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Iron Labeling of Mesenchymal Stem/Stromal Cells for Magnetic Resonance Imaging:

Studies on Poly-L-Lactic Acid ï Iron Nanoparticles

Dissertation

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Presented by

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Just for me ï it is never too late!

With deep love and gratitude to my husband.

Also thanks to the other usual suspects, my kids and dogs.

List of Content

Abbreviations

7-AAD	7-aminoactinomycin D
APC	allophycocyanin
APC-Cy7	allophycocyanin coupled with the cyanine dye Cy7 (tandem fluorochrome)
CD	cluster of differentation
CLSM	confocal laser scanning microscopy
CTMA-Cl	cetyltrimethylammonium chloride
d	day
FACS	fluorescence activated cell sorter
FCS	fetal calf serum
FDA	Food and Drug Administration
FL	fluorescence
FITC	fluorescein isothiocyanate
FSC	forward scatter (FACS)
ICP-OES	inductively coupled plasma optical emission spectrometry
IRB	Institutional Review Board
ml	milliliter
mg	milligramm
min	minutes
nFL	normalized fluorescence
nm	nanometer

MSC	mesenchymal stem/stromal cell
MRI	magnetic resonance imaging
hBM	human adult bone marrow
PBS	Phosphate buffered saline
PE	phycoerethrin
PECy7	R-phycoerethrin coupled with the cyanine dye Cy7 (tandem fluorochrome)
PerCP	peridinin Chlorophyll Protein
PMI	perylenmonoimide
PL	platelet lysate
PLL	poly-L-lysin
PLLA	poly-L-lactic acid
SDS	sodium n-dodecyl sulfate
SPIO	superparamagnetic iron oxide particles
SSC	side-/orthogonal scatter (FACS)
TEM	transmission electron microscopy
T_1	T_1 relaxation time (longitudinal relaxation time)
T_2	T_2 relaxation time (transverse relaxation time)
T_{2^*}	T_2 star relaxation time
µm	micrometer
1.5 T; 3 T	1.5 Tesla; 3 Tesla

1. Introduction

1.1. Mesenchymal stem/stromal cells

In the last years increasing scientific interest focuses on mesenchymal stem cells (MSCs), also called mesenchymal stromal cells [9, 10, 34, 49]. Identified sources for MSCs include the adult bone marrow, compact bone, peripheral blood, adipose tissue, amniotic fluid, and other fetal tissues [13, 15, 51, 54]. MSCs are present in a low frequency. In human bone marrow they appear as 1 MSC in about 3.3×10^4 nucleated cells [63, 112]. Minimum criteria for the *in vitro* characterization of MSCs include that they are plastic adherent when maintained in standard culture conditions and that they express CD105, CD73 and CD90. They lack expression of CD45, CD34, CD14 or CD11b, CD79alpha or CD19, and HLA-DR surface molecules. MSCs differentiate to osteoblasts, adipocytes, and chondroblasts *in vitro* [36, 43, 44]. However, there is no unique marker which characterizes the MSCs and the criteria above define a minimum set of characteristics obtained in vitro under nonphysiologic conditions. These easy accessible and *in vitro* expandable multipotent cells are investigated in promising cell therapeutic settings such as tissue and wound repair and immunomodulation [1, 2, 18-20, 28, 31, 42, 53, 57-61, 83, 88, 93, 115]. As a flip side of the coin there are even strong hints that MSCs play a role as precursors of tumor stroma and in the growth of epithelial solid tumors, which on the other hand could also be used to use MSCs as drug carriers into the tumor [25, 26, 45, 64, 73, 74, 92, 97]. A lot on MSCs remains to be learned. As they are used in cell therapy settings, a better understanding of their behavior after extracorporal expansion and transplantation is crucial. In this context observation of the trafficking and homing of the expanded MSCs in a physiological environment and in a relatively unmanipulated state is of high interest.

1.2. Magnetic resonance imaging

Magnetic resonance imaging (MRI) appears to be an appropriate method for *in vivo* observation of MSC. This method is non-invasive, does not apply ionizing

radiation and has a high spatial resolution which in theory allows imaging of structures ranging from a near cellular level (50 µm) in animals to up to 300 µm in clinical grade scanners and therefore would allow studies in the living animal and even in humans [105]. To reach the required sensitivity and specificity labeling of the MSCs with an MRI contrast agent is necessary.

The criteria which have to be fulfilled by the ideal contrast agent for specific cell labeling are the following:

- amount of (intracellular) uptake of contrast agent has to be sufficient to alter a MRI signal,
- it should stay intracellular in a concentration which alters MR signal for some time to allow a sufficient in-vivo observation period,
- it has to be non-toxic,
- it must not alter the specific function of the target cell,
- it must not affect the growth kinetic of the cell,
- it should not interfere with the ex-vivo MSC expansion process.

Magnetic resonance imaging uses powerful magnetic fields to align the nuclear magnetization of (usually) hydrogen atoms in water in the body. Radiofrequency fields are used to systematically alter the alignment of this magnetization, causing the hydrogen nuclei to produce a rotating magnetic field detectable by the scanner. This signal can be manipulated by additional magnetic fields to build up enough information to reconstruct an image of the body. Frequently used terms to describe the behavior of different tissues in the magnetic field are T_1, T_2 and T_{2^*} [77, 113]: T_1 relaxation time (also called spin-lattice or longitudinal relaxation time) is a biological parameter that is used in MRI to distinguish between tissue types. This tissue specific time constant for protons will indicate how quickly the spinning nuclei will emit their absorbed radiofrequency into the surrounding tissue. As the high-energy nuclei relax and realign they emit energy which is recorded to provide information about their environment. The realignment with the magnetic field is termed ñlongitudinal relaxationò and the time percentage of the tissue nuclei to realign is termed T_1. Due to the slow molecular motion of nuclei in fat tissue, longitudinal relaxation occurs rather rapidly and longitudinal relaxation is regained quickly, leading to a short T_1 time for fat. Water is not as efficient as fat in T_1 recovery due to the high motility of the water

molecules. Water molecules do not give up their energy to the surrounding tissue as quickly as fat, and therefore take longer to regain longitudinal magnetization, resulting in a long T_1 time.

T_2 relaxation time (also called spin - spin relaxation time or transverse relaxation time) is also a biological parameter used in MRI to distinguish between tissue types. It is a tissue specific time constant for protons and is dependent on the exchanging of energy with nearby nuclei. T_2 weighted images rely upon local dephasing of spins following the application of a transverse energy pulse. T_2 is the decay of magnetization perpendicular to the magnetic field (in an ideal homogenous field). Due to interactions between the spins they lose their phase coherence, which results in a loss of transverse magnetization and MRI signal. Fat has a very efficient energy exchange and therefore has a relatively short T_2. Water is less efficient than fat in the exchange of energy and therefore has a long T_2 time [68].

When the radiofrequency pulse is turned off, the transverse vector component produces an oscillating field which induces a small current in the receiver coil. The signal is called free induction decay (FID). In an idealized nuclear magnetic resonance experiment, the FID decays approximately exponentially with a time constant, T_2 but in practical MRI small differences in the static magnetic field at different spatial locations (ñ i n h o m o g e n e i t i e s ò) c a u s e L a r m o
across the body creating destructive interference which shortens the FID. The time constant for the observed decay of the FID is called T_{2^*} (ñ T 2 s t a r ò) m e r e l a x a
and is always shorter than T_2 [113].

1.3. Magnetic resonance imaging contrast agents

Two major classes of contrast agents exist: paramagnetic agents primarily based on gadolinium (Gd(III)). These contrast agents have relatively more effect on T_1 relaxation and create hyperintense contrast on conventional T_1-weighted spin-echo or spoiled gradient-echo MR sequences. The second class of contrast agents is based on superparamagnetic iron oxide (SPIO) particles. These particles have magnetic moments that, because of the small crystal size, are unhindered by lattice orientation and therefore do not exhibit hysteresis, hence the term ñ s u p e r p a r a m a g n e t i c ò . B e s i d e s t h e i r T_1 effect, in an applied magnetic field, the

individual moments are free to align along the field, resulting in the formation of a single spin with a net moment of at least 4 orders of magnitude higher than a comparable ensemble of paramagnetic spins. This creates extremely large microscopic field gradients for dephasing nearby protons. This in turn, dramatically shortens the magnetic resonance T_2 relaxation time and thus leads to usually create a hypointense contrast on conventional spin-echo MR sequences, in particular when agglomerated within cells. On gradient-echo images, where T_{2^*} effects dominate, these (intracellular) particles induce an even larger hypointense contrast effect [23]. Given the greater sensitivity of MR imaging for detecting superparamagnetic nanoparticles and the fact that iron is biodegradable, these contrast agents are the natural choice for labeling cells.

Several studies on cell labeling for MRI imaging are published [3-6, 8, 17, 38, 65, 81, 86, 98, 99, 109, 116]. Iron oxide contrast agents are commercially available and approved for human use (i.e. Resovist®, Feridex®/Endorem®, Combidex®). Their clinical approved labels include mainly liver and lymph node imaging. They are not approved for cell labeling. For cell labeling they are being combined with different commercially available transfection agents (i.e. poly-L-lysine, protamine sulfate, Superfect, Lipofectamine Plus, cytRoti-Fect etc.). Transfection agents are macromolecules possessing an electrostatic charge and are used for the nonviral transfection of DNA into the nucleus. One problem for using transfection agents to shuttle DNA into cells is endosomal capture of DNA-transfections agent complexes and thus inefficient release of the transported material into the nucleus ï however this process can be used to trap contrast agents in the endosomes [3]. Relevant MRI signal changes could be obtained even in vivo allowing visualization of clusters of cells [3, 94, 105, 107]. Also new approaches of intracellular contrast agents are being developed and show promising results [22, 33, 39, 40, 55, 56, 66, 70, 76, 95, 96]. The settings of the studies vary widely. It got apparent that iron uptake and thus the resulting MRI imaging properties depend strongly on the cell type. Further a variety of cell labeling protocols was applied varying in the use of different iron compounds, concentrations and incubation times. Also the type, concentration, and incubation time of different transfection agents used to enhance cell labeling varies widely. The current hypothesis is that the commercially available iron oxides form micelle-like positive charged complexes with the polycationic transfection agents which then can be taken up by the cells

[39, 56, 62, 94]. However, there are hints that the transfection agents coat the cellular surface and thus interfere with surface antigen expression/detection [90]. The commercially available contrast agents (Resovist®, Feridex®/Endorem®) are mainly approved for liver imaging. When used for hepatic MRI, the iron is phagocytosed and accumulates in the Kupffer cells and other reticuloendothelial cells. The particles are biodegradable in that they are metabolized by cells and enter into the normal whole body iron metabolism, as evidenced by transient increase or serum iron values within one day and an increase of serum ferritin values seven days after administration [5, 37, 111]. It could be shown that the Resovist® (generic name: ferucarbotran) in contrast to Feridex®/Endorem® (generic name: ferumoxides) is taken up by HeLa cells also without transfection agent, when incubated with high concentrations. It is hypothesized that the reason can be seen in the different chemical structure: in Resovist® the magnetite nanoparticles aggregates are stabilized by carboxydextran (i.e. positively charged) whereas in Feridex® by dextran only [70]. However, whether this uptake suffices for MRI cell imaging remained open.

1.4. Iron loaded poly-L-lactide nanoparticles

Nanoparticles are small clusters of atoms or molecules with a size of about 1 ï 100 nm. Nanoparticles are larger than individual atoms and molecules but are smaller than bulk solid. They have unique properties and can be used as drug carriers, which may open new diagnostic and therapeutic opportunities in medicine. It has been shown that nanoparticles are taken up in a variety of cells, utilizing more or less all known cellular uptake mechanisms. Intracellular metabolism, excretion, and influence on cellular function of nanoparticles is of high relevance, as not all biological effects of intracellular nanoparticles are elucidated yet [17, 29, 30, 32, 65, 79, 80, 117-119].

It has been shown that nanoparticles synthesized via the miniemulsion technique show a favorable cell uptake with a toxicity profile depending on the polymer, the surfactant and the surface functionalization applied [32, 66, 69, 76, 110]. Figure 1 illustrates the key processes of the synthesis which in a nutshell is based on the mixture of aqueous and non aqueous phases and followed by ultrasonication which provides the necessary energy or formation of the respective nanoparticles.

In this case iron oxide nanoparticles are encapsulated in poly-L-lactic acid (PLLA), further referred as iron-PLLA particles.

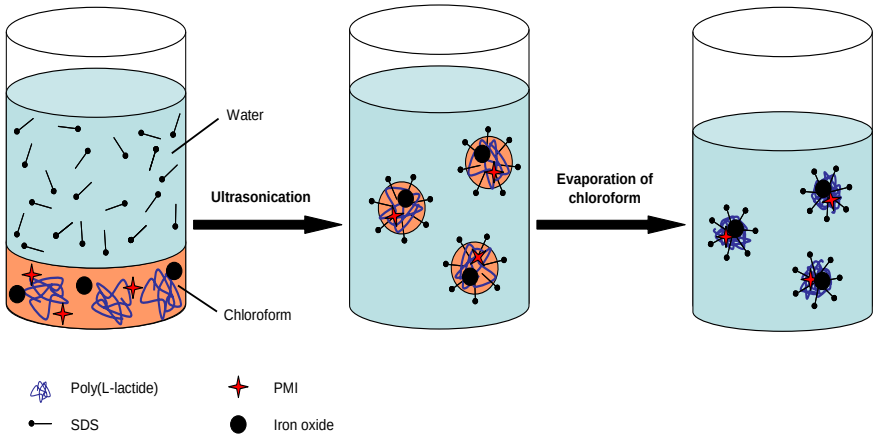


Figure 1: Miniemulsion process for synthesis of iron loaded PLLA particles. Courtesy Markus Urban [102].

With the miniemulsion technique it is possible to create biodegradable nanoparticles with a defined size, defined iron content, and a defined iron distribution on/in the particle. It is anticipated that biodegradable particles created by this technique in a uniform size improve the labeling of the MSCs by creating a high intracellular iron load, avoidance of particle aggregates on the cell surface, avoidance of alteration of cell surface as suspected when labeling the cells with the help of transfection agents. As a further advantage a fluorescent dye is integrated into the polymer. This allows the direct detection of the nanoparticles via confocal laser scanning microscopy (CLSM) and fluorescence activated cell sorting (FACS) and MRI. As iron itself is utilized by the organism, biodegradability of the nanoparticles´ polymer is seen as an advantage also to keep toxicity and interference with cellular processes and behavior at a minimum. It was chosen to take poly-L-lactic acid (PLLA) as the polymer to build the particle to be investigated. PLLA has a long standing history of safe use in medical applications, such as pins, screws, plates, intra-bone and soft-tissue implants, and as vectors for sustained release of bioactive compounds [14, 95]. An injectable medical device based on PLLA was FDA approved in 2004. PLLA microparticles are metabolized to carbon dioxide and water [66, 76, 95].

1.5. Scope of the dissertation

The scope of this dissertation was to evaluate:

- the uptake of different iron-PLLA particles into MSCs,
- the intracellular persistence of iron-PLLA particles in MSCs,
- the influence of iron-PLLA particles on MSCs with regard to cell viability, differentiation capability and surface antigen expression,
- MR properties of iron-PLLA particles alone and after MSC uptake in comparison to Resovist®/PLL.

These results shall allow selecting an appropriate particle which is suitable for efficient MSC labeling and further give guidance for improved particle design and synthesis.

2. Materials and methods

2.1. Devices

Analytical balance	Sartorius, Göttingen
Achieva 1.5T (1.5 T MR scanner) used with Sense Flex Coil Medium	Philips, Eindhoven, Netherlands
Achieva 3.0T (3 T MR scanner) used with Sense Flex Coil Medium	
FACScan (Fluorescence Activated Cell Sorter) Programme: Cell Quest 3.0	Becton Dickinson, Heidelberg, Germany
FACS Aria Programme: FACS DIAVA 6.1.2	Becton Dickinson, Heidelberg, Germany
Biofuge A ï centrifuge	Heraeus, Hanau Germany
Cytospin - centrifuge	Shandon-Elliot, England
CLSM (confocal laser scanning microscope) Fluoview on an IX71 equipped with 2 lasers (488 nm and 543 nm) and a 60x oil lens	Olympus Hamburg, Germany
Eppendorf research pipettes	Eppendorf, Hamburg, Germany
Incubator for cell cultures	Heraeus, Hanau Germany
Laminar Air® laminar flow bench	Heraeus, Hanau, Germany
Neubauer cell counting chamber	Neubauer Brandt, Germany
PolarStar Omega	BMG LabTech, Germany
TEM (transmission electron microscope) Philips	Fei, Philips Industries, Eindhoven, Netherlands
Varifuge GL and RI - centrifuges	Heraeus, Hanau , Germany
Zeiss ï microscope and camera	Zeiss, Germany
Leica ï microscope and camera	Leica Microsystems, Germany

2.2. Plastic materials

BD FALCON, 5 ml Polystyrene Round Bottom Tubes (for FACS measurement)	BD Biosciences, Bedford, MA, USA
Corning, centrifuge tubes, sterile, polypropylene 15/50 ml	Corning Incorp.. Corning NY, USA
Eppendorf tubes	Eppendorf, Hamburg, Germany

NUNClon™, surface, disposables for cell culture	Nunc, Roskilde, Denmark
NUNC multidish 6 wells	Nunc, Roskilde, Denmark
BD FALCON, 5 ml polystyrene round bottom tubes (for FACS measurement)	BD Biosciences, Bedford, MA, USA
Slide Flask®	Nunc, Roskilde, Denmark

2.3. Chemicals, solutions and buffers

7-Aminoactinomycin-D	Sigma Aldrich, St. Louis, USA
Agarose for routine use	Sigma Aldrich, St, Louis USA
CellMask™ orange plasma membrane stain 5 µg/ml in DMSO	Invitrogen, USA
Dimethyl-sulphoxide (DMSO)	Sigma Aldrich, St. Louis, USA
Eosin-Hematoxylin solution according to Ehrlich	Fluka, Sigma-Aldrich, Steinheim, Germany
2.5% glutaraldehyde	Fluka, Switzerland
Gibco 14190 DPBS without $CaCl_2$ and $MgCl_2$	Invitrogen, Grand Island, NY, USA
Potassium hexanocyanoferrate(II)trihydrate	Sigma Aldrich, St. Louis, USA
2.5% Trypsin Gibco®	Invitrogen, Canada
Trypan blue solution 0.4%	Sigma, Germany
Poly-L-lysine hydrobromide	Sigma, Germany

2.4. Cell culture media and supplements

Adipogenic induction medium	Lonza, Verviers, Belgium
Adipogenic maintenance medium	Lonza, Verviers, Belgium
Bio Whittaker® Alpha MEM	Lonza, Verviers, Belgium
Ciprofloxacin Kabi 200mg/100ml	Fresenius Kabi, Germany
ChondroDiff medium	Miltenyi Biotec, Germany
Gibco fetal bovine serum ï EU approved origin	Invitrogen, Grand Island, NY, USA

Lot 30591711	
OsteoDiff medium	Miltenyi Biotec, Germany
Pen Strep Gibco®	Invitrogen, Grand Island, NY, USA
PL (human platelet lysate)	Institute for Clinical Transfusion Medicine (IKT), Ulm
Sodium pyruvate 1 mM solution	Sigma, Germany

2.5. Antibodies

Anti-human mouse antibodies:

CD3-APC-Cy7	BD Biosciences Pharmingen, USA
CD9-APC	BD-Biosciences, USA
CD13-APC	Caltag Labs, USA
CD11b-PE	BD-Biosciences, USA
CD14-PE	BD-Biosciences, USA
CD16 ï PE	BD Biosciences Pharmingen, USA
CD19 ï PECy7	BD-Biosciences, USA
CD29-PE	BD-Biosciences, USA
CD45-PerCP	BD-Biosciences, USA
CD61 ï PerCP	BD-Biosciences, USA
CD71-APC	BD-Biosciences, USA
CD73-PE	BD Biosciences Pharmingen, USA
CD90 ï PE	BD-Biosciences, USA
CD105 ï APC	Caltag Labs, USA
CD105-FITC	BD-Biosciences, USA
CD33-APC	Miltenyi Biotec, Germany
CD166 ï PE	BD-Biosciences, USA
CD271-APC	Miltenyi Biotec, Germany
HLA A, B, C - APC	BD Biosciences, USA
HLA DR ï APC	eBioscience, USA
SSEA ï 4 ï APC	R&D Systems, USA

Isotype Controls:

Anti-mouse IgG1-APC	BD Biosciences, USA
Anti-mouse IgG1-PE	BD Biosciences Pharmingen, USA
Anti-mouse IgG1-PECy7	BD Biosciences, USA

Anti-mouse IgG1-PerCP	BD Biosciences, USA
Anti-mouse IgG1-APC-Cy7	BD Biosciences Pharmingen, USA

2.6. Test compounds

Resovist®

Resovist® 0.5 mmol Fe/ml; 1 ready to use syringe and filter for i.v. injection; 0.9 ml; Batch: 71048C; Expiry Date 01.2010; Pharmaceutical Manufacturer: Bayer-Schering-Pharma, Berlin, Germany.

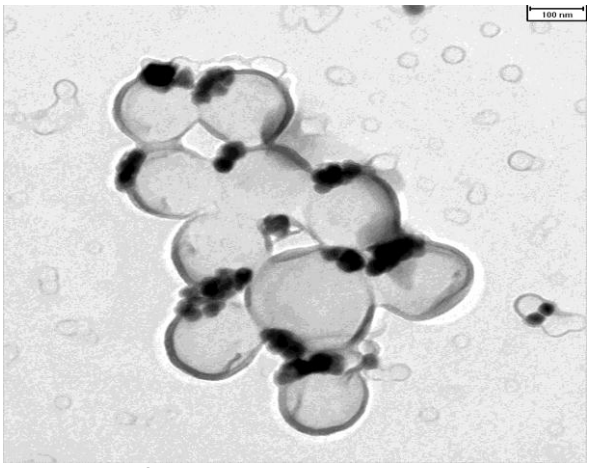
Iron-PLLA nanoparticles

The nanoparticles investigated in this thesis were synthesized, analyzed and provided by Markus Urban (Department for Organic Chemistry III; University of Ulm; Germany; head: Prof. Dr. Katharina Landfester/Max-Planck-Institute for Polymer Research; Mainz; Germany). Particles vary with regards to the surfactant used, iron content, surface charge and size. Particles were stored between +4 and +8 ^{o}C in a refrigerator.

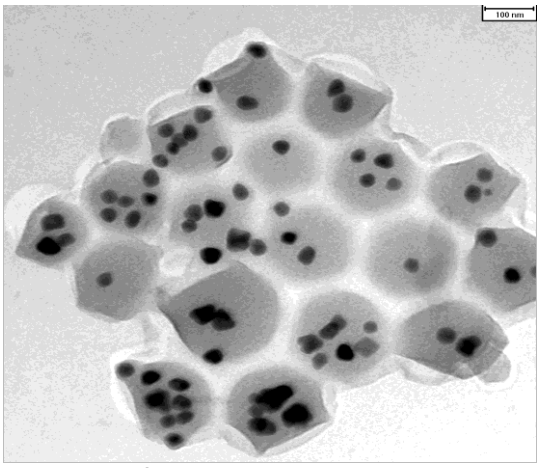
Table 1: Overview on characteristics of particles investigated in this thesis.

Particle Name	Biomer	Surfactant	Iron oxide used for Prepara-tion	PMI in mg/g solid content	Iron Content per ml Dispersion (ICP-OES) (mg per ml dispersion)	Particle Diameter (D_z) (nm)	Zeta Potential ï surface charge (mV)	Solid Content (%)
MU119-1zN2	PLLA ï - Polymer Biomer L9000 300 mg	SDS 72 mg	60 mg wustite (25 nm)	0.371	1.26	111	-55	1.64
MU119-1zLN2	PLLA - Polymer Biomer L9000 300 mg	SDS 72 mg exchanged by Lutensol AT50	60 mg wustite (25 nm)	0.180	0.90	124	-8.6	1.19
MU119-1zdN2	PLLA - Polymer Biomer L9000 300 mg	SDS 72 mg	60 mg wustite (25 nm)	0.419	1.00	115	-44	1.13
MU128-1zd	PLLA ï Polymer Biomer L9000 300 mg	CTMA-Cl 125 mg	60 mg wustite (25 nm)	0.312	0.40	149	+59	0.92
MU119-5zdz	PLLA ï Polymer Biomer L9000 300 mg	SDS 72 mg	150 mg wustite (25 nm)	0.312	2.35	113	-49	1.17
MU119-5zdzN2	PLLA - Polymer Biomer L9000 300 mg	SDS 72 mg	150 mg wustite (25 nm)	0.400	2.68	112	-32	1.36
MU130-5zdz	PLLA ï Polymer Biomer L9000 300 mg	SDS 72 mg	150 mg magnetite (10-25nm)	0.401	2.57	113	-36	1.42
MU119-5zdzN3+N4	PLLA ï Polymer Biomer L9000 300 mg	SDS 72 mg	150 mg wustite (25nm)	0.412	2.73	134	-28	1.26

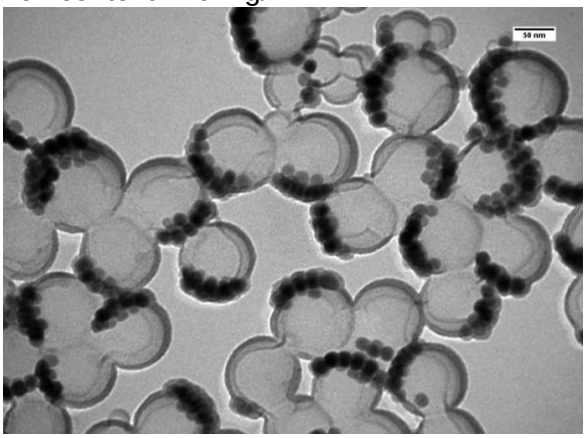
Figure 2 shows TEM images of the different particles used (courtesy Markus Urban, MPI Mainz) to demonstrate the iron distribution and configuration in/on the PLLA particle polymer.



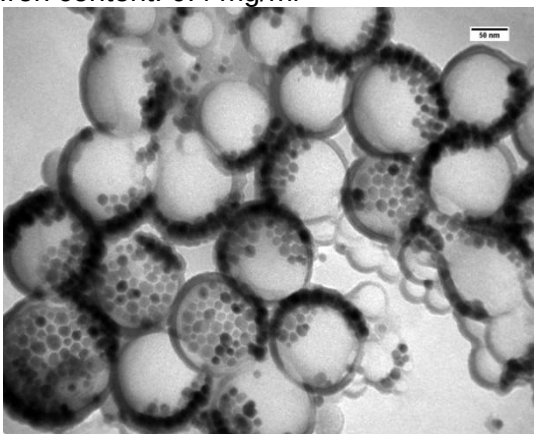
MU119-1zdN2
Iron content: 1.0 mg/ml



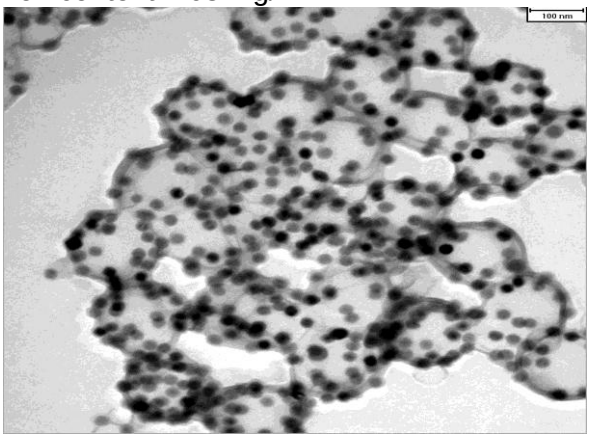
MU128-1zd
Iron content: 0.4 mg/ml



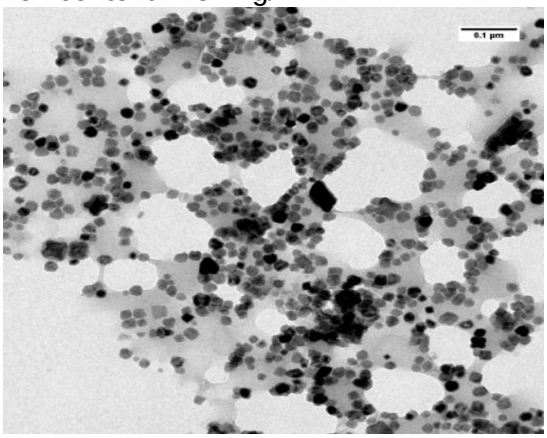
MU119-5zdzN2
Iron content:2.68 mg/ml



MU130-5zdz
Iron content: 2.57mg/ml



MU119-5zdz
Iron content: 2.35 mg/ml



MU119-5zdzN3+N4
Iron content: 2.73 mg/ml

Figure 2: TEM Images of the particles used in different MR phantom tests ï courtesy Markus Urban.

2.7. Mesenchymal stem/stromal cells (MSCs) used

Deep frozen in vitro-expanded MSCs were provided by the Institute of Transfusion Medicine of the University of Ulm. Informed consent was gained for gathering of the original probe and covered by IRB approval. Cells provided had shown osteogenic, chondrogenic and adipogenic differentiation.

An overview on origin and culture conditions of the MSCs that were used in this thesis is given in Table 2. In the experiments performed additionally to the name of the MSC line the passage number (px) is given indicating the proliferative age of the respective MSCs.

Table 2: Overview on origin and culture conditions of MSCs used in this thesis.

Name	Origin	Growth Medium + Additive (FCS or PL)*
Sark02	human spongiosa	alpha-MEM + 20% FCS
RKU01	human spongiosa	alpha-MEM + 20% FCS
RKU02	human spongiosa	alpha-MEM + 20% FCS
ULGeb-CH-03	human spongiosa	alpha-MEM + 10% PL
IKT14	human bone marrow aspirate	alpha-MEM + 10% PL
UL0094	human bone marrow aspirate	alpha-MEM + 10% PL

* cells are grown with either fetal calf serum (FCS) or platelet lysate (PL) as supplement.

2.8. Methods

2.8.1. Cell culture of MSC and passaging

MSCs were maintained in NUNC sterile plastic flasks for cell culture in alpha-MEM medium supplemented either with 20% FCS or 10% PL, depending on the primary conditions in which the cells were cultured and deep frozen. Alpha-MEM medium with 10% PL (supernatant after centrifugation at 5000 rpm) was supplemented with 2 iU Heparin and 1.2 mg ciprofloxacin per ml medium. Alpha-MEM medium with 20% FCS was supplemented with 100 iU penicillin, 100 µg streptomycin, 1.2 mg ciprofloxacin and 0.1 mg sodium pyruvate per ml medium.

Cells were grown in a humidified incubator at 37 ^{o}C and 5% CO_2. Medium was changed once weekly.

For passaging/harvesting of the cells, medium was removed, cells were once washed with PBS and were incubated with 0.5% trypsin for 4 ï 8 min at 37 ^{o}C and detachment was visually checked before trypsin activity was neutralized with the addition of equal volumes of medium. The cell suspension was centrifuged at 1800 rpm for 5 min and resuspended with medium. In order to determine cell viability, 20 µl cells suspension were mixed with 20 µl trypan blue and incubated for 1 min. Subsequently, cell count was performed with a Neubauer chamber. All 4 squares were counted by separating between unstained (viable) and stained (dead) cells. The cell concentration (cells/ml) of viable cells was determined by the following formula:

$$\text{Cells/ml} = \frac{\text{number of unstained cells}}{4} \times \text{chamber factor } (10^4/\text{ml}) \times \text{dilution factor (2)}.$$

Cell numbers were calculated and for labeling tests cells were re-seeded at a density of 2 x 10^4 cells per cm^2.

2.8.2. Resovist®/PLL labeling protocol

A PLL stock solution was prepared with PLL dissolved in distilled water at a concentration of 1.5 mg/ml. Aliquots were frozen at -20 ^{o}C and thawed up immediately before use. MSCs were seeded in 6-well plates in a concentration of 2 x 10^4 cells/cm^2 24 h before labeling. After 24 h first PLL was added to the incubation medium in a concentration of 0.75 µg/ml and mixed thoroughly. Then Resovist® was added to the medium in a concentration of 50 µg iron/ml and also mixed carefully. Incubation time was exactly 24 h in a humidified incubator at 37 ^{o}C and 5% CO_2. After 24 h, supernatant was removed, PBS washed and trypsinated.

2.8.3. Iron-PLLA nanoparticle labeling protocol

MSCs were seeded and incubated as described above. Iron-PLLA particles were added in defined concentrations and thoroughly mixed. The standard incubation time was 24 h if not otherwise specified. In the beginning of the experiment, the

iron content of the particle solution was not known and thus pre-published concentrations, based on the solid content of the particles were used [66, 70]. As the focus of interest is cellular iron uptake, incubation concentrations were adjusted to the iron content/ml incubation medium as soon as iron content of the particle suspensions got available. Harvesting was done identically as described for Resovist®/PLL.

2.8.4. Flow cytometric analysis

Flow cytometry can be used to distinguish cells according to their size, their granularity, their surface properties, their internal composition, and their viability.

In a flow cytometer, cells are moving in a laminar flow and pass one or more laser beams which cause light to scatter and fluorescent dyes to emit light at various frequencies. Light that is scattered in the forward direction is collected by a lens known as the forward scatter channel (FSC). The FSC intensity roughly equates to the cell´s size and can be also used to distinguish between cellular debris and living cells. The side scatter channel (SSC) provides information about the granular content within a cell. Fluorochrome labeled antibodies or other compounds (i.e. nanoparticles, 7-AAD) are used to detect cellular surface structures or the cellular content. Fluorochromes are essentially dyes, which accept light energy (i.e. from a laser) at a given wavelength and re-emit it at a longer wavelength (excitation and emission). The process of emission follows extremely rapidly, commonly in the order of nanoseconds, and is known as fluorescence. Flow cytometers use separate fluorescence (FL) channels to detect light emitted [72].

In this dissertation FACScan was used for particle detection (PMI included into the particle ï ñ d u a l r e p o r t e r p-PLL particles) and assessment of iron cell viability (7-AAD staining). This device is equipped with 1 laser with a wavelength of 488 nm.

For determination of the surface antigen panel, CD71, and for cell sorting for MR phantom generation, FACS Aria® was used. Measurements of the provided samples at the FACS Aria® and respective data evaluation were done by Dr. Markus Rojewski, Institute for Transfusion Medicine, University of Ulm.

Sample preparation and measurement of iron-PLLA particle uptake:

MSCs were harvested as described above. Cells were resuspended in 300 ï 500 µl PBS. Measurements were performed on FACScan. MSCs were gated by sideward scatter vs forward scatter plots (SSC/FSC) plots. Fluorescence measurements were done in the FL1 channel. To allow comparison of uptake of different particles, the FL1 values were normalized (normalized FL1, nFL1). The fluorescence signal was divided by the concentration of incorporated PMI (mg/g solid content) of the iron-PLLA particle as provided in the particle data sheet. For each value 3 independent labeled samples were measured and the fluorescence intensity is presented with ± standard deviation of the mean.

Cell viability testing: 7-AAD staining and measurement:

7-amino actinomycin D (7-AAD) is used to discriminate viable cells from dead or apoptotic cells. It is efficiently excluded by intact cells and has a high DNA binding constant. It emits in far red and thus can be easily separated from PMI, FITC and PE dyes [82, 91]. MSCs were harvested as described above, 10 µg 7-AAD in 50 µl distilled water (from stock solution) was added and resuspended with 300 µl PBS and incubated for 20 min at room temperature in the dark. Then cells were centrifuged and prepared for FACS measurement as described above. 7-AAD was measured in the FL3 channel. For each value 3 independent labeled samples were measured. Results are presented with ± standard deviation of the mean.

Determination of surface antigens and cell sorting:

These measurements were done with the FACS Aria®. This device is equipped with 2 lasers of 488 and 633 nm wavelength and for cell sorting. Antibodies as listed above were added to about 100 µl cell suspensions and were incubated for at least 20 min in the dark at room temperature. Then cells were washed and prepared for measurement/sorting. For these measurements only single probes were done.

2.8.5. Confocal laser scanning microscopy

Confocal laser scanning microscopy is a technique for obtaining high-resolution optical images. The key feature of confocal microscopy is its ability to produce in-focus images of thick specimens (i.e. cell layers) with structures labeled with fluorescent markers [71].

For confocal laser scanning microscopy (CLSM) imaging 5 x 10^4 MSCs were incubated overnight on poly-L-lysine-coated coverslips placed in 6-well plates. Iron-PLLA particles were added in defined concentrations and incubated for 24 h. Images were taken with Fluoview software on a Fluoview 300 equipped with an IX71 with two lasers, 488 and 543 nm and a 60x oil lense. PMI was excited by 488 nm laser light. For imaging of the cell membrane, 1 µl CellMaskÊ Orange was added, which was excited by 543 nm laser light. Images were taken in the Kalman filter mode.

2.8.6. Transmission electron microscopy (TEM)

Transmission electron microscopy (TEM) is a technique whereby a beam of electrons is transmitted through an ultra thin specimen, interacting with the specimen as they pass through. An image is formed from the interaction of the electrons transmitted through the specimen, which is magnified and focused onto an imaging device. With this technique it is possible to get a resolution a thousand times better than with light microscopy [78, 114].

Cells were fixed with 2.5% glutaraldehyd containing 1.5% saccharose and 0.1 M phosphate buffer in PBS (pH=7,3) and post fixed in 2% aqueous osmium tetroxide. The samples were dehydrated in a 1-propanol series, block stained in 1% uranyl acetate and embedded in Epon. Ultra thin sections were imaged in a Philips EM400 TEM, which was operated at a voltage of 80 kV. TEM probes and images were prepared in cooperation/technical assistance of Julia Dausend (Organic Chemistry III, University of Ulm) and F. Schmid (Central Facility of Electron Microscopy, University of Ulm).

2.8.7. Cytospin and Prussian blue staining

Cytospin is a method to spin a cell suspension onto a slide for viewing under the microscope. Prussian blue reaction involves the treatment of sections with acid solutions of ferrocyanides. Any ferric ion (Fe^{3+}) present in the cell combines with the ferrocyanide and results in the formation of a bright blue pigment called Prussian blue, or ferric ferrocyanide [50]:

$4Fe^{3+} + 3[Fe(CN)_6]^{4-} \rightarrow Fe_4[Fe(CN)_6]_3$ insoluble, blue

MSCs were harvested as described before a cell suspension with 3 ï 5 x10^5 cells/ml in PBS was prepared. For each cytospin slide 100 µl of cell suspension was injected and centrifuged at 5000 rpm for 5 min. Slides were air dried for at least 30 min. Slides were then fixed in methanol for 10 min and then incubated for 17 min in freshly prepared potassium hexacyanoferrate(II) solution 2% w/w, with 0.1 N HCl and washed in distilled water, then counterstained with hematoxylin-eosin for 5 min, washed 3 times in distilled water and then air dried.

2.8.8. Cell proliferation test

To determine whether cell proliferation rate is altered by iron labeling of the MSCs, CyQuant®Cell Proliferation Assay (Invitrogen C7026) was performed. The basis of the test is the green fluorescent dye which exhibits strong fluorescence enhancement when bound to cellular nucleic acids. Cells were trypsinated and 200 cells per well were seeded per 24-well plate in 0.5 ml standard medium per well. In parallel triple negative controls with 200 cells and 2000 cells are centrifuged at 3000 U/min, supernatant is discarded and pellets are frozen at -80 ^oC and stained and measured together with the cultivated cells. At day 7 optical microscopic judgments were made, then medium was removed and adherent cells were washed with PBS. Then samples were frozen at -80 ^oC for at least 1 h. For staining and lysis 19 ml distilled water was mixed with 1 ml of the kit buffer and 50 µl of the dye. 0.5 ml of the mixture were added to each plate and then prepared for optical measurement via multidetection microplate reader Polarstar Omega, BMG Lab Tec.

2.8.9. In vitro differentiation assays

Differentation assays into osteoblasts, chondroblasts and adipocytes were performed for 2 concentrations of MU119-5zdzN3+N4 labeled cells, for Resovist®/PLL labeled cells and for the negative controls of the same cell line to assess whether the iron labeling of the cells influences the differentiation capacity of the MSCs.

2.8.9.1. Osteogenic differentiation and staining

For the osteogenic differentiation assay iron-labeled and unlabeled MSCs were plated in Slide Flasks® in a density of 4.5 x 10^4 cells per flask with 1.5 ml NH OsteoDiff Medium (Miltenyi Biotec 130-091-678) supplemented with 100 units penicillin and 100 µg/ml medium streptomycin. Differentiation medium was changed every 3 or 4 days. At day 10 cells were Fast blue® stained. At the same time negative controls of iron-labeled and unlabeled cells were employed by plating the same number of cells in a Slide Flask® and keeping them in culture for the same time using the standard medium alpha-MEM with 20% FCS and the respective supplements. Alkaline phosphatase (AP) is a biochemical marker for osteoblast activity. Therefore, staining for intracellular AP activity serves as qualitative proof of osteogenic differentiation [84]. AP staining was performed using the staining kit SIGMA FAST BCIP/NBT. 1 tablet of the staining kit was dissolved in 10 ml distilled water 15 min before staining. Cells were washed with PBS and incubated for 1 h with 10% formaldehyde solution. The fixation solution was removed and the cells were then incubated for 30 min in the dark with the staining solution. The staining solution was removed and distilled water was added for 2 minutes. The slides were air dried.

2.8.9.2. Adipogenic differentiation and staining

For the adiopogenic differentiation assay iron-labeled and unlabeled MSCs were plated in Slide Flasks® in a density of 2 x 10^5 cells per flask cultured in 2 ml standard alpha-MEM Medium with 20% FCS and the standard supplements. When cells reached confluence, adipogenic differentiation was induced using adipogenic induction medium (Lonza, PT-3102B). After 3 ï 4 days induction medium was removed and substituted by adiopogenic maintenance medium for 3

ï 4 days (Lonza, PT3102A). This cycle was repeated 3 ï 4 times. Following the last cycle, cells were cultured 3 - 4 days in maintenance medium. Then the cells were stained with Oil-Red-O. At the same time negative controls of iron-labeled and unlabeled cells were employed by plating the same number of cells in a Slide Flask® and keeping them in culture for the same time using the standard medium alpha-MEM with 20% FCS and the respective supplements. Adipogenic differentiation of MSC is phenotypically defined by formation of lipid vacuoles which contain triglycerides. Lipid vacuoles are one characteristic of mature adipocytes [35]. Oil-Red-O is a hydrophobic stain that accumulates in the lipid vacuoles and stains them red. The Oil-Red-O working solution consisted of a 67% Oil-Red-O solution in distilled water (Oil-Red-O stock solution: 600 mg Oil-Red-O added to 200 ml Isopropranol 99%). After completion of the adipogenic differentiation cycles, cells were washed with PBS and fixated with 10% formaldehyde solution for 45-60 min at room temperature. The formaldehyde was removed and cells were washed with distilled water, then cells were covered with Isopropranol 60% for 5 min and thereafter with Oil-Red-O working solution for another 5 min. Excessive staining solution was removed with tap water and the nuclei were counterstained with Harris hematoxylin solution.

2.8.9.3. Chondroblast differentiation and staining

For the chondrogenic differentiation assay iron-labeled and unlabeled cells were plated in Slide Flasks® in a density of 4.5 x 10^4 cells in 1.5 ml NH ChondroDiff Medium (Miltenyi Biotec 130-091-679) supplemented with 100 units penicillin and 100 µg/ml medium streptomycin. The differentiation medium was changed after 3 or 4 days. After 24 days staining with methylene blue according to Löffler was done. At the same time negative controls of iron-labeled and unlabeled cells were employed by plating the same number of cells in a Slide Flask® and keeping them in culture for the same time using the standard medium alpha-MEM with 20% FCS and the respective supplements. Differentiation into chondroblasts is phenotypically described [84] as development of a multilayered matrix rich morphology and increased proteoglycan rich extracellular matrix. Methylene blue stains the proteoglycan rich extracellular matrix. Cells were PBS washed then methylene blue was added for 90 min. Staining solution was removed and acetous distilled water (6 drops of acetatic acid in 100 ml distilled water) added for a few

seconds. Then +4 ^{o}C distilled water was added. After this the slide was washed two times with distilled water.

2.8.10. MRI ï phantom preparation

24 h before MR imaging a probe mold was casted with agarose 2% in a glass container with placeholders set for the te
was heated with PBS until boiling and clearing.

At the day of test sample preparation, particle suspension or labeled/unlabeled MSCs were worked up as defined in the respective test protocols. Required dilution series were prepared. For cell test samples freshly prepared, cleared, liquid agarose 2% in PBS was pipetted in a vial. At a temperature of about +42 ^{o}C the respective cell solution was added and thoroughly dispersed with the help of a pipette. It was assured that always the same amount of PBS/fluid was added to the respective agarose test sample of a phantom. Then test sample was poured in the respective molds of the phantom mother. At the end the top of the phantom was covered with another layer of agarose 2%.

2.8.11. MR ï phantom imaging

Phantoms were delivered immediately after preparation to cardiac MR (head: Prof. Dr. Volker Rasche), Department of Internal Medicine II, University Hospital of Ulm, where imaging was started on 1.5 T or 3 T clinical grade scanners by Sonu Sharma, PhD candidate department of cardiac MR, normally within the next 2 - 6 h after delivery. Details of the special MR techniques and sequences applied can be found in the respective method description prepared by Prof. Rasche in Appendix I of this dissertation.

2.8.12. Statistics

Only descriptive statistics was used. Mean and standard deviation of the mean was calculated with Excel 2007 and/or GraphPad Prism 5.

3. Results

3.1. Cell - particle interaction

Iron-PLLA particles tested in this thesis vary with regard to surfactant used in the preparation process, their surface load, their iron content, and their iron distribution in/on the particle. In these experiments it was tested whether these differences influence uptake behavior and MSC behavior (viability) after uptake. Iron-PLLA particles which showed increased apoptotic/dead cells after incubation with MSCs where excluded from further tests as they disqualify by this for MSC labeling.

Cell particle interaction was approached from the following perspectives:

Short term ï evaluations of iron-PLLA particle MSC uptake directly after incubation:

- Demonstration of intracellular iron (III) after particle-incubation by Prussian blue staining (see chapter 3.1.1).
- TEM imaging of MSCs to show whether the iron-particle were taken up as a whole or whether the iron detached from the PLLA particle polymer before or during uptake (see chapter 3.1.2.).
- Confirmation of intracellular localization of iron-PLLA particle by confocal laser scanning microscopy (see chapter 3.1.3.).
- Quantification and comparison of iron-PLLA particle uptake with regard to different particles, incubation time and particle concentration by FACS measurements (see chapter 3.1.4.).

After encouraging ñshort term uptakeò re persistence over time was evaluated utilizing again Prussian blue staining, TEM imaging, and FACS measurements (see chapter 3.1.5.).

For demonstration of the MSC integrity after labeling, 7-AAD FACS tests were done for cellular viability testing, MSC surfaces markers were checked via FACS measurements and cell proliferation and differentiation tests were done to test the biological properties of the labeled MSCs (see chapter 3.1.6.).

As demonstrated elsewhere cellular MR imaging is feasible with Resovist®/PLL labeled cells [3-6, 23]. Comparative tests of Resovist®/PLL and iron-PLLA nanoparticles were done whenever feasible.

3.1.1. Prussian blue staining of MSCs for intracellular iron

To show the presence of intracellular iron (III), Prussian blue staining of MSC cytospin samples was performed with cells either incubated for 24 h with different concentrations of MU119-5zdz or with varying concentrations of Resovist®/PLL. Figure 3 demonstrates that MSCs incubated for 24 h with MU119-5zdz in concentrations from 150 µg to 450 µg (solid content) per ml incubation medium clearly show relevant amounts of intracellular iron. A perinuclear granular pattern can be observed with singular cells showing extra high iron load. The number of these highly loaded cells seems to increase with higher particle concentrations. Most of the visible iron appears to be intracellular located. Only in the highest concentration (600 µg solid content = 140 µg Fe) few extracellular iron precipitates may be assumed.

Prussian blue staining was done with Resovist®/PLL labeled cells as well in different concentrations and combinations. As published before, iron labeling with Resovist® alone is possible using high incubation concentrations [70]. The issue with this method is that a relevant amount of the iron sticks to the extracellular cell membrane as demonstrated in Figure 4 (a and b). To reduce extracellular iron adherence and to increase intracellular Resovist® uptake pre-published labeling protocols were tested using Resovist® in combination with the transfection agent poly-L-lysine (PLL) in various concentrations [39]. It turned out that with Resovist®/PLL in a concentration of 50 µg Fe and 0.75 µg PLL per ml incubation medium robust intracellular MSC labeling with good MR signals could be achieved (see image c and d in Figure 4). This concentration was used as a standard for all future experiments with Resovist®/PLL labeled MSCs.

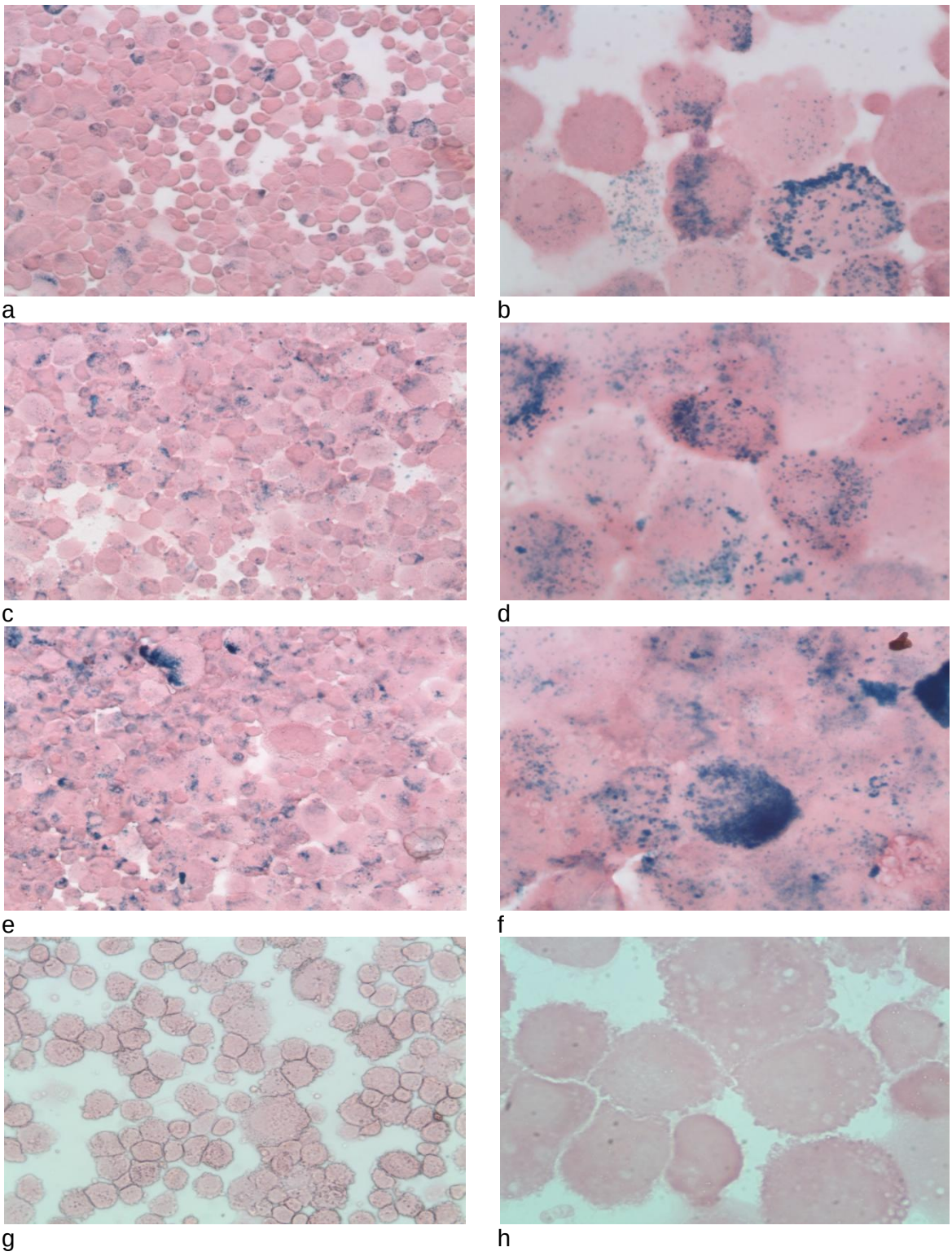


Figure 3: Prussian blue stained MSC (Sark02p11) cytospin slides after 24 h incubation with various concentrations of MU119-5zdz: a) and b) after 24 h incubation with 150 µg MU119-5zdz 20x and 100x magnification, respectively; c) and d) after 24 h incubation with 450 µg MU119-5zdz 20x and 100x magnification, respectively; e) and f) after 24 h incubation with 600 µg MU119-5zdz; g) and h) negative controls 20x and 100x magnification, respectively.

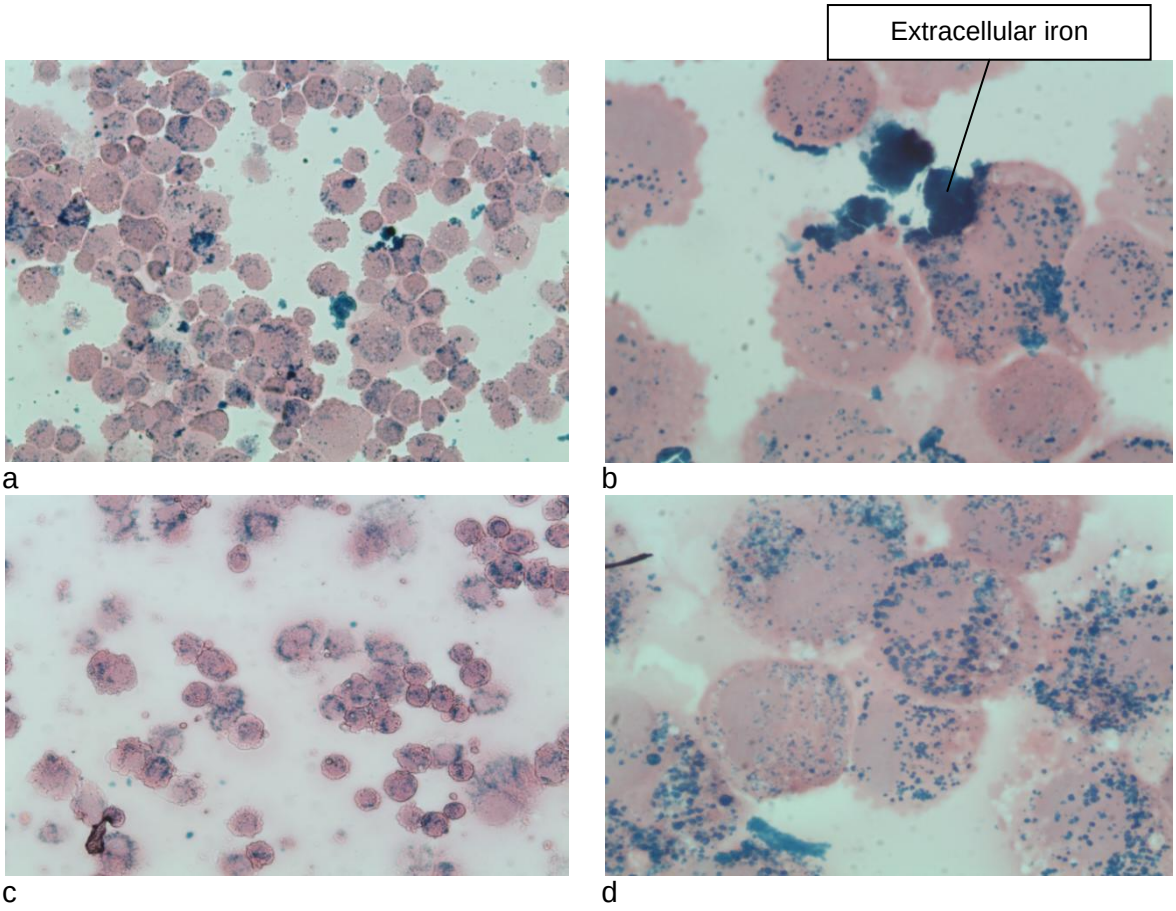


Figure 4: Prussian blue stained MSC (Sark02p11) cytospin slides after 24 h incubation with various Resovist®/PLL concentrations. a) and b) MSCs incubated with Resovist® alone in a concentration of 200 µg Fe/ml incubation medium, 20x and 100x magnification, respectively, showing extracellular iron aggregates; c) and d) MSCs incubated with Resovist®/PLL in a concentration of 50 µg Fe and 0.75 µg PLL per ml incubation medium, 20x and 100x magnification.

With this experiment it could be demonstrated that iron is taken up into the MSCs with more iron-PLLA particle uptake with increasing particle concentrations. Individual MSCs show more iron uptake than others after 24 h incubation.

3.1.2. TEM examinations ï intracellular location, structural integrity and uptake mechanisms

TEM examinations were done with MU119-5zdz, MU119-5zdzN3+N4, MU119-5zdzN2 and MU130-5zdz to evaluate whether the iron-PLLA particle keep their structural integrity during incubation and uptake and to get information on the intracellular location. Figure 5 shows that the iron-PLLA particles MU119-5zdz are taken up into the cell as a whole, i.e. that they mainly keep their structural integrity. In the TEM images the PLLA particle polymer appears as a white sphere and the

iron nanoparticles appear as black spheres, which makes a differentiated image analysis of the particles and their structural integrity in TEM possible. The images give also hints that a part of the iron oxide detaches from the particle surface intracellular (Figure 5e). Particles are mainly located in smooth vacuoles, which represent endosomes. Some images give hints that the particle are in an acidified endosomes (i.e. lysosomes), which present as equally dark vacuoles (Figure 5d). The darkening occurs by a reaction of the fixation medium with acid.

This finding is relevant as it validates the FACS and LSM findings which are based on the fluorescent properties of PMI included in the polymeric PLLA particles.

For MU119-5zdzN3+N4 some insight into uptake mechanisms could be gained. Figure 6 shows endocytosis of the particles. It appears that uptake occurs by multiple endocytosis mechanisms as described before [32]. Appearance in Figure 6a) suggests a lipid raft uptake, b) suggests clathrin dependent uptake, c) suggests macropinocytosis (Figure 6).

In TEM images not all particles present identically. As shown in Figure 7 in MSCs labeled with particles MU119-5zdzN2 and MU130-5zdz more iron agglomerates, which are not PLLA particle bound are visible (Figure 7a and 7c) than with particle MU119-5zdz (Figure 5) and MU119-5zdzN3+N4 (Figure 7e). Figure 7 below also shows that more free iron can be detected extracellular with particles MU119-5zdzN2 and MU130-5zdz (see Figure 7b and 7d), whereas with particle MU119-5zdzN3+N4 mainly iron connected to the PLLA shell can be seen (Figure 6 and Figure 7e). This observation suggests that additional stability/disintegration tests for the different particle batches may be useful.

TEM evaluations were done with Resovist®/PLL incubated MSCs as well. Intracellular iron could be shown (see Figure 8a). As already indicated by Prussian blue staining substantial amounts of extracellular iron agglomerates on the cell surface of Resovist®/PLL labeled cells could be detected (Figure 8c and 8d).

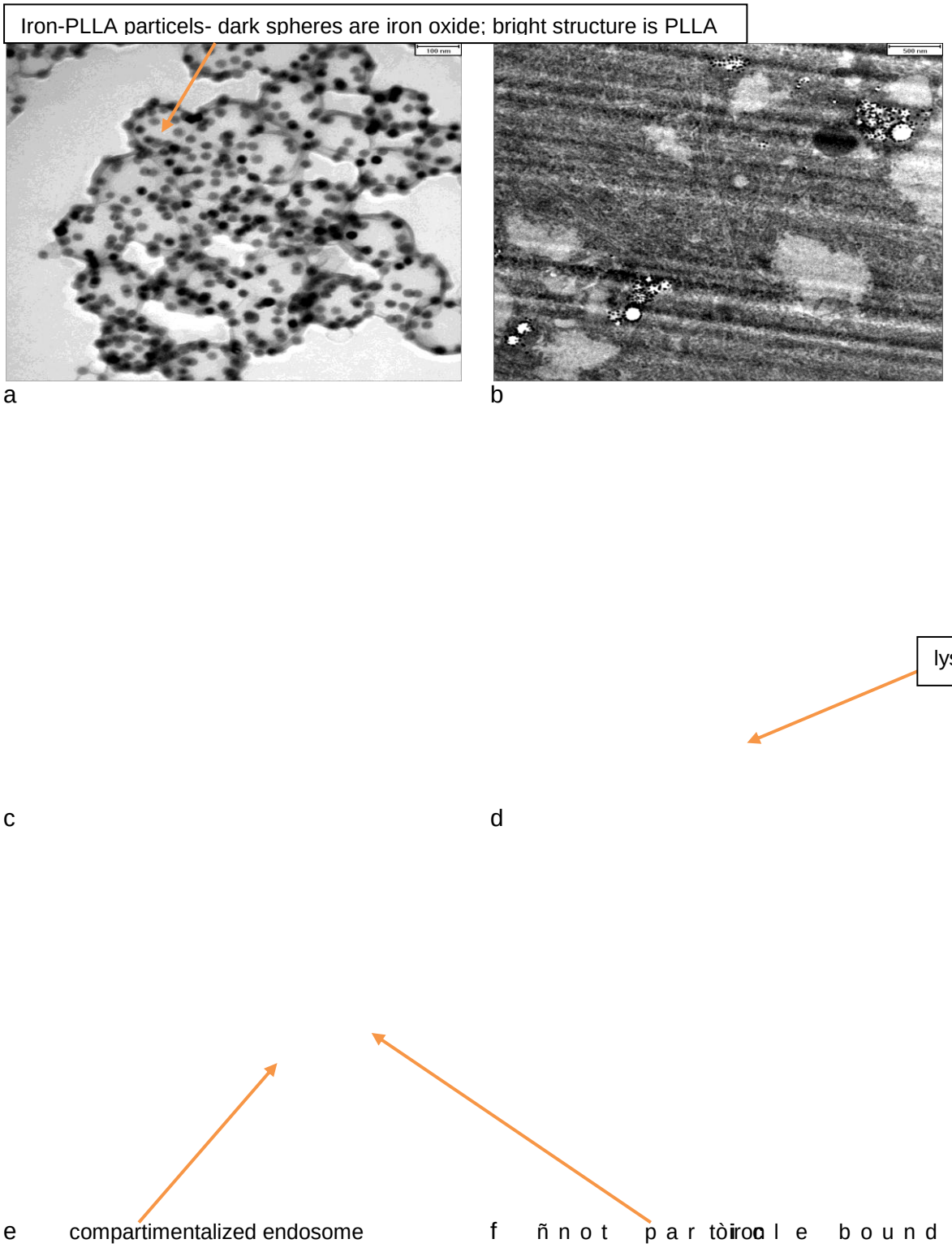


Figure 5: TEM Images of MSCs with intracellular intact MU119-5zdz. a) Native particle MU119-5zdz - courtesy Markus Urban; b) ï f) MSC (Sark02p11) with MU119-5zdz after 24 h incubation with 300 µg/ml of the particles.