parameters to correctly apply SPECT/CT using tissue-mimicking phantoms. The development of a multimodality phantom to be used in ultrasound imaging, MRI and SPECT was crucial to perform the *in vitro* analysis. Various experiments have been performed in order to find adequate materials, chemicals, concentrations, dimensions and shapes. To reach the objectives of WP6 several multimodal phantoms mimicking both tissues and vessels of different sizes were designed and developed. In particular, DKFZ constructed phantoms using agarose-sephadex (Figure 10). These phantoms were suitable for all considered imaging modalities, both in size and image requirements, and allowed the *in vitro* characterization of MBs. Different sets of *in vitro* experiments were carried out considering Plain MB, Type A and Type B, Type C.

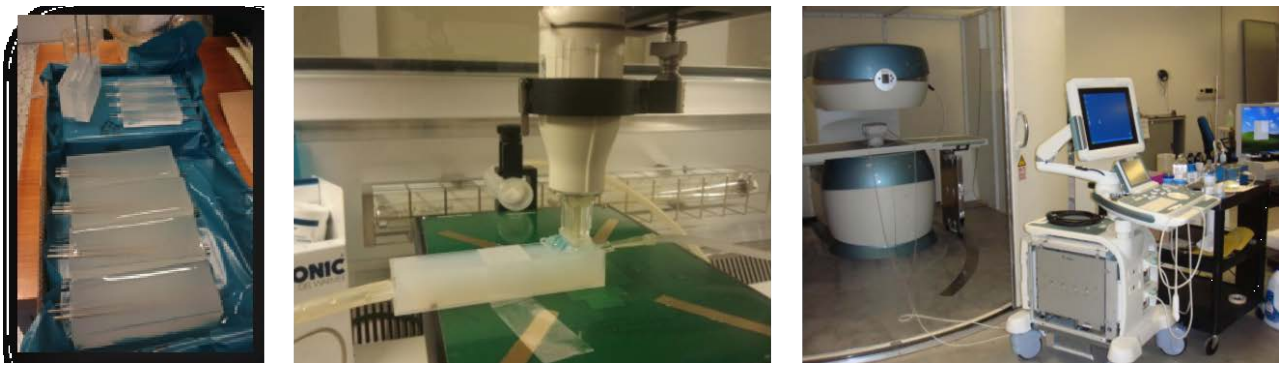

Figure 10. Phantoms and different experimental set-up

Task 6.2 We were able to study several signal processing techniques in order to maximize the SNR as well as the contrast to tissues ratio. As far as ultrasound was concerned several signal processing techniques were studied. To increase the visual detection of contrast agents, specific or custom imaging methods were applied. These methods are based on signal processing strategies that exploit the nonlinear signal backscattered by the MBs to differentiate them from surrounding tissues. If MBs are excited by an appropriate acoustic pressure, an asymmetry in the contraction and expansion phases occurs and a nonlinear response is generated. On the other hand, for low/moderate pressures the biological tissues mainly act as a linear system. The major drawback of polymer-shelled MB is related to the low elasticity of their shell, which can result in a loss of contrast agent echogenicity and consequently a lower nonlinear response. In addition, the introduction of SPIONs could also modify the acoustic response. Considering PVA MB, it was necessary to use higher acoustic pressures (i.e. > 120 kPa) in comparison with lipidic MBs (assumed pressures 60-100 kPa) to correctly detect them.

This finding was considered as a starting point to correctly excite the magnetic MBs developed during the 3MiCRON project. Pulse inversion (PI) technique, a contrast pulse sequence based on the transmission of three pulses in temporal succession (CPS3) and a non-conventional technique based on the combination of multi-pulse excitation and chirp coding (Chirp CPS3) were considered.

Results presented in Figure 11 allowed ultrasound to conclude that PI was not to be considered to image 3MiCRON MBs, only CPS3 and Chirp CPS3 were taken into account. Finally a good compromise between significant MB detection and the aim of maintaining as low as possible the acoustic pressure was reached by assuming pressures ≥ 180 kPa and ≤ 320 kPa, whereas batch concentrations in the range 10^5 - 10^6 MBs/mL resulted to be suitable to obtain a significant contrast in the ultrasound images.

Moreover, a further custom made ultrasound detection technique was designed to optimize the adoption of Type C MBs. The stiffness and the thickness of the shell should be the cause of the poor performance of MB Type C when CEUS imaging techniques based on a nonlinear MB response are assumed. To address this issue the new custom made technique was developed by Sintef. It was found that such a technique was able to detect MB Type C at high acoustic pressures.

Task 6.3 The multimodal contrast agent required an experimental setup (phantom, pump, RF coil, etc.) which taken into account the following critical points: a) The flow must be continuous, to prevent possible sedimentation of MBs; b) No flow speed variations must be present, to avoid flow artefacts in the MRI images; c) The piping must be long enough to keep the peristaltic pump and Ultrasound system at a safe distance from the magnet (i.e., outside the 0.5 mT iso-contour line). Therefore, the approach followed to characterize the new multimodal contrast agent has been to estimate the optimal concentration useful to be used with all imaging modalities, in particular ultrasound and MRI. This estimation has been performed by using a 0.25 T MRI system. SPIONs embedded in the MBs shell increased the transverse relaxivity (R_2*).

By imaging using a custom-made version of the gradient echo sequence, which has a high sensitivity to T_2*, it was possible to evaluate the influence of different concentrations of MBs (Figure 12a). In this custom-made version, no slice selection and phase encoding gradients were used; in this way, the signal from the whole sample was acquired, and the repetitions normally used for phase encoding were used as averages to increase the SNR. A simple fitting to the signal model equation gave the measurement of T_2* at each concentration, therefore allowing the determination of the optimal contrast agent concentration. In addition, two phantom experiments for evaluation of T_2-relaxivity and detection limit for the Type B at a 9.4 T MRI research system have been performed.

Task 6.4 It has been investigated that the novel system of the 3MiCRON MBs could be functionalized with ligands to bind ^{99m}Tc, which makes them visualized using fusion SPECT/CT. However to be able to develop a new contrast agent, animal tests are necessary before trying the substance in man. At the same time there is a desire to decrease the number of animals used in research.

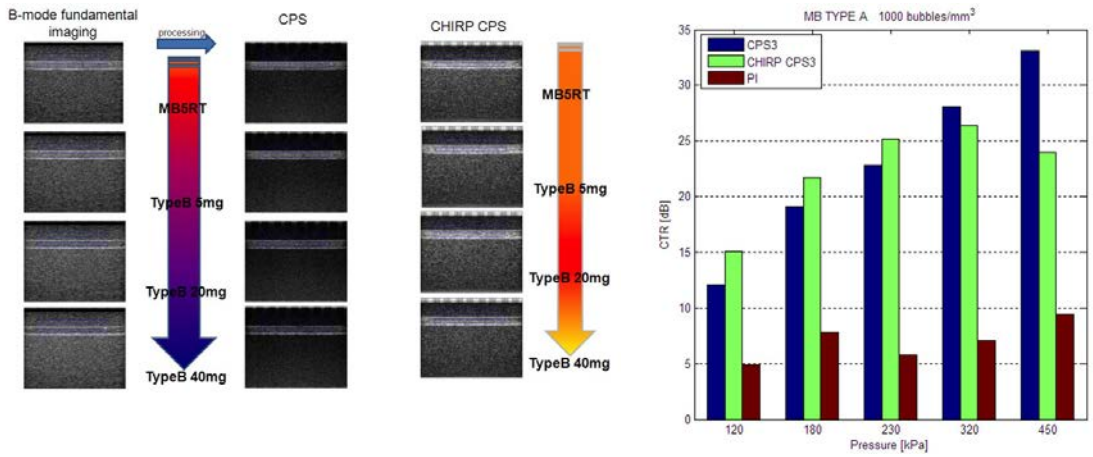

Figure 11. Ultrasound images of an MB (left) and the contrast-to-tissue ratio applying different signal processing techniques (right).

Task 6.5 It was designed and developed a new quantification tool to support diagnosis and therapy based on contrast agent uptake and dynamics. The new approach was based on the development of new algorithms to reduce artefacts due to noise and movement in ultrasound images and to extract and quantify the main parameters. The addressed issues concerned the movement artefacts and patient displacements (during acquisition), the solution to noise problem concerning the pixel-based estimation of clinical parameters and implementation of measurement methods of parameters. The results obtained underlined the feasibility of such an approach, thus allowing ultrasound to quantify parameters (such as MV: maximum value of dynamic speed of contrast agent uptake, REE: slope of enhancement curve and ME: maximum value of enhancement) for supporting diagnosis and therapy (Figure 12 b and c). The approach took into account the new multimodal contrast agent by addressing a correlation of parameters obtained by different modalities.

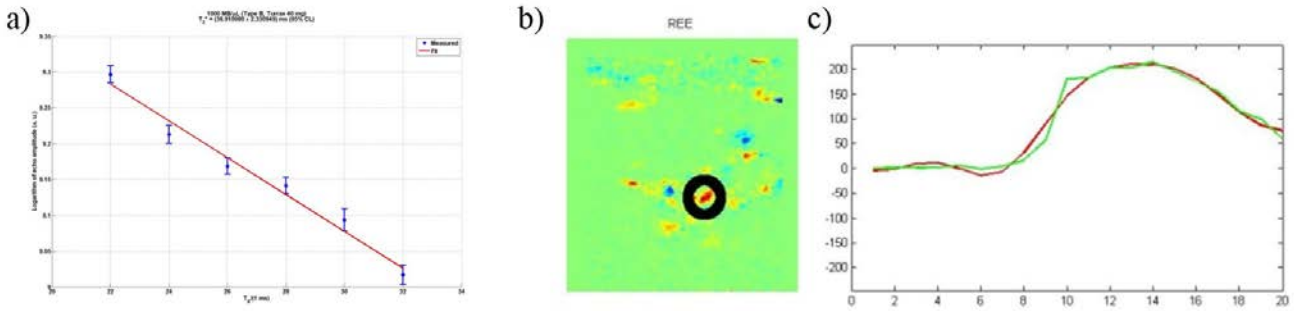

Figure 12. (a) The concentration curve shows how the T_2* behaviour of MBs is as expected. The values found are lower than the T_2* value of blood that is around 300 ms, and this confirms that MBs can be detected inside the blood (T_2*evaluation - Type B 40mg (106 mL-1; (b-c) Contrast agent enhancement: time/intensity curves of a region of interest.

Conclusions – WP6

The activity was fundamental to understand the properties of different MBs considered within the project framework. The study started from the construction of a multimodality phantom to be used in ultrasound, MRI and SPECT. About ultrasound, the innovative magnetic MBs have been analysed and compared considering a well-known and a non-conventional (not commercially available)

ultrasound signal processing technique. The measurement and calculation of the contrast-to-tissue ratio have shown that the structure of the magnetic MBs can modify the detection sensitivity and the performance of the technique. About MRI (from low field (< 0.5 T) to high fields (from 1.5 T to 9.4 T)) the phantom study allowed the *in vitro* characterisation of potential contrast agents by using different concentrations, and above all the definition of a robust protocol for assessment of relaxometric properties of flowing media, whose qualitative results were confirmed by quantitative measurements taken in static experiments. The detectable concentration limit was set around 10^6 MBs/mL, which confirms that the T_2/T_2* relaxation process can be used as a nearly field-independent contrast mechanism. The good results seen at low field with this kind of contrast let ultrasound foresee that further sequence parameter optimization at high field could result in even lower detection threshold, therefore making high-quality *in vivo* examination possible with moderate amounts of contrast agent injected, reducing toxicity issues. Considering the SPECT, ^{99m}Tc-labelled Type A can be visualized using a clinical SPECT/CT simulating MBs circulating in the rat aorta. The results indicate that the concept of multimodal imaging using MRI and SPECT/CT to image magnetic MBs in small animals using clinical MRI and SPECT/CT equipment is feasible.

1.3.5 WP7: Advanced biocompatibility and biological impact

Objectives – WP7

Given their proposed use as contrast agents *in vivo*, MBs need to exert an excellent safety profile in order to be suitable for development for clinical use and *in vivo* applications. Thus, a significant focus with the 3MiCRON project is given to addressing the safety, toxicity and side effects (degradation products) of the MBs being developed within the project. The main tasks were:

Task 7.1 Characterization of the MB protein corona

Task 7.2 Determination of protein residence times

Task 7.3 Recovery of MBs from tissues following *in vivo* exposure

Task 7.4 Assessment of the *in vitro* biocompatibility of new polymeric and LbL MBs in normal fibroblasts and inflammatory cell models

Results – WP7

Tasks 7.1 and 7.2: It is now becoming more evident that nano-micro particles in contact with biological fluid interact with the biomolecules of the media. Particles will bind, with different extent, to the material surface forming the protein corona that will affect the nano-micro particles biodistribution, toxicity and macrophage recognition. This can have consequences on nanoparticle biological fate, biological trafficking and targeting efficacy while the pristine surface remains hidden and non-accessible. In this WP, we have evaluated the MBs behaviour after exposure with different ranges of biological fluid that mimic laboratory experimental condition (e.g. cell culture media in 10% bovine serum) or different physiological condition of the *in vitro/in vivo* scenario (i.e. 6-10% (v/v) and 100% (v/v) human blood plasma or serum). The study was initially focussed on the MB behaviour in relevant media with physiochemical approach and then on the identification of the biomolecule protein corona binders to predict the MBs behaviour in cellular milieu. Until now MBs properties were evaluated by an imaging approach that has shown to be effective but time

consuming. Here we develop a new approach, based on flow cytometry, as a high throughput tool to characterize the MB's dispersion both after synthesis and after exposure to different biological media. Forward and side scatter values are directly correlated to the object diameter and granularity, respectively, which has provided information regarding the MBs dispersion.

Protein corona assessment has shown that the protein interaction with the MB surface is relatively weak suggesting that the proteins are easily displaced by other binders. Several desopsonin proteins, such as serum albumin, have been identified as major protein corona binders indicating good biocompability, while a stronger association with depsosponin proteins, like immunoglobulins or complement proteins would promote macrophages recognition and blood clearance. These findings suggest that MBs are not likely to be recognized by macrophages in the blood stream and thus should have a prolonged circulation life in the system, which is an excellent result in terms of their potential for use as contrast agents where circulation time will be a critical factor.

Task 7.3 As an ulterior challenge for this work, we investigated whether flow cytometry could be used to detect, accurately count and isolate MBs in organs recovered from animals exposed to the MBs. Rat blood, lung and liver, which have been previously shown to be main regions of MBs accumulation have been harvested and shipped to UCD after an early exposure injection of 10 minutes. Thanks to the use of flow cytometry, detection of MBs in the blood was investigated by simply run the blood sample in the flow cytometer and using the fluorescence and forward scatter intensity. Flow cytometry showed that no MBs could be detected in the blood, indicating MBs clearance from the main arteries and veins (Figure 13 b). MBs detection in liver and lung required tissue disruption to promote MB release into solution. A high number of MBs was detected in lung tissue indicating rapid translocation and accumulation in this organ while lower numbers of MBs were detected in other organs (Figure 133 c-d). These findings were in partial agreement with WP9 infusion studies and this approach has then shown to be successful in providing accurate quantitative MBs accumulation in organs providing useful biodistribution information after exposure and suggested that flow cytometry can be used successfully not only to recover and detect the protein corona associated with the MBs but also to provide key information on bio-distribution data. Moreover while biodistribution analysis performed by SPECT and MRI does not allow determining the number of MBs in the different organs, flow cytometry can give clear indications on MBs concentrations and could be further developed to obtain exact MBs numbers.

Task 7.4 Based on physiochemical characterization and protein corona study, the majority of the MBs tested appeared monodispersed and intact after exposure with biological fluid, with the exception of LBL05 and LBL08 that appear highly aggregated (Table 2). Additionally, the protein corona proteomics study have identified several desopsonin proteins, and immunoglobulin with lower extent, to be associated with the MBs surface, suggesting that the MBs will unlikely be recognized and sequestered by macrophages.

In vitro toxicity studies performed showed a dose response cellular activity after incubation with MBs after 3 and 7 days exposure and cell cultures were examined for cell morphology and density by phase contrast microscopy. MBs type and a clear outer layer composition have a clear effect on the cellular proliferation and Table 2 summarizes the *in vitro* biocompability findings (10% human plasma) where MBs toxicity was considered where cell viability exceeded 80%.

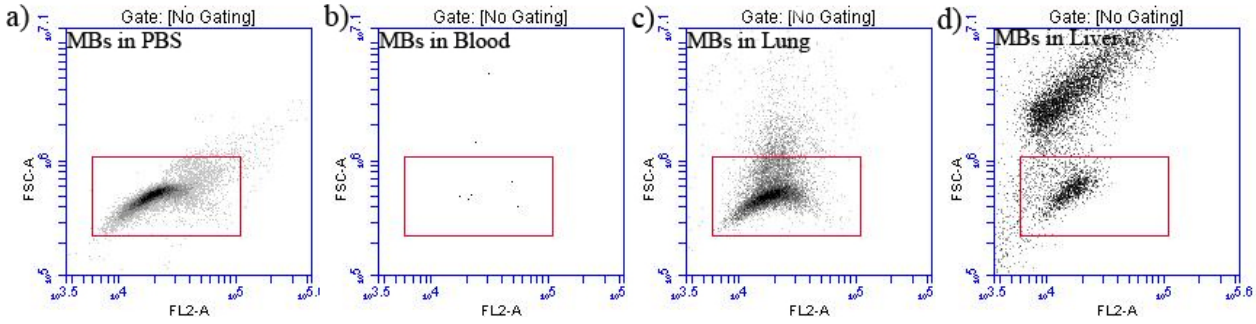

Figure 13. MB detection in PBS and organs of rat injected with MBs. Double scatter plot of FSC versus fluorescence intensity of MBs in PBS (a), and blood (b), lung (c) and liver (d) tissues recovered from rats after injection of MBs. 10^8 MBs/ml Type C MBs were injected in adult rats.

Conclusion – WP7

In vitro biocompability tests indicated that most of the MBs were stable and monodispersed after exposure to different biological fluids. Protein corona assessment showed that the protein interactions with the MB' surface are relatively weak suggesting that the proteins are easily displaced by other binders. Serum albumin, have been identified as major protein corona binders indicating that MBs in blood are likely to be biocompable while a stronger association with immunoglobulins would promote macrophages recognition and blood clearance. These findings suggest that MBs are likely not to be recognized and sequestered from macrophages and that they can potentially have prolonged circulation time.

Table 2. *In vitro* biocompability tests

MBs	**Name**	**Biocompability in vitro study**	**Cell growth inhibition**
Plain	Plain MB	Not toxic	0
SPIONS:CHITOX	Type A (magnetic)	Toxic at high dose	20-40%
SPIONS	Type B (magnetic)	Toxic at high dose	20-50%
NOTA-PVA	TypeC (magnetic)	Toxic	80-95%
LBL01	Unmodified	Modest toxicity	15-30%
LBL02	Cationic MBs	Toxic at high dose	15-40%
LBL03	AG-MB/PSS	Modest toxicity	15-30%
LBL04	AG-MB/PMAA	Not toxic	0
LBL05	AG-MB/Carrageenan	Not toxic	0
LBL06	AG-MB/Albumin	Modest toxicity	10-20%
LBL07	AG-MB/PSS/PAH	Toxic	30-40%
LBL08	AG-MB/PSS/Chitosane	Toxic	30-40%
LBL09	AG-MB/PSS/PEI	Toxic at high dose	20-40%
LBL10	AG-MB/PSS/PaOEt	Not toxic	0
LBL11	AG-MB/PSS/PDA	Modest toxicity	10-15%

1.3.6 WP8: *In vivo* testing on multimodality MBs

Objectives – WP 8

In WP8 the *in vivo* characteristics of the 3MiCRON MBs were addressed. The key features of the different MBs like the *in vivo* contrast enhancement, the biodistribution and the determination of the respective elimination pathways were explored. However, one has to keep in mind that any alteration regarding the total, or the local surface charge, due to chemical modifications on the different MBs, will most likely lead to a change in the characteristic biodistribution and therefore also effect the respective elimination pathways. Predictions on the pharmacokinetic behaviour or the transfer of one finding from one MB to another is thus limited and extensive experiments regarding body clearance and elimination pathways had to be focused on selective MBs. The tasks of WP8 are listed below.

Task 8.1 Evaluation of the contrast enhancement in different organs *in vivo* using ultrasound, MRI and SPECT.

Task 8.2 Evaluation of the distribution of the MBs in the different organs by using MRI, SPECT, scintillation counting and microscopy

Task 8.3 Assessment of the biological half-life of the MBs using inductively coupled plasma mass spectrometry (ICP-MS) measurements and histological analysis

Task 8.4 Evaluation of the biological elimination pathways using ICP-MS measurements and histological analysis

Results – WP8

Task 8.1 Using ultrasound the Plain MB can be visualized with high frequency ultrasound and contrast specific sequences. Female New Zealand white rabbits were used to optimize and verify a custom made ultrasound MB detection technique developed in the current project. Plain MBs with a concentration of $9x10^8$ MBs/ml was used. Figure 14 shows long-axis renal contrast images from a rabbit at two different time points in the first passage of a bolus injection. It can be seen that the custom made detection technique has good specificity in detecting MBs because little tissue signal is present in the images. The sensitivity with this technique is also good and adequate for visualization of individual Plain MBs which is well demonstrated in the renal medulla in the right panel of Figure 14. Plain MBs demonstrate good *in vivo* signal enhancement and exhibit a longer blood half-life time than the market leader SonoVue.

Task 8.2 For all contrast agents a strong increase in signal intensity, more than 100%, was observed in carotid artery after the injection (Figure 15) when using ultrasound. The signal intensity returned quickly back to baseline when using Type C and SonoVue. The results show a blood half-life time below five minutes for SonoVue and Type C. However, after injection of Plain MBs the signal did not return to the baseline level even after 10 min post injection. Nevertheless, even though the signal enhancement in blood had returned to its starting level after 10-15 minutes (blood elimination time), depending on the dosage and the animal model, MBs are probably still circulating in the

bloodstream, because the lowest detection limit probably includes signals from MBs as well as noise from the surrounding.

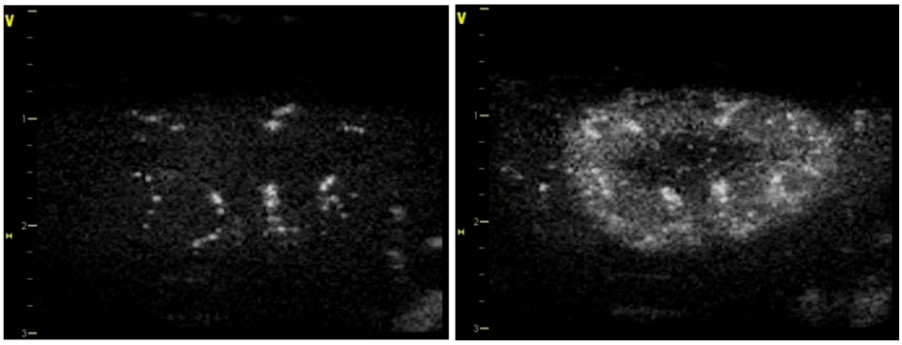

Figure 14. Rabbit renal contrast images with the custom made contrast detection technique after bolus injection of 0.5 ml Plain MBs; imaging frequency 8 MHz. Left panel: shows MBs arriving in the renal arteries. Right panel: 5 seconds after MBs arrive in the renal arteries.

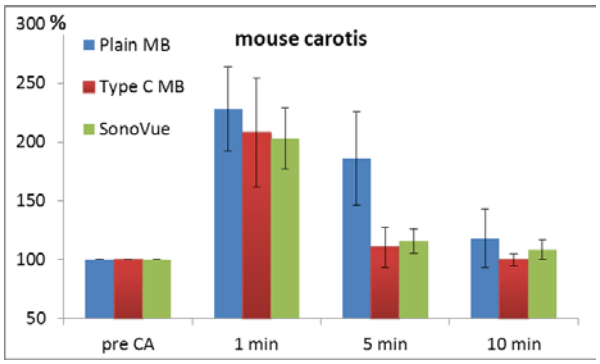

Figure 15. Signal intensity of a mouse carotid artery when using ultrasound. Normalized greyscale intensity over time post injection of Plain MB, Type C and SonoVue. (n=4) (Vevo 770, VisualSonics)

Task 8.3 MRI *in vivo* imaging of multi-functional MBs Type A, Type B and Type C, which all contain SPIONs, provided a strong negative contrast in organs accumulating respective MB (Figure 16). This allows detecting even small amounts of MB in different organs over time. The negative contrast was induced within several minutes and could easily be visualized by various MRI scanners with field strength reaching from one up to 3T. A dose of 100µl Type B (1.17x 10^9 MB/ml) were injected into the tail vein of mice resulting in a strong signal decrease in T_2w MRI images of liver and kidney (Figure 16, yellow arrows) compared to the signal intensities before MB administration. No signal decrease could be observed in either muscle or brain. A complete restoration of T2 times after injection of SPION containing 3MiCRON MBs was not complete after one month post injection in liver tissue of mice and rat. For Type B MB the elimination half-life was calculated to be 448 ± 123 hours. Dynamic PET imaging was performed after labelling of MB Type C with ^{68}Ga (Figure 17). For the alignment of the activity to the respective organs µCT imaging was performed to supply the PET images with the anatomical information of the mouse skeleton. An immediate strong accumulation of Type C MBs post injection in lung tissue could be observed which is typical for 3MiCRON MBs. In a similar set up, using planar dynamic scintigraphy and CT, hybrid imaging was performed. Relocation of ^{99m}Tc-labeled DTPA-SPION MB from lung in liver tissue within 24 h is demonstrated in Figure 18. However, SPECT/CT and MRI showed that the distribution of ^{99m}Tc -labeled ligand-functionalized MB varied with the type of ligand.

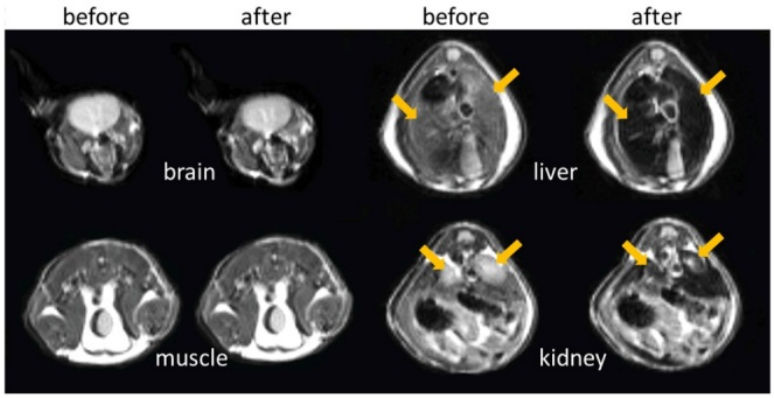

Figure 16. MRI: T2 weighted images of a mouse imaged at 1.5 T before and 5 min after i.v. injection of 100 µl Type B MB (1.17x 109 MB/ml).

Task 8.4 Histology of mouse tissue samples collected at different times after MB administration identified the spleen as the major organ concerned in the final degradation of Type C MBs. Already after one day the first signs of MB degradation could be observed. Furthermore, it was demonstrated that intact MBs can be found even after three months and that intact MB can relocate *in vivo*. Also through histology it could be demonstrated that the lungs are strongly affected by the accumulation of MBs, which are distributed uniformly over the entire sections of the organ (Figure 19). The MBs remain in a great number until three days post injection. After this time, the number decreases gradually but after three months single large aggregates of MBs can still be observed sporadically, and several iron deposits are distributed throughout the tissue section. Tissues like the brain and lymph nodes show nearly no occurrence of Type C MBs and the heart and kidneys only show low MB content. By using ICP-MS the iron content of different tissues of mice injected previously with SPION-containing Type C MBs at different time points was quantitatively determined. In line with the findings from histology and gamma counting, ICP-MS shows an acute and lasting accumulation of the bulk of injected Type C MBs in the mouse lung. Most of the SPION-containing Type C MBs relocate from the lungs to liver and spleen within three days. During the following week the values acquired from liver and spleen decreased to baseline. No relevant changes in iron content in the kidney, brain, lymph node and heart could be detected.

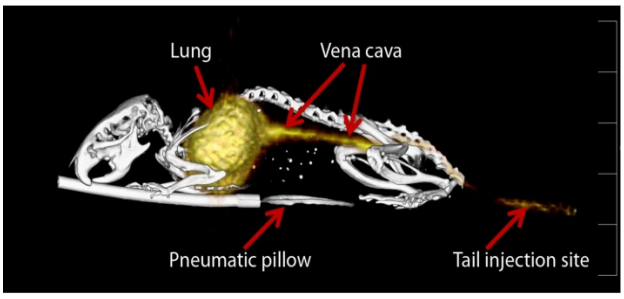

Figure 17. PET/CT: Mouse imaged immediately after injection of 70 µl 68Ga labeled Type C MB (5x108 MB/ml) (Administered activity: 19,2 MBq, µCT: Inveon SIEMENS, set up: 120 RS, 50 KeV, 500 mA, 50 ms exposure time)

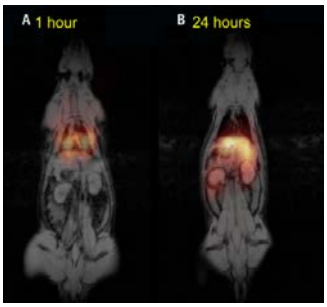

Figure 18. SPECT/CT: rat infused with 99mTc-labeled DTPA-SPION MB. Initially MB are accumulated in lungs, but redistribute to liver within 24h.

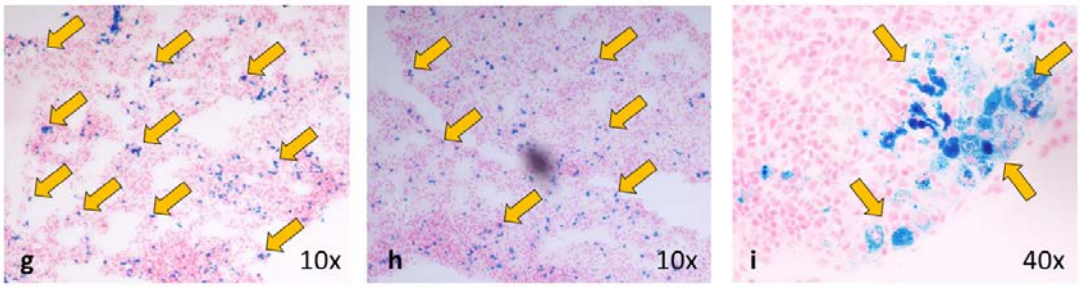

Figure 19. Histology of mouse lung (Prussian Blue and nuclear fast red) g: 1 day, h: 3 days, i: 3 months post injection of Type C MB. Yellow arrows point to intact MB (g, h) and to MB degradation products containing SPION (i).

Conclusion – WP8

It could be demonstrated that all types of the 3MiCRON MBs can be successfully utilized *in vivo* by standard radiological imaging technology like ultrasound, MRI and the nuclear medicine techniques as PET and SPECT. Plain MBs are only visible *in vivo* via ultrasound and the blood half-life time of Plain MBs extends past that observed for SonoVue *in vivo*. Plain MBs are well tolerated by mouse, rat and rabbit models, even when high doses are used (up to 1 ml of $1.9x10^9$ MBs/ml to rat). At high frequency ultrasound, Type B MBs demonstrated an almost as good curve progression and visibility as Plain MBs. This is a very positive finding as Type B MBs are loaded with large amounts of SPION which are otherwise known to decrease the echogenicity of MBs. Type C MBs produced using the LbL technology were very stable and easy to couple with ligands and/or therapeutics. They are well detectable by MRI and high frequency ultrasound techniques, PET and SPECT. Due to a high biostability, intact Type C MBs can still be found in various organs three months post injection.

To obtain sufficient contrast by various SPION containing MBs (Type A, B and C), the necessary dosage repeatedly resulted in severe and fatal side effects due to a rapid accumulation of MBs in lung tissue only in mice models. An enhanced body clearance would also be desirable in order to facilitate repetitive measurements *in vivo*.

1.3.7 WP9: *In vitro* and *in vivo* detection of inflamed tissue using MBs

Objectives – WP9

WP9 uses confocal microscopy, flow cytometry and visualization of MBs to study MB attachment to endothelial cells and uptake by macrophages in relation to inflamed tissue. Various inflammation models were used, comprising inflammation of the outer ear, carotid denudation, zymosan peritonitis, and zymosan induced skeletal muscle inflammation. These studies were performed in rats. We also evaluated aortic atherosclerosis in gene modified mice. Different imaging techniques were applied: ultrasound, PET, SPECT, MRI and confocal microscopy. Regarding ultrasound, the acquisition techniques were modified in several ways to improve the possibility to detect small amounts of MBs. SPECT was applied using a clinical scanner, while PET and a 9.4 T MRI were designed for small animal studies. Standard protocols were applied for these modalities. The tasks for WP9 are listed below.

Task 9.1 Behaviour in relation to surfaces and cells: Targeted contrast behaviour in relation to specific surfaces and cells

Task 9.2 Affinity to target: Influence of the MB construction, specific for single or multimodal imaging, regarding affinity to target

Task 9.3 Identification of inflamed tissue: Findings regarding identification of inflamed tissue by single or multimodal contrast

Task 9.4 Contrast in inflamed tissue: The expected increase of targeted contrast in pathologic/ inflamed tissue. Tissues transferred to WP7 and results on proteomics of targeted MBs.

Results – WP9

Task 9.1 Macrophage cells have a natural ability to phagocytise foreign matter. We found that Plain MBs were taken up to a certain extent, but only after minimum 3 hour incubation, while the MBs modified with SPION were phagocytised more rapidly. SPION chemically attached to the surface of chitosan coated MBs (Type A) caused an uptake within 30 min. The MBs with SPION physically adsorbed in the PVA-shell (Type B) were taken up at a slightly slower rate. Experiments using endothelial cells showed no significant uptake or adhesion of native, unmodified MBs. Further, they did not adhere strongly enough to withstand cautious washing. It was not unexpected that endothelial cells did not phagocytise the MBs. However, endothelial cell models become more relevant when stimulus to over-express certain adhesion molecules was applied to mimic inflamed tissue.

Task 9.2 The efficiency of inflammation targeting by injectable MBs was evaluated. For this purpose, the ability of lipopolysaccarides (LPS)-activated macrophages to over-express adhesion molecules, e.g. ICAM-1, on their membrane surface was exploited. The MB shells were decorated with antibodies or peptides to increase attachment to inflamed cells. This coating also caused more MBs to attach to inflamed cells compared to the non-specific attachment of IgG2b isotype conjugated MB. We have further managed to activate endothelial cells and macrophages with pro-inflammatory cytokines (TNF-α/IL-1β) and target inflammatory markers. A particular challenge for comparison is to maintain control cells unstimulated, but we managed to overcome this problem. All experiments showed that MBs tagged with antibodies against ICAM-1, VCAM-1, Selectin or with cRGD-peptide adhered stronger to stimulated endothelial cells, but also to non-stimulated cells, compared to a PSS-coated negative control MB. However, non-specific adherence was seen to some extent between streptavidin coated control MBs and endothelial cells. Our findings suggested that ICAM-1, VCAM-1 and cRGD might be used for *in vivo* experiments.

Task 9.3 MBs were shown to be versatile in terms of multi-modality illustrated by ultrasound, MRI, SPECT, PET and fluorescence (see also WPs 8 and 10). The MBs functionalized with antibodies or peptides by a streptavidin-biotin linkage showed the best and most promising results using ultrasound, nuclear imaging and fluorescence techniques due to their high sensitivities. With ultrasound, the sensitivity of the modality was high enough to visualize single MBs *in vivo* as well as imaging areas of inflammation. With the nuclear techniques small amounts of MBs can be visualized, but results are limited by scan time, MBs radio-labelling efficiency and background levels. Via MRI it was possible to identify the inflamed areas but more difficult to identify changes in contrast in small areas. The detection limit of approximately 10^6 MBs/ml is likely a main cause for this (see WP6). Since we found that a large proportion of injected MBs accumulated in the lungs

(see WP8), which had the effect of reducing the amount of MBs reaching any pathologic area, a new strategy for administering the contrast agent had to be developed to avoid this problem. By slow infusion during up to 1 h, using an MB concentration of 40-100-fold lower than applied with bolus injection, it was possible to reduce the lung accumulation in rats by around 80%. Redistribution of MBs in the body is a slow process, 12-24 h, as shown and discussed further in WP8. The infusion technique increased the chance for the contrast agent to reach inflamed areas more rapidly and accumulate at enough concentrations for visualization by the imaging modalities. Although ultrasound results were promising, other imaging techniques need further optimisation in order to increase the sensitivity using these MBs as a contrast agent. The specificity of the MBs also needs to be increased in order to reach high enough concentration in tissues of interest.

Task 9.4 The applied MBs are potentially useful as a model system of a multi-modal contrast agent for ultrasound, MRI, SPECT, PET and fluorescence. Targeting the MBs to specific cells or pathologic tissues with a strength that prevents detachment and continued circulation poses a great challenge. The strategy to utilise targeting ligands such as antibodies or peptides is common in existing research efforts but has not proven to be truly specific and efficient for our disease models and MB systems. Nevertheless, promising experiments have shown potential accumulation in inflamed tissue and possibilities to target these tissues. New ultrasound techniques were successfully developed by Sintef within the project and optimised for our polymeric-shelled MBs. This was of particular importance for the possibility to detect single MBs, a prerequisite for identification of small inflamed areas. For MRI more developmental work will be necessary both on the imaging side and on the MB property side. Higher targeting specificity will be beneficial to all imaging modalities. Tissues from these experiments were transferred to WP7 for determination of MB content and the influence by inflammation on the composition of an MB protein corona.

Conclusion – WP9

MBs are phagocytised by macrophages. MBs containing SPION were phagocytised at a faster rate. Unmodified MBs did not adhere to endothelial cells, nor were they taken up by such cells. LPS stimulated macrophages over-express adhesion molecules such as ICAM-1, and MBs coated with antibodies towards such epitopes caused increased attachment to cellular attachment compared to MBs with isotype control antibodies. Compared to negative control MBs, antibody coated MBs adhered more strongly to cytokine stimulated endothelial cells but also to unstimulated cells, and nonspecific adherence was noted for streptavidin. For *in vivo* nuclear imaging techniques, NOTA was found preferable for tracer attachment. Nuclear techniques and ultrasound were found most sensitive, and the latter could even detect single MBs applying a dedicated technical solution. The sensitivity of MRI was lower. The administration technique of MBs was crucial for the distribution and infusion of low concentration MBs, reducing the lung trap and thus allowing more time for MB accumulation within inflamed areas. Pegylation did not improve circulation of MBs to an extent increasing uptake in inflamed areas. Increased uptake of targeted MBs in such areas was clearly evidenced by ultrasound techniques.

1.3.8 WP10: Pre-clinical testing of multimodality MBs in animals

Objectives – WP10

The aim of this WP was to evaluate if the 3MiCRON MBs could be used to detect and treat disease. This was a very ambitious goal as, prior to the start of the project, the MBs had never been tested *in vivo*. The success of this part of the WP was therefore dependent on a favourable biodistribution, i.e. a long circulation time of MBs *in vivo*, and on a successful functionalization of the MBs. Another goal of the WP was to use the theoretically appealing ultrasound capabilities of the MBs to improve the visualization of the heart at cardiac ultrasound studies. The tasks for WP10 are listed below.

Task 10.1 Visualization of targeted MBs using ultrasound, MRI, SPECT

Task 10.2 Visualization of accumulation of magnetic MBs by using external magnets

Task 10.3 Release of air and fluorescent material

Task 10.4 Treatment effect

Task 10.5 Improved segmentation with contrast in cardiac and vascular applications

Task 10.6 Perfusion studies with contrast in cardiac and vascular applications

Results – WP10

Task 10.1 and 10.2 It has previously been shown, by other groups, that muscular inflammation induced from LPS can be reduced by dexamethasone. A model where muscular inflammation was induced by direct intramuscular injection was therefore used. That model would make it possible to compare the inflammation with the contralateral leg, i.e. having an internal reference. A study with four different arms was initiated: a) controls, b) i.v. treatment with the study drug, c) targeted MBs loaded with the drug, and d) magnetic but otherwise not targeted MBs loaded with the drug. In the latter group a strong magnet (0.25T) could be applied to the skin in order to increase the local concentration of magnetic MBs. For targeting (group c) streptavidine could be used. By drawing blood samples and performing ultrasound, MRI and SPECT it should be possible to compare the different treatment arms. A separate model was also developed where muscle inflammation was induced in rabbits using Zymosan. The rationale for this study was to use the developed optimized ultrasound sequence in order to visualize local binding of single MBs and also to show the feasibility of releasing gaseous MB content. Such content could, for instance, be NO localized inside the MB. In previous experiments, prior to this project, we have shown that NO released from our MBs prevents blood clotting. A theoretical use could be to remove local blood clotting in a transplanted organ, e.g. in kidney transplants that do not function properly after the surgery. For cardiac experiments a porcine model was developed. Different concentrations of MB were compared with that of SonoVue using analyses of wash-in and wash-out curves.

Using a Zymosan animal model, targeted MBs attaching to diseased tissue could be visualized using ultrasound (Figure 20). The concentration of MBs was increased locally by using streptavidin MBs, and MBs targeted with streptavidine biotinylated antibodies. Unexpectedly the LPS model turned out to cause unsatisfactory inflammation and unacceptable side-effects in the rat strain used. Further testing with MRI and SPECT with histopathological correlation must therefore be performed

after the end of the 3MiCRON project. Local accumulation of MBs by using external magnets is a theoretically appealing approach. However, our pilot experiment could not demonstrate such an accumulation, probably due to a low local blood flow and/or low local concentration of MBs in the hind leg muscle of the anaesthetized rat.

Task 10.3 Streptavidin coated Type C MBs can be destroyed by applying an ultrasound pulse with a MI greater than 1.0. The custom-made MBs detection sequence is able to detect the destruction of a single MB. The results attest to the notion that 3MiCRON MBs have a potential to be used as a drug delivery platform, for example in targeted cancer therapy, where the release of therapeutic doses in defined local areas is desired. The possibility to inject a therapeutic agent into the systemic circulation and then non-invasively selectively activate the drug release at the area of the pathology would be of high medical impact.

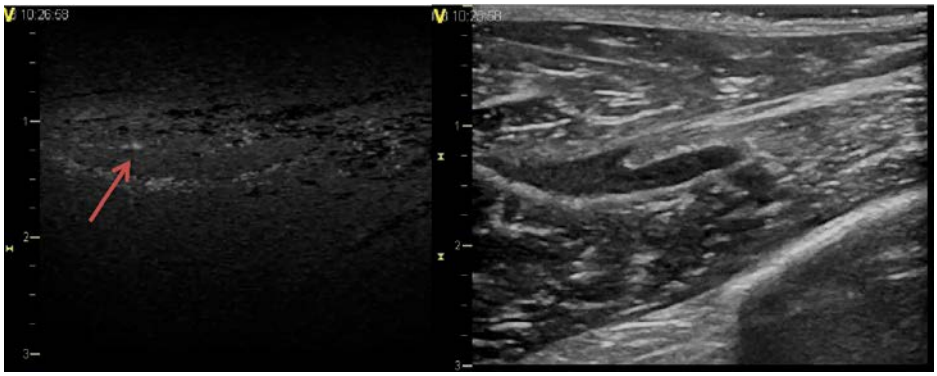

Figure 20. Contrast image (left panel) and B-mode image (right panel) 48 hours after Zymosan injection. MBs with streptavidine on the shell were injected 24 hours prior to imaging. In the left image, a significant number of MBs can be seen as bright spots in the tissue surrounding the pus-filled area. The red arrow indicates the position of a few of these MBs attached to the tissue surrounding the pus-filled region.

Task 10.4 As described in Task 10.1, the LPS model unexpectedly turned out to cause unsatisfactory muscular inflammation and it had unacceptable side-effects. The animal model can fairly easily be corrected by changing the inflammation drug from LPS to Zymosan. However, the descriptions in our animal ethical applications did not include the Zymosan model. An amendment to the previously approved animal ethical application would therefore have been necessary. Due to the great effort within WP8 to achieve MBs with satisfactory *in vivo* properties, the decision on which type of MB to use for the development of a treatment MB was delayed. It was therefore not possible to achieve an amendment to the animal ethical approval within the time constraints of 3MiCRON. The planned experiments will however be performed and published after the project.

Task 10.5 Using a higher dose of Plain MBs, the efficiency of the endocardial border delineation in a porcine model was comparable to that of the commercially available contrast agent SonoVue. This was evidenced by equal visual scores at evaluation, observed time for clinically sufficient contrast enhancement and the ability to automatically segment the left ventricle. Moreover, neither contrast agent affected the physiological parameters in the pigs.

Task 10.6 The uncertainty in the approximation of wash-out curves was higher for our MBs compared with that of the commercially available contrast agent SonoVue. Moreover, the values of volumetric flow, time to peak concentration and mean transit time varied to a considerably large

extent between our MBs and those of SonoVue. However, it should be kept in mind that the suppression of the tissue signal and the enhancement of the contrast agent signals are important in contrast perfusion imaging. Commercially available sequences for CEUS are tuned for the acoustic properties of SonoVue. The comparison is therefore somewhat biased. Specific contrast sequences, optimized for polymer-shelled MBs, would probably yield more stable results for the 3MiCRON MBs in studies on myocardial perfusion. Such sequences have been developed within this project, but it was not possible to implement those to cardiac imaging within the limited time-frame.

Conclusion – WP10

This WP was the endpoint of a very ambitious project to develop a multimodal theranostic MB. The final candidate fulfils all the requirements to successfully complete WP10. The effort needed in terms of chemical design, feedback from *in vitro* tests and biodistribution tests, as well as the further improvements by alterations in SPION inclusion procedures, coupling ligand and storage (freeze drying of samples), finally rendered too little time for full optimization of the animal models. The further tests of local treatment using the developed multifunctional theranostic MBs must await a formal amendment to the animal ethical application which could not be received within the time limits of the project. Despite these time limitations and despite the well-known great risks associated with the development of new drugs and treatments, the project could successfully show local increase of the developed contrast agent in the targeted area and the successful local release of gaseous contents.

1.3.9 Overall conclusions

The overall conclusions of 3MiCRON corresponding to the objectives in Section 1.2.3 are listed below:

1) Multimodal 3MiCRON MBs with enhanced biodegradability and desired properties were synthesized and characterized.

2) A quality control procedure including characterization of structural, magnetic, mechanical and interaction properties of MBs was developed, which allowed optimization of the MB production process and performance with respect to the imaging modalities investigated in the project.

3) The preparation of 3MiCRON MBs was upscaled in accordance with the ISO 9001 standard enabling a delivery of MBs with reproducible properties, such as shell thickness, diameter, and magnetic content.

4) The 3MiCRON MBs were visualized *in vitro* and *in vivo* with several clinical available imaging modalities, such as ultrasound, MRI, SPECT, PET, fluorescent visualization.

5) Several desopsonin proteins, such as serum albumin, have been identified as major protein corona binders indicating good biocompatibility of 3MiCRON MBs.

6) The macrophages were responsible for the elimination of 3MiCRON MBs. The biodistribution of MBs in different organs was determined by contrast enhancement evaluation using different imaging modalities. The biodistribution was shown to be dependent on the attached ligand type,

indicating promising steps towards multimodal molecular imaging. The blood half-life time of 3MiCRON MBs was longer than for commercially available contrast agents, indicating potential use in tissue specific targeted imaging.

7) Initial results demonstrate that 3MiCRON MBs have diagnostic potential to be applied in cardiovascular diseases for quantification of myocardial perfusion and endocardial border delineation. In addition, increased uptake of targeted MBs was shown in inflammation models demonstrating the possibility of molecular imaging of inflammation, which is pathophysiological mechanism of development of various diseases from atherosclerosis through rheumatoid arthritis and cancer.