



3MiCRON

FINAL PUBLISHABLE SUMMARY 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, several new multimodal contrast agents consisting of microbubbles (also known as microbubbles, MBs) with a diameter of 3 μm have 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.

A new medical and technological approach of CEUS concerns *theranostics*, i.e. the combination 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 still several problems when performing clinical tests using this technique. The physician who acquires and interprets the image needs to be trained and experienced in using the technique. Another problem is concerns inhomogeneity and instability of the physical properties of the commercially available contrast agents that are 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) using 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

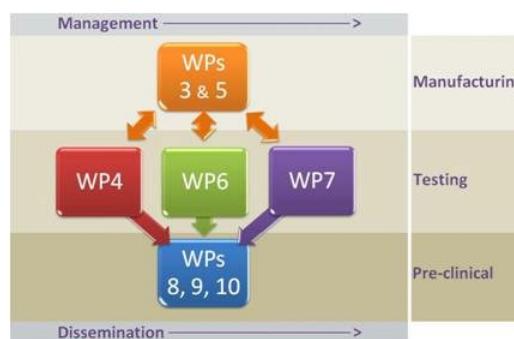


Figure 1. The scientific WP structure of 3MiCRON

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.

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 polymer shelled MBs with enhanced functionality 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 microparticle 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 MB was designed for targeting inflamed cells and, for this purpose, specific molecules which recognize markers of inflammation were conjugated on the MB surface. 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 $^{99\text{m}}\text{Tc}$

Results - WP3

Task 3.1 All partners participating to this WP received training by UNITV on 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 perfluorocarbon (PFC) hydrophobic liquid (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 shown that these vesicles are also temperature responsive when using N-isopropyl acryl amide (NiPAAm) as co-monomer in the polymer shell formation (point b of Task 3.2). For these research results an Italian patent (RM2012A000290, 2012) was filed and a paper was 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 for loading drugs that can be locally delivered to specific organs/tissues by bursting the MBs with a high intensive ultrasound pulse. With regard to point (c) of Task 3.2, UBT proceeded with the use of high-power ultrasound for the electrochemical formation of polypyrrole MBs with precise control of shell thickness and diameters. This could be a promising option for the design of microbubbles with combined imaging and drug delivery functions.

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 adhesion of 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

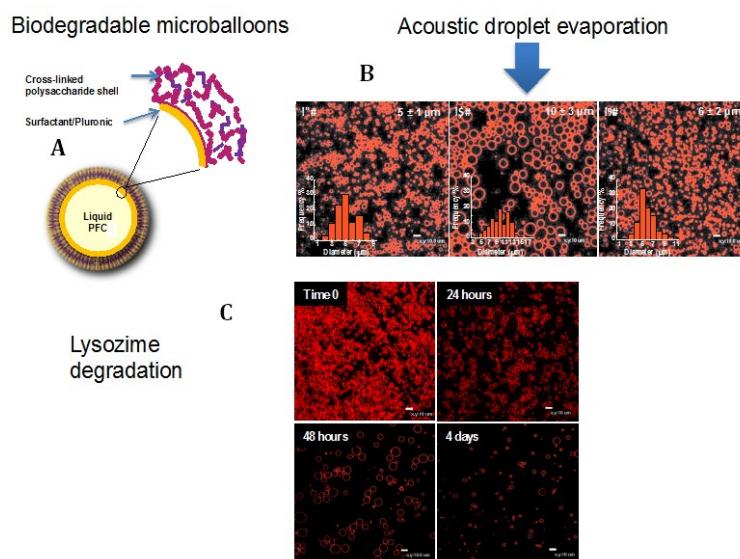


Figure 2. (A): Scheme of the vesicle containing a liquid PFC core stabilized by a lipid monolayer and a cross-linked biopolymer based shell. (B): A confocal micrograph sequence of vesicles (left) before ultrasound irradiation, (center) immediately after ultrasound irradiation (ADV), (right) after return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

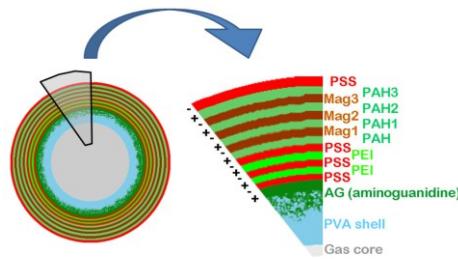


Figure 3. Type C MBs obtained with LbL technology

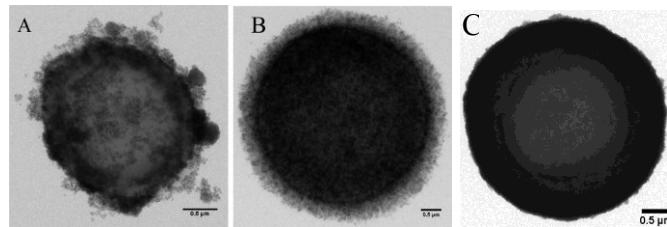


Figure 4. Electron micrographs of A) chemically coupled Type A, B) physically entrapped Type B and C) LbL immobilized Type C, SPION/MBs

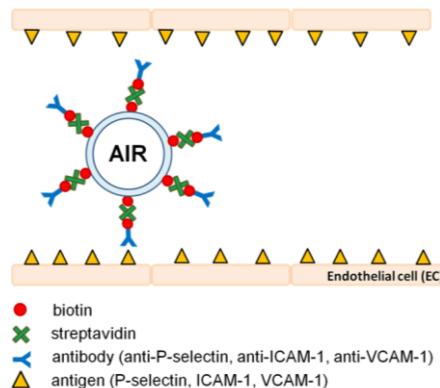


Figure 5. MB biotin – streptavidin bridging to antibody

oxide nanoparticles (SPIONs) were attached to the shell of the MBs using three different methods: with the SPIONs chemically attached onto the external surface of the shell (Type A), with the SPIONs directly embedded in the PVA shell (Type B) or the LbL technology with the SPIONs sandwiched between PAH layers (Type C) (Figure 3). Transmission Electron Microscopy (TEM) micrography of the different types of MBs is reported in Figure 4a-c. The results of the study are described in the paper in *Biomacromolecules*, 2012 (see Section 2.2).

Task 3.3 MBs with surface modifications 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 (RBTC), cyanine 3 (Cy3), AlexaFluor 488, the near Infrared (NIR) dye VivoTag 680XL. NIR fluorescence imaging is a powerful diagnostic approach in transdermal applications for tracking in preclinical models. 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.

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 individually or in combination.

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 a new technique was developed to increase the storage time by freeze-drying the MBs.

1.3.2 WP4: Structural/physical characterization of MBs

Objectives – WP4

The activities in WP4 aimed at 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. WP4 supported the optimization of the MBs' manufacturing process, the development of quality assurance criteria during upscaling, and it provided the data needed 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.

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

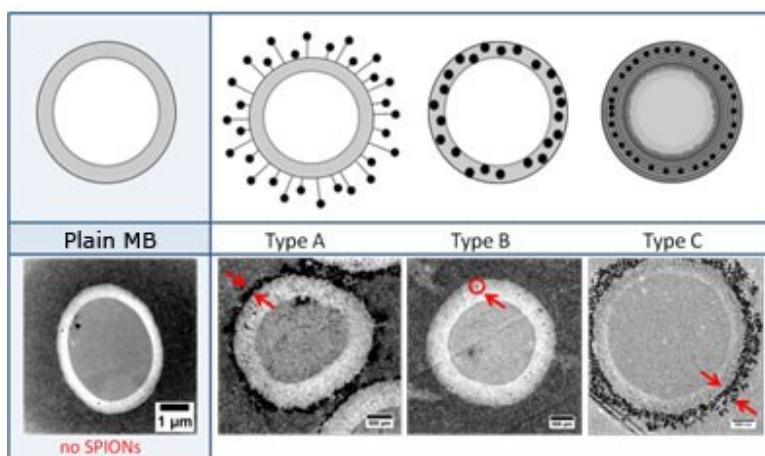


Figure 6. Different structural designs of MBs produced in the 3MiCRON project. (Top) schematic of the structure of US/MRI hybrid probes and (bottom) Ultrathin sections of MBs imaged with TEM. Red arrows indicate SPIONs.

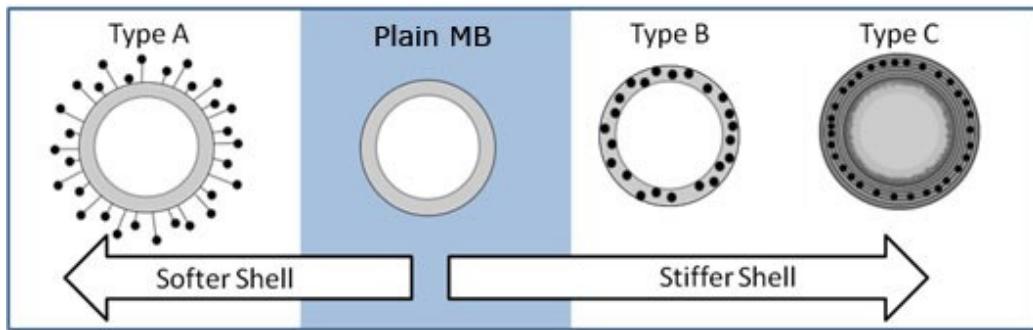


Figure 7. Overview of shell structure and shell mechanics investigated in the low and high frequency deformation regime

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 surface modifications were characterised and quantified. An example of 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 .

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. The structural parameters such as shell composition, shell thickness, and shell mechanical stiffness are inputs for the models predicting imaging performance and thus allow bridging the gap between characterisation, 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.

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.

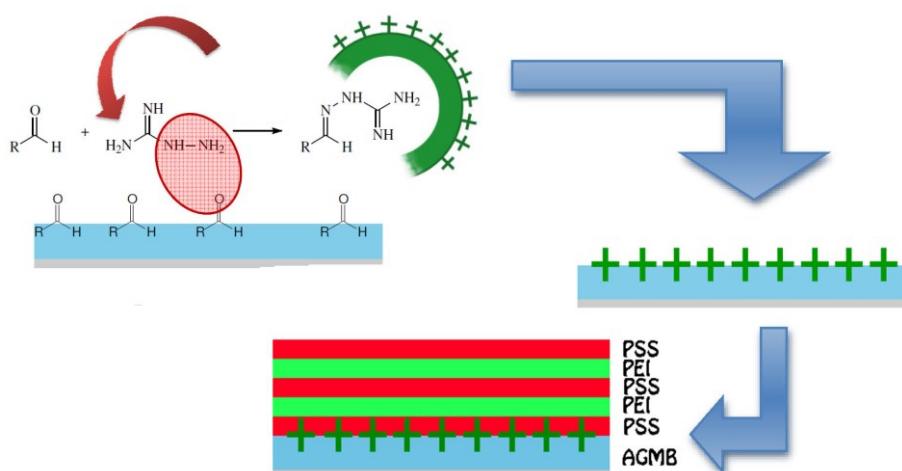


Figure 8. Top: priming the surface by aminoguanidine (hydrazine chemistry), bottom: adding the first 5 layers for stabilizing purposes and for additional charge.

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 2×10^{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. A 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.

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.



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 5.4 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 types of MBs: Plain MBs, LbL-coated MBs, magnetic MBs, and multimodal MBs; at $1-2 \times 10^{11}$ 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, enabling delivery of MBs with reproducible properties. A method to apply the LbL-coating to the inert MBs was developed. Using the LbL-technology a modular system was developed allowing the simultaneous functionalization for several imaging modalities, in the desired combinations. Furthermore, these MBs were 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/upscaleing 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 imaging quality with the new contrast agents 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 systems in order to maximize the signal-to-noise ratio (SNR) for the new contrast agents

Task 6.4 The Nuclear Imaging (SPECT) activity is focused on the tuning of the isotope imaging to the contrast agent and the 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

Combining imaging modalities to use the advantages of each modality is of great interest for clinicians. 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.

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

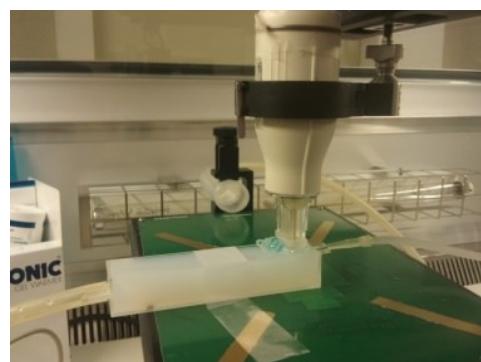


Figure 10. Phantoms and different experimental set-up

polymer-shelled MB is 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 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 to conclude that PI could not to be used to image 3MiCRON MBs and therefore only CPS3 and Chirp CPS3 sequence 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 using pressures ≥ 180 kPa and ≤ 320 kPa, whereas batch concentrations in the range 10^5 - 10^6 MBs/mL were suitable to obtain a significant contrast.

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 were the cause of the poor performance of MB Type C with the CEUS imaging techniques based on a nonlinear MB response. To address this issue testing of a new custom made technique was performed by Sintef. This technique was based on pulse subtraction using two identical ultrasound pulses. 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). SPIONs embedded in the MBs shell increased the transverse relaxivity (R_2^*).

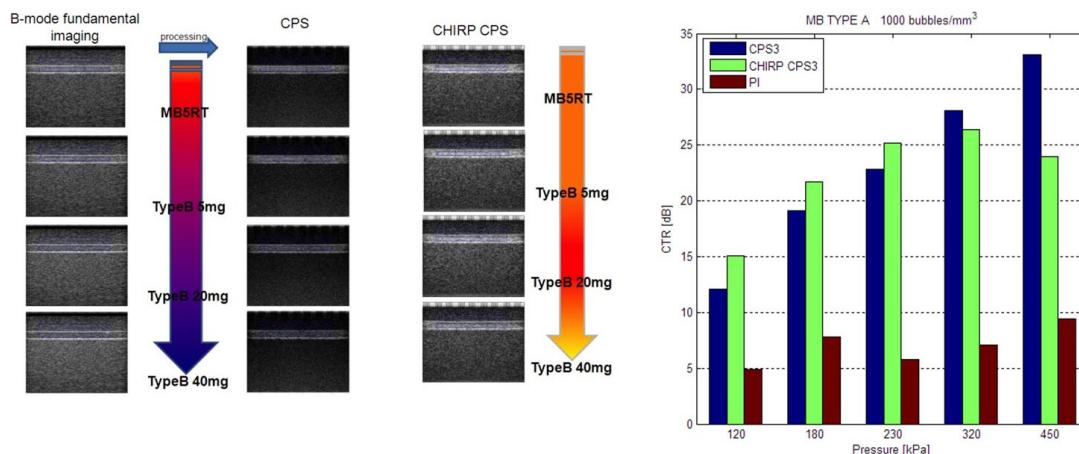


Figure 11. Ultrasound images of an MB (left) and the contrast-to-tissue ratio applying different signal processing techniques (right).

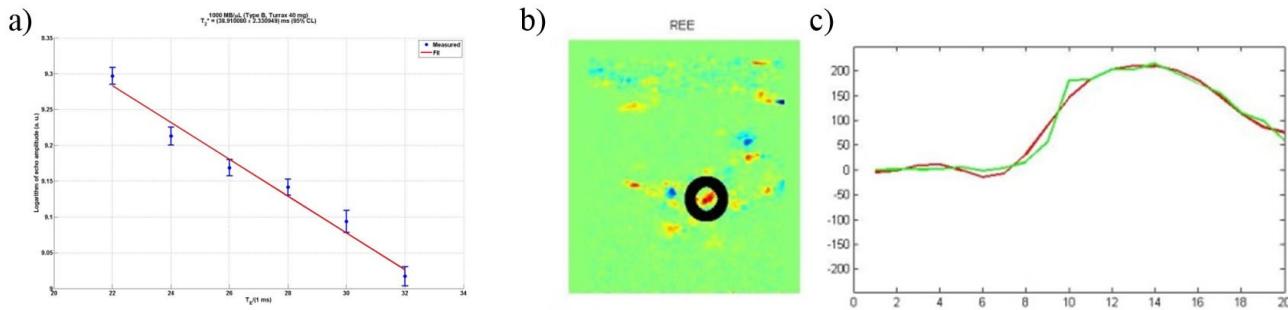


Figure 12. (a) The concentration curve shows how the T_2^* behaviour of MBs is as expected. The values found are lower than the T_2^* value of blood that is around 300 ms, and this confirms that MBs can be detected inside the blood (T_2^* evaluation - Type B 40mg (106 mL⁻¹); (b-c) Contrast agent enhancement: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

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 The novel system of 3MiCRON MBs could be functionalized with ligands to bind ^{99m}Tc , which makes them visible using fusion SPECT/CT.

Task 6.5 A new quantification tool was designed and developed 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 issues addressed concerned the movement artefacts and patient displacements (during acquisition), the solution to noise problems caused by the pixel-based estimation of clinical parameters and implementation of measurement methods of parameters. This approach was feasible, 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.

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. 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. For 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 agent let 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 intended use as contrast agents *in vivo*, MBs need to exert an excellent safety profile in order to be suitable for clinical use and *in vivo* applications. Thus, a significant focus in the 3MiCRON project was on the safety, toxicity and side effects (degradation products) of the MBs. 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 physicochemical 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 biocompatibility, while a stronger association with desopsonin 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.

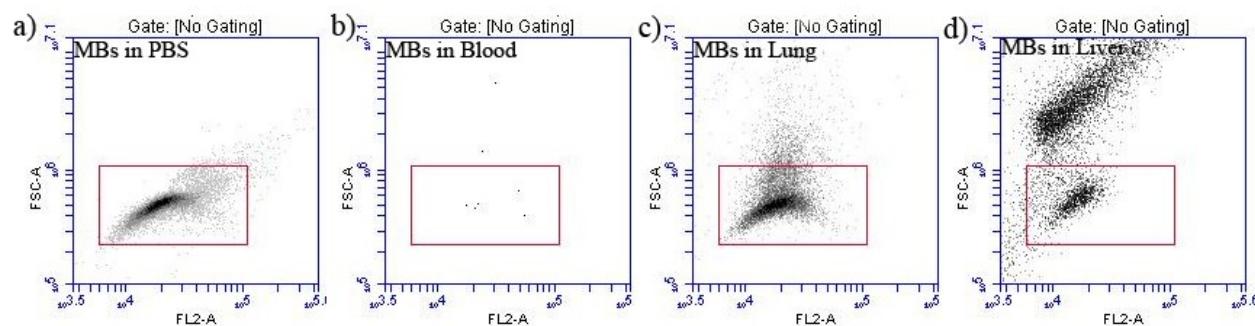


Figure 13. MB detection in PBS and organs of rat injected with MBs. Double scatter plot of FSC versus fluorescence intensity of MBs in PBS (a), and blood (b), lung (c) and liver (d) tissues recovered from rats after injection of MBs. 10^8 MBs/ml Type C MBs were injected in adult rats.

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 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* biocompatibility findings (10% human plasma) where MBs toxicity was considered where cell viability exceeded 80%.

Conclusion – WP7

In vitro biocompatibility 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 biocompatible 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* biocompatibility tests

MBs	Name	Biocompatibility <i>in vitro</i> 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 9×10^8 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.

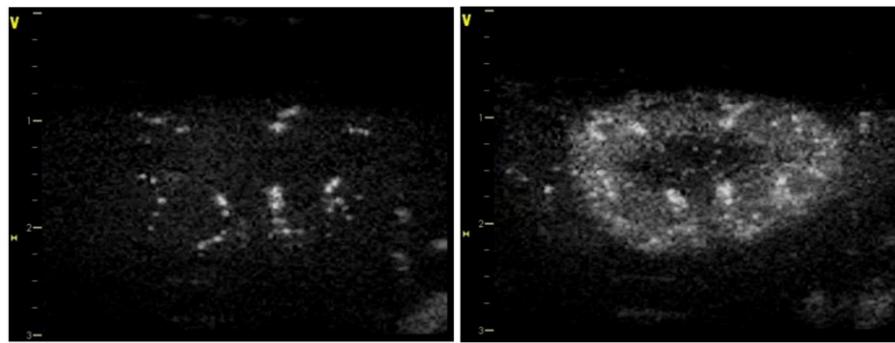


Figure 14. Rabbit renal contrast images with the custom made contrast detection technique after bolus injection of 0.5 ml Plain MBs; imaging frequency 8 MHz. Left panel: shows MBs arriving in the renal arteries. Right panel: 5 seconds after MBs arrive in the renal arteries.

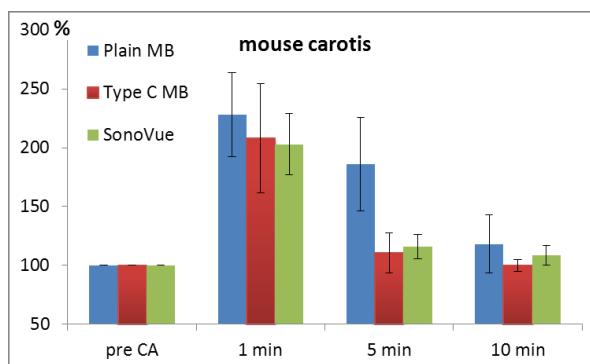


Figure 15. Signal intensity of a mouse carotid artery when using ultrasound. Normalized greyscale intensity over time post injection of Plain MB, Type C and SonoVue. (n=4) (Vevo 770, VisualSonics)

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 100 μ l Type B (1.17×10^9 MB/ml) were injected into the tail vein of mice resulting in a strong signal decrease in T₂w 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 ⁶⁸Ga (Figure 17). For the alignment of the activity to the respective organs μ CT 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.

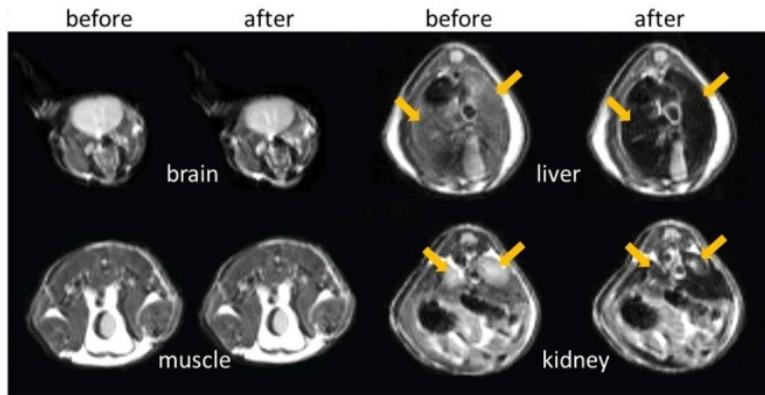


Figure 16. MRI: T2 weighted images of a mouse imaged at 1.5 T before and 5 min after i.v. injection of 100 μ l Type B MB (1.17x 109 MB/ml).

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.

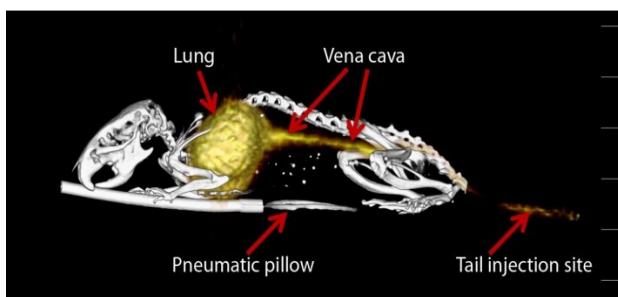


Figure 17. PET/CT: Mouse imaged immediately after injection of 70 μ l 68Ga labeled Type C MB (5x108 MB/ml) (Administered activity: 19,2 MBq, μ CT: Inveon SIEMENS, set up: 120 RS, 50 KeV, 500 mA, 50 ms exposure time)

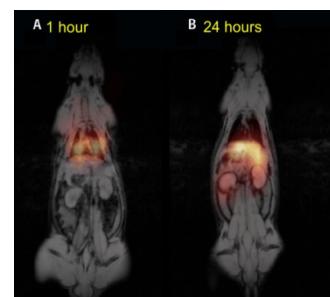


Figure 18. SPECT/CT: rat infused with 99mTc-labeled DTPA-SPION MB. Initially MB are accumulated in lungs, but redistribute to liver within 24h.

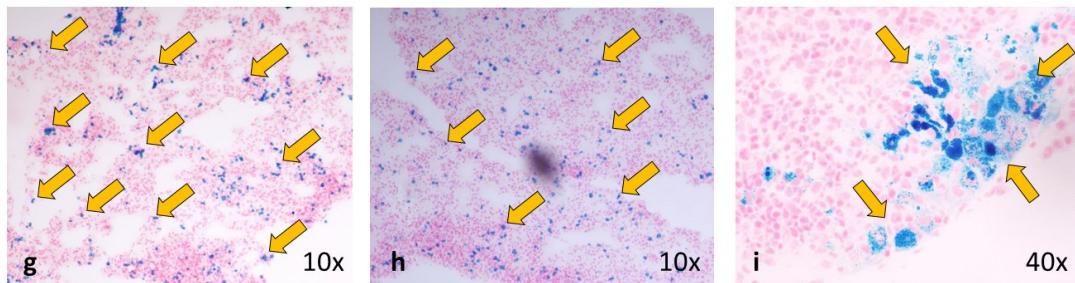


Figure 19. Histology of mouse lung (Prussian Blue and nuclear fast red) g: 1 day, h: 3 days, i: 3 months post injection of Type C MB. Yellow arrows point to intact MB (g, h) and to MB degradation products containing SPION (i).

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 1.9×10^9 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 lipopolysaccharides (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-1 β) 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 by 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

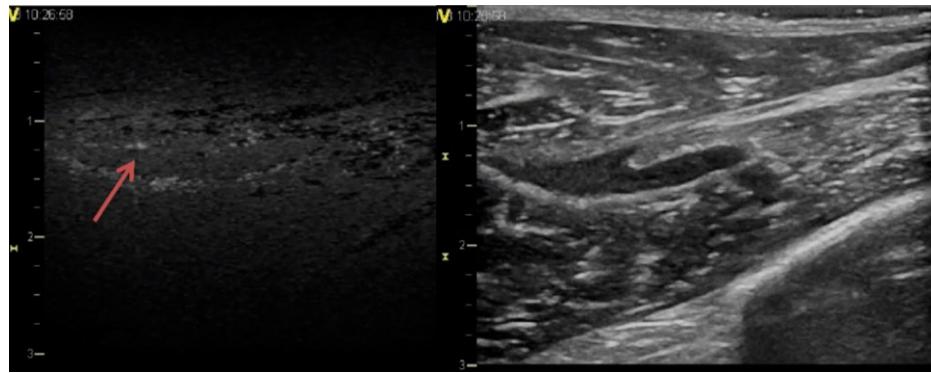


Figure 20. Contrast image (left panel) and B-mode image (right panel) 48 hours after Zymosan injection. MBs with streptavidine on the shell were injected 24 hours prior to imaging. In the left image, a significant number of MBs can be seen as bright spots in the tissue surrounding the pus-filled area.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

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.

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.

1.4 Potential impact

1.4.1 Socio-economic impact

The developments outlined above will lead to new technologies, methodologies and approaches for non-invasive prediction, diagnosis, monitoring and possibly even prognosis of diseases. The 3MiCRON project will contribute to the evaluation and monitoring of therapies, and will aid the development of improved planning and guiding of therapeutic interventions. The results will be of benefit to patients in terms of potentially earlier and less invasive diagnostic procedures. The 3MiCRON project also improves European competitiveness as it involves European industry and an SME, who have developed and refined their know-how and product portfolios.

As stated in the Strategic Research Agenda of the European Technology Platform for Nanomedicine, it is important to avoid fragmentation and lack of coordination across academia and industry in this young and very fast growing discipline. Thus, a European approach to these developments is clearly indicated. 3MiCRON project with multidisciplinary competences from well-known universities includes expert knowledge in several fields - from chemical manufacturing to clinical applications of different imaging modalities and therapy of atherosclerosis and cancer. For many years the trend for the development of diagnostic scanners goes to the combination of single modalities into multimodal imaging. The reason lies in the extremely specific and narrow nature of the information obtained by each single radiological modality. By combining several modalities it is possible to achieve both function and image resolution, thereby increasing both sensitivity and specificity.

Dual modality scanners, for example PET/CT or PET/MRI scanners are widely known and well established in clinical routine. Using such combinations it is possible to combine the anatomical information gained by CT or MRI with specific tissue metabolic activities detected by PET. However, purchasable contrast agents are monomodal and do not support the diagnostic capability of both of the radiological modalities. Contrast agents are powerful diagnostic tools in modern medicine. Due to their contrast giving features it is possible to discriminate pathological tissues and morphologies. Moreover, the use of various radionuclides allow the assessment of physiological parameters.

The ambition in 3MiCRON has been to push this development one step ahead and to develop a multifunctional contrast agent suitable for ultrasound, SPECT or PET and MRI. Based on Plain MBs developed during the previous Sight project (within FP6) new types of MBs have been developed by

coupling of additional contrast enhancing particles and tracers. The MBs have undergone biocompatibility, toxicity and distribution studies both in cell cultures, small and big animal models. Such studies are absolutely necessary prior to human studies. It is crucial that every new substance incorporated into the shell is carefully evaluated through all these steps, because even a small change in the electrical properties of the MBs will affect the distribution and metabolic effects.

Various challenges had to be conquered in the process of developing these multifunctional MB. Besides the chemical challenges in order to stably coupling the different components, the ratio of the contrast enhancing particles had to be fine-tuned to match the needs of each radiological modality without changing the properties of the Plain MBs for the other modalities, e.g. ultrasound.

During the project several single, dual and also three modality MBs were successfully developed and tested. The multimodal Type C MBs can be detected by using ultrasound, MRI and nuclear medicine techniques such as PET or SPECT. The development of multimodality contrast is expected to be of particular interest for medical applications. Societal advantages are directly related: lowering costs of marketed ultrasound contrast agent due to longer shelf- and circulation-life, handiness in device preparation, localized drug delivery with a subsequent increased bio-availability, patient acceptability and potential healthcare cost reduction, and the possibility of multi-modal imaging enabling the strengths of several different imaging modalities to be utilized simultaneously or in parallel. The developed ability to enrich the local concentration of drugs by using drug loaded MBs targeted with ligands or magnets and to deliver gaseous and intramural contents (e.g. NO and dexamethasone) also shows the concept of local delivery. Such treatment can potentially reduce side-effects of drug treatment.

Synergistic effects with therapeutic applications

As the present project is focused on the diagnostic application of MBs with special emphasis on multimodality, therapeutic approaches was not pursued within 3MiCRON. Nevertheless, the concepts and materials developed in the framework of the project provide new options for therapeutic applications. A new class of multimodal, well defined MBs could be provided to groups and companies pursuing therapeutic applications. It will offer advantages as compared to existing MBs with regard to (lower) polydispersity, more well-defined bursting points for triggering release, well defined magnetization, and multimodal imaging options.

Impact on health and quality of life

An ageing population, higher expectations for better quality of life and the changing lifestyle of European society raise the need for innovative, more efficient, less invasive and affordable healthcare. Early diagnosis and smart treatment approaches are the two main challenges. Advancement of *in vivo* diagnostics relies on molecular imaging and on the development and application of minimally invasive devices. Molecular imaging aims to create highly sensitive and reliable detection agents that can also deliver therapies and monitor the progress of the therapy. These newly conceived molecular imaging devices are intended to make molecular processes visible, quantifiable and traceable over time *in vivo* or under physiological conditions. This concept has been defined as theranostics and combines the separate disciplines of diagnostics and therapeutics. As theranostic technologies become available, physicians will be effectively

empowered to bring the hospital to the patient's bedside in real time; tissue of interest is first imaged using a targeted contrast agent, in addition the targeting device loaded with a pharmacologically active agent can be used for therapy. Finally, monitoring of the results of this therapy over time is possible by sequential imaging.

In this context, the final aim of these devices is to allow discovery of a pre-disease state in a patient when the first genomic and proteomic mid-steps in a pathway or cascade of event occurs during disease onset. This, in turn, allows for a better (more efficient) treatment monitoring. A recent report from the FDA highlights imaging technologies as offering powerful insights into the distribution, binding and effects of drugs and drug candidates.

The 3MiCRON project is committed to the development of devices and MBs, and of fabrication processes with beneficial fallouts for society. Improvements to quality of life, lowering mortality rates, (one out of four heart strokes is fatal even in countries with advanced medical assistance), safety and low environmental impact are the background conditions from which the project is developed. In this respect, several aspects of marketed contrast agents can be improved in terms of shelf-life, convenience of preparation, echogenic ability, multi-modal imaging capacity and combined therapeutics and diagnostics.

Non-medical health issues

Another potential use of MBs is as a 'filler' in several applications, due to the significant weight reduction compared to the usual filler formulations based on talcs, aluminates, silicates. Hollow micro-spheres offer a means of introducing controlled, small voids in a closed-cell configuration. We should mention here that this is a difficult task in viscous and non-viscous fluids, resins, coatings and cements, where the foaming process is responsible for unequal cell growth, time and temperature-dependent gas diffusion, and cell coalescence. Colloidal hollow micro-particles are traps for gases or liquids.

Gas MBs based on surfactants (colloidal gas aphrons) are described in the literature as having distinctive features such as large interfacial area, relatively high stability, easy separation from the bulk medium, flow properties close to those of water. Such properties have suggested a number of applications in diverse fields, identified as, e.g. drilling technology, gas storage, the food industry (protein separation, as MBs are convenient micro-'phials' for storage and delivery of flavours/fragrances), and as fillers for several industrial products, e.g. thermoplastics resins, sealants, pipe insulation, coatings and inks, polymer medical concretes and synthetic medical cements.

1.4.2 Wider societal implication of the project

Impact on European competitiveness

Diagnostic and therapeutic imaging is not only important for the EU from the patient welfare point of view but also for its economic potential and development of the knowledge economy. The development of innovative products and technologies, such as drug-carrying targeted-MBs, or the combination of microcapsules and MBs for combined visualization and delivery, could boost the uptake of UCAs by clinical practices and emergency rooms, and the economic impact will involve

biotech companies, especially SMEs, actively working on developing drug-delivery platforms, innovative diagnostics and imaging solutions. This is a niche where European research can transform traditional industry, promote science-based industry, and foster scale-intensive and specialised suppliers industry, thereby gaining prestige and a corresponding share of the world market in the development, production and marketing of such intelligent devices for a sustainable supply industry.

Contribution to standards

The fact that the MBs are multi-modal may also enable ultrasound to contribute to the development of standards for imaging diagnostics, by cross-validating the various techniques and cross-calibrating the sensitivities and imaging planes.

Contributions to regulation and policy

These contributions come under the remit of medical devices, and if they contain a drug also, may come under the remit of the European Medicines agency. Since these multi-modal approaches are new, they could contribute knowledge and expertise, and discussion on issues related to regulation and policy to the various agencies. For example, the effects of cavitation of drug targeting and efficacy, synergistic effects etc. The primary purpose of this project is focused research in the areas described. Where it is consistent with EC policy, efforts were made to disseminate to other stakeholder groups, and contribute to policy development. The results obtained in our project can potentially assist on a scientific level in the planning and development of reference technical instruments needed for EU policies such as the revision of Environment Directives, REACH, protection of health, food safety, etc.

Networking

This project has gathered several European research institutes and companies. This collaborating platform will be very useful in future research projects. Moreover, the research that has been performed within 3MiCRON has also attracted other institutes and companies that have initiated new promising research collaborations.

Young researchers

3MiCRON was particularly keen to support young researchers (e.g. PhDs and post-docs) within its activities. The project was structured so that young researchers were employed, and received research training, within the project lifetime. We foresee a large interest from young researchers in this project and in the outcomes. Training activities, which were provided by all partners included research supervision, university level courses, support with developing writing skills, diagnostic equipment skills training, multimodality aspects, and more.

1.4.3 Main dissemination activities

Dissemination and exploitation promotion of the results and technologies developed within the project falls into the objectives of WP11. This WP was a collaboration between KTH, UNITV, UCD and Esaote. The tasks for WP11 are listed below.

Task 11.1 Ensure wide dissemination and public awareness of the results, concepts and solutions

Task 11.2 Identify of relevant target industry sectors

Task 11.3 Establish effective strategies for partnership

Task 11.4 Convert the knowledge from academia to industrial product

Task 11.5 Identify technological applications in different arena

Task 11.6 Establish collaborations with other EU research projects

Task 11.7 Develop self-sustainable strategies for spin-off activity

Results

Results with potential for economic exploitation were patented according to the guidelines provided by the partner's technology transfer offices. Results were disseminated through participation in national and international scientific conferences and networks, such as the European Technology platform in Nanomedicine (ETPN). The project and results were communicated as part of courses and through lectures to undergraduate and graduate students at academic partners. Knowledge, experience and results gained from the project position collaborating partners for future calls within the field of nanomedicine, for example in EUs FP8, Horizon 2020. The main activities which occurred within WP11 were:

- 3MiCRON Info-Day and Workshop
- Cluster meetings (WP2)
- Public activities (conference contributions, external activities, educational work, scientific publications)
- Business plan development seminar
- Webpage and exchange of information

3MiCRON info-day and workshop

On the 27th October 2011 KTH STH (School of Technology and Health) together with Innovationsplatsen (at the Karolinska University Hospital) organized a scientific info-day entitled 'Contrast enhanced medical imaging – A novel multimodality approach' covering both the activity of 3MiCRON and state of the art approach in both industrial and clinical practices. During the 3MiCRON info-day invited speakers from industry (GE, Esaote) and academy (KTH, KI, UNITV, Uppsala University, Sintef, Karolinska University Hospital) presented education activities and the current research initiatives at KTH; illustrated the collaboration between Research Institutes, Clinical practice and Industry; described the history and the successful development of the micro-device; discussed strategy of manufacturing nanomaterials; described new initiatives at GE and how they are intend to integrate them into current clinical solutions; described the history and evolution of ultrasound contrast agents; spoke about the causes of inflammation disorders; highlighted the advantages of fusion of imaging techniques and went on to talk about the importance of the multimodality approach; informed on the regulations that are currently applied for contrast imaging in cardiology practice.

The workshop concluded with a panel discussion where questions from both the audience and the panel led to a fruitful exchange of ideas with constructive comments and suggestions for further actions and activities covering not only 3MiCRON but new research and collaborative initiatives.

The examples of such collaborations are 1. the possible future collaboration between Innovation-platsen, Sweden represented by Dr. Christian Östberg and the Italian Innovation platform represented by Dr. Sergio Paddeu and 2. the possible collaboration between KTH and GE Healthcare on a future project dealing with Portable Cyclotron.

The 3MiCRON workshop was located at one of the largest echocardiographic meetings in the world, EUROECHO & other Imaging Modalities, in order to attract end-users of contrast agent such as medical doctors, sonographers and nurses. EUROECHO & other Imaging Modalities is the annual meeting of the European Association of Cardiovascular Imaging (EACVI) and the congress took place in Athens, Greece, 5-8th December 2013. Over the years, the meeting has grown more and more to become one of the largest echo meetings in the world with over 3,000 attendances and has also expanded its focus to other imaging modalities.

During the 3MiCRON workshop representatives from 3MiCRON and Karolinska University Hospital presented clinical benefits of contrast-enhanced ultrasound imaging as well as the 3MiCRON concept for end-users. The workshop was in the format of an 'imaging campus session', meaning that the same session (1.5h) was presented four times during the congress. The workshop attracted persons from around 20 different countries.

Public activities

Figure 21 demonstrates all public activities performed within 3MiCRON. From various types of public activities performed several important points should be mentioned. In particular it became evident that single imaging modality often has a limited diagnostic power. By performing hybrid or multimodal imaging, which can be viewed as a simple and powerful integration of two or more imaging methods (e.g. PET-CT; PET-MRI), improved diagnostic efficiency can be achieved. Moreover with the help of a contrast agent and potentially a multimodal contrast agent the diagnostic efficiency can be further improved.

Web page and exchange of information

In order to communicate the results of the project with the broader community project website was set up and continuously updated. Web address: www.3micron.eu. Traditional communication paths such as project webpage and internal platform did not fully match the need of rapid exchange



Figure 21. a) Peer-reviewed publications. b) Conference/Congress contributions and Workshops/Cluster meetings. c) Educational work (student lab courses are listed as workshops in Template A2)

of the information. Email and telephone conversations are proven to be effective but include rather limited amount of participants. As a result a number of other communication methods were introduced:

- Regular teleconferences with strict agenda and notes distributed shortly thereafter
- For synchronized and systematic information upload and exchange Google Docs was employed
- A web-based file storage system was employed in order to facilitate information exchange
- Social media was used (e.g. the Facebook page www.facebook.com/3MiCRON.FP7) with a news-line updated on regular basis
- A Skype conference facility was utilized

A project leaflet that summaries the objectives and goals of the project as well as project booklet that highlight major results achieved during first half of the project has been published.

Other dissemination activities

In order to further facilitate industry collaboration, a requested contribution and published material was sent to the exhibition 'SMART Systems integration' hosted in Dresden, Germany on 22-23 March 2011, and organized by the COWIN initiative. This initiative is dedicated to: a) the commercial exploitation of advanced technologies developed in the framework of European collaborative research projects; b) the creation of start-up companies; c) supporting investment in companies, key technologies and IP.

The KTH Life Sciences Technology Platform, one of the coordinating university's five strategic research platforms, offered the possibility for projects to participate in a three-year exposition at Arlanda Airport Conference & Business Center. The exhibition displayed novel methods and technologies in healthcare to key people in media, business and society visiting the Business Center. Posters for the exhibition, highlighting major objectives of 3MiCRON were submitted to the organization committee.

Last, but not the least, it should be noted that involvement in EU FP7 project 3MiCRON triggered several unforeseen initiatives and acquisitions. In particular, KTH in collaboration with clinical partners KI and Karolinska University Hospital purchased high frequency ultrasound equipment with photoacoustic application from VisualSonics. In addition, KTH bought programmable open-source ultrasound equipment from VeraSonix. Both these items open new possibilities in development of innovative ultrasound based imaging sequences to answer most challenging clinical questions. Contribution in several EU and national proposals were triggered by involvement in 3MiCRON:

- EU FP7 - CATAMARAN project (19 partners)
- IMI6 initiative – Nano-LOGISTICS project (11 partners)
- Uppsala Bio, BioX framework – CADiLiC project (3 partners)
- ALF medel, Sweden – CellFit project (4 partners)
- Heart and Lung foundation, Sweden - Thrombolysis project (3 Partners)

Three patent applications have been filed:

- RM2011A000022, 21.01.2011 by UNITV on fabrication of MBs TypeA
- DE102011000264.2, 21.01.2011 by SURFLAY on fabrication of MBs TypeB
- RM2012A000290 0357-12 by UNITV on fabrication of biodegradable MBs

1.4.4 Exploitation of results

An **Exploitation Strategy Seminar** (ESS), held on 3rd May 2011 in Stockholm, was provided by European Commission and facilitated by CIMATEC S.r.l and Dr. Mauro Caocci, whose contribution is kindly acknowledged. Two persons from each partner organization attended the seminar. The major objective of the seminar was to identify, review and characterize exploitable project results; analyse the intellectual property rights situation; and assess the non-technical risks. After the fruitful discussion, an 'Action Plan' was prepared, which aids the 3MiCRON consortium to improve the exploitation strategy and risk management of the project. Several points of the plan deserve special attention, such as enrolling the patent service company for assessment of the IP situation, strengthening management by increasing the frequency of teleconferences between WP leaders and increasing the number of research meetings complemented by reports submitted to the project coordinator.

A **Business Plan Development Seminar** (BPD) was provided by European Commission through ESIC2 (Exploitation Strategy and Innovation Consultants) initiative and held on the 17th of October 2012 in Genoa. The BPD seminar was moderated by Nina Mazgan (Meta Group) and external biotech expert Elizabeth Robinson (Nicox). 3MiCRON was represented by one industrial partner (Dr. Sergio Paddeu, Esaote), two academic partners (Prof. Lars-Åke Brodin and Dr Dmitry Grishenkov, KTH, Prof. Gaio Paradossi, UNITV) and one clinical partner (Associate Prof. Torkel Brismar, KI). The major objective of the seminar was to identify main critical issues related to commercialization strategy, readiness to start the company, identification of the product/service and its market potential.

After productive discussions partners agreed that a small targeted group should be formed to revise the patent-publication strategy and identify the unique application for multimodal imaging platform that has been developed within 3MiCRON project.

More detailed information regarding ESIC2 initiative, business plan preparation, financial schemes and investor readiness in general as well as specific to the 3MiCRON project recommendations from the expert regarding composition of the research team, exploitable results and market needs, IPR and patenting strategy, technological and commercial properties of the developed product is presented in the report delivered by META Group.

Each partner has acquired the following knowledge and has identified strategies for dissemination and exploitation of the MBs.

Design of MB

A PVA MB was the platform used throughout the project. It was derived from the background know-how of UNITV concerning their synthesis. These MBs are microparticles characterized by a gas core and a cross-linked PVA shell, with an average diameter of 3 μm and limited size distribution.

Starting from this platform PVA MBs were equipped with different functionalities for imaging purposes (ultrasound, MRI, PET, SPECT), which can be added in a singular way or in combination. The surface of MBs can be functionalized 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 can be 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 these materials. Last but not least the new technique to increase storage time by freeze-drying the MBs has been developed. The wide range of surface and structural medications can be used in a broad way, not only for the functionalization of the PVA MBs but in the same manner also for other particles or colloidal systems.

The multimodal MB enables loading of different contents rather than air (as currently done), which can provide a new drug delivery system. We believe that the MBs developed within 3MiCRON represent a new generation core for future drug delivery applications and vehicles enabled by its stability, a prolonged shelf-life and the possibility of large-scale production. It is now possible to characterize these objects in a large throughput using new approaches such as modified flow cytometry and to monitor their stability over time and in different media. Overall during the 3MiCRON it has been possible to achieve a new and versatile platform where, by altering the MBs surface, it is possible to characterize intrinsic (such as shell thickness, morphology, shell stiffening) and extrinsic MB properties (such as colloidal stability, biomolecules adsorption, cellular toxicity) in a systematic way as strong protocols have been developed for several applied techniques. This framework has been shown to be fully functional in the invention and production of a multimodal platform. Acquired knowledge can be further applied and transferred to the production of other devices with engineered properties. The SME partner, Surflay, has upscaled the preparation of plain PVA-bubbles in accordance with the ISO 9001 standard in the project, enabling in the future the delivery of MBs with reproducible properties to interested companies or research institutes.

Phantom design

At Esaote, DKFZ, KTH and KI techniques to make multimodal phantoms have been further improved. Such phantoms are valuable when evaluating contrast media and when making research on new image fusion techniques. The intention is to use such phantoms for demonstrations and to introduce such interactive training material into the academic process. For instance, KTH already employs Plain MBs in combination with the ultrasound phantom, mimicking human tissue with incorporated vessels, for laboratory sessions. This makes it easier for the students to grasp the basic principles of wave scattering and enables simulations of *in vivo* experiments.

Software design

Esaote, Sintef and KTH have developed new imaging sequences specifically tailored to improve the detection of polymer-based MBs. The knowledge gained in pulse sequence development and programming can be used for future development for other, not yet developed MB. Development in T2* and T1 quantification with MRI is more modest, because the partners already had a great knowledge in this area.

Molecular imaging

In addition KI, Sintef and UCD provided evidence of specifically targeting of defined pathologies using vectorized 3MiCRON MBs coupled with specific antibodies and peptides. As mentioned earlier, it has also been demonstrated that 3MiCRON MBs have the capability to be used as a drug delivery platform.

IP and patents

The following strategy has been identified regarding IP and patenting. All partners agreed to revise their patent/publication strategy to increase the likelihood of knowledge transfer between academia and industry. Each partner agreed to check within its organization regarding the possibility of getting a patent related to realized results and to perform a survey to gain knowledge about, and to respect, any previously presented patients within the field. The option of whether to file for a national, European (PCT) or a patent on the American market should also be considered carefully. All partners have expressed their willingness to further advertise and disseminate the knowledge related to the plain and multimodal MBs through their individual websites, in research meetings, conferences and business fairs, in order to attract potential collaborators, customers or to find partners for the further development and exploitation. If new products will be developed by such collaboration, or if there will be production in a larger scale, the consortium will discuss the possibility to transfer the production and technology to the new partner, for example a big pharmaceutical company, and try to use the patent rights (IP) and the generated know-how for achieving licence fees. As a final remark it should be noted that it is the first time, to our knowledge, that one contrast agent combines multimodal imaging, targeting and drug delivery features. However, several characteristics have been identified that have to be improved in order to lift the 3MiCRON MBs to marketability. Even though these points could not be solved within the project lifetime, the MBs can be otherwise utilized in several fields, for example:

- Development and calibration of a new family of multimodality scanners to be used in human medicine
- Serving as a standard for the development of future multimodality contrast agents
- Serving as a reference for the development of future drug delivery systems
- Use in cutting edge *in vivo* experiments where the combined detectability of one contrast agent is crucial

1.5 Website and contact details

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