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Amino-polyvinyl Alcohol Coated Superparamagnetic Iron Oxide Nanoparticles are Suitable for Monitoring of Human Mesenchymal Stromal Cells In Vivo

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6 Amino-polyvinyl alcohol coated superparamagnetic iron oxide nanoparticles are suitable for
7 monitoring of human mesenchymal stromal cells *in vivo*

8

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32 polyvinylalcohol, magnetic resonance imaging, cell based therapies

33

34

35 **Abstract**

36

37 Mesenchymal stromal cells (MSCs) are promising candidates in regenerative cell-therapies.

38 However, optimizing their number and route of delivery remains a critical issue, which can be39 addressed by monitoring the MSCs' bio-distribution *in vivo* using super-paramagnetic iron-

40 oxide nanoparticles (SPIONs).

41 In this study, amino-polyvinyl alcohol coated (A-PVA) SPIONs were introduced for cell-42 labelling and visualization by magnetic resonance imaging (MRI) of human MSCs.43 Size and surface charge of A-PVA-SPIONs differed depending on their solvent. Under MSC-44 labeling conditions, A-PVA-SPIONs had a hydrodynamic diameter of 42 ± 2 nm and a45 negative Zeta potential of 25 ± 5 mV, which enabled efficient internalization by MSCs46 without the need to use transfection agents. Transmission X-ray microscopy localized A-47 PVA-SPIONs in intracellular vesicles and as cytosolic single particles. After identifying non-48 interfering cell-assays and determining the delivered and cellular dose, in addition to the49 administered dose, A-PVA-SPIONs were found to be non-toxic to MSCs and non-destructive50 towards their multi-lineage differentiation potential. Surprisingly, MSC migration was51 increased. In MRI, A-PVA-SPION-labelled MSCs were successfully visualized *in vitro* and *in*52 *vivo*.53 In conclusion, A-PVA-SPIONs had no unfavorable influences on MSCs, although it became54 evident how sensitive their functional behavior is towards SPION-labeling. And A-PVA-55 SPIONs allowed MSC-monitoring *in vivo*.

56

57 1. Introduction

58
59 Mesenchymal stromal cells (MSCs) have gained much interest as a promising source for cell-
60 based therapies. Their potential to regenerate damaged tissue has been attributed to their
61 ability of self-renewal, differentiation into a variety of specialized cell types (e.g. in bone
62 MSCs are able to differentiate into bone-forming osteoblasts) and migration towards gradients
63 of growth factors secreted by damaged tissue.^[1] Experimental cell-therapy approaches in
64 animals using MSCs led to promising results for a number of neurological, myocardial and
65 musculoskeletal disorders (e.g. femoral head necrosis, osteogenesis imperfecta, and
66 osteoarthritis).^[2-7] Even though numerous clinical trials have been initiated and some
67 revealed a degree of success, a broad clinical application of such therapies is still not
68 available.^[8, 9] Critical parameters for successfully transferring results from animal
69 experiments to clinical application include the number of transplanted cell and their
70 cultivation and delivery process. Visualizing and monitoring the temporal and spatial
71 distribution of transplanted cells can provide valuable insight into understanding how to
72 optimize cell delivery and/or dosing. Unfortunately, methods for non-invasive tracking of
73 transplanted cells *in vivo* are still limited.

74 Visualization of cells *in vivo* can be achieved by using different molecular imaging modalities
75 such as magnetic resonance imaging (MRI), radionuclide imaging (positron emission
76 tomography (PET), single-photon emission computed tomography (SPECT)) and optical
77 imaging.^[10, 11] Although none of these imaging techniques is optimal, MRI is still the
78 preferred imaging modality for visualization of exogenously delivered cells, because of its
79 non-destructive and non-invasiveness, deep penetration and high spatial resolution.^[12]

80 The most commonly used imaging agents for MRI application are superparamagnetic iron-
81 oxide nanoparticles (SPIONs), which were introduced several decades ago and have become a
82 part of daily clinical routine use such as in imaging liver metastasis. SPIONs are nanoscaled

83 (5 - 15nm) crystals that consist of the biodegradable iron oxides magnetite (Fe_3O_4) or
84 maghemite ($\gamma\text{-Fe}_2\text{O}_3$) or a mixture of both phases and exhibit magnetism only under the
85 influence of an external magnetic field (superparamagnetism).^[13] In MRI, SPIONs exhibit a
86 negative enhancement on T2- and T2* weighted sequences, thus generating a signal change
87 that is several magnitudes stronger compared to other contrast agents (e.g. gadolinium).^[14] To
88 improve colloidal stability, solubility, and biocompatibility, SPIONs are coated with polymers
89 such as dextran.^[15]

90 Most previous studies on cellular tracking of MSCs used commercially available dextran- or
91 carboxydextran-coated SPIONs (Endorem/Feridex or Resovist, respectively).^[16-22] However,
92 manufacturing of both products was discontinued in 2008 and 2009, which prevents their
93 future applications. But more importantly, these nanoparticles were originally developed to be
94 taken up by phagocytic cells from the reticuloendothelial system (e.g. monocytes,
95 macrophages and osteoclasts) but not by non-phagocytic cells such as MSCs. To overcome
96 this limitation, transfection agents (TA) were used.^[23] However, some TAs are reported to be
97 toxic under certain circumstances and their influence on MSCs biology is an issue of
98 debate.^[19, 24-26] In addition, the colloidal stability of dextran- or carboxydextran-coated
99 SPIONs is impaired in cell culture media, making *in vitro* labeling difficult.^[27] ~~Furthermore,~~
100 ~~the dextran coating itself raises problems as it is susceptible to lysosomal degradation,~~
101 ~~resulting in exposure of cellular compartments and the cytosol to uncoated iron oxide~~
102 ~~nanoparticles and ions causing cytotoxic effects.~~^[27, 28] Therefore, it is necessary to develop
103 SPIONs with non-toxic coatings that meet the physiochemical need for efficient cellular
104 uptake by MSCs *in vitro*.

105 In recent years, several studies focused on the development of novel SPION-coatings for
106 MSC-labeling.^[28-33] Unfortunately, most of the previous studies suffer from missing
107 information on either one or more of the following aspects: (1) characterization of the
108 physiochemical properties of SPIONs, (2) exclusion of SPION-interference with the applied

109 methods (especially fluori- and colorimetric toxicity assays), (3) proof of SPION-
110 internalization, (4) information on the correct dosimetry, which includes not only the
111 administered, but also the delivered and effective cellular dose, (5) analysis of possible
112 secondary effects introduced by SPIONs on MSC beyond their key characteristics and (6) the
113 proof of principle for MRI visualization of SPION-labeled MSCs *in vitro* and *in vivo*. It is
114 thus difficult to accurately interpret the results and compare them between different studies.

115
116 In this study, we describe a novel approach to address the above-mentioned challenges. Our
117 aim was to label MSCs with amino-polyvinyl alcohol coated SPIONs (A-PVA-SPIONs) and
118 to find a balance between cellular uptake without TAs for MRI visualization and low
119 toxicity/impact on MSC cellular functionality. In particular, we aim (1) to develop an efficient
120 A-PVA-SPION-labeling procedure for MSCs based on particle internalization, (2) to analyze
121 the influence of A-PVA-SPIONs on MSC viability, proliferation, adipogenic, osteogenic and
122 chondrogenic differentiation as well as migration and (3) to provide proof of principle for
123 visualization of A-PVA-SPION-labeled MSCs in MRI *in vitro* by using MRI-phantoms and *in*
124 *vivo* by using animal models. We hypothesize that A-PVA-SPIONs are suitable to label
125 MSCs without provoking cytotoxicity allowing their visualization and monitoring in MRI.

126

127

128

129 2. Results

130

131 2.1. Development of an efficient A-PVA-SPION-labeling procedure for MSCs

132

133 Developing an efficient A-PVA-SPION labeling procedure is crucial for subsequent
134 visualization of MSCs in MRI and requires information about the nanoparticles
135 physiochemical properties. For example, the extent to what nanoparticles are internalized by
136 cells is determined by characteristics like size and surface charge, i.e. hydrodynamic diameter
137 and Zeta potential.^[15] Furthermore, the size of the nanoparticle is needed for dosimetry
138 calculations and for correct identification when confirming internalization by nanoscale-
139 resolution imaging methods. The physiochemical properties of polymer coated SPIONs can
140 change in response to pH (i.e. osmotic swelling) and protein concentration (i.e. formation of
141 protein corona).^[49] It is thus important to thoroughly characterize the A-PVA-SPIONs under
142 conditions that are identical to the read-out experiments.^[50] Therefore, we characterized size
143 and surface charge of A-PVA-SPIONs not only in their solvent (HNO₃ 10mM, pH 2) but also
144 in cell culture media (physiological pH 7.4) with and without fetal calf serum (FCS)
145 supplementation. The iron oxide crystal mean diameter was 7.2 ± 2.5 nm (**Figure S1Figure**
146 **1**). The SPIONs hydrodynamic diameter measures 14 ± 2 nm for the uncoated and 25 ± 3 nm
147 for the A-PVA-coated SPION in its solvent HNO₃ (10mM, pH2). The Zeta potential of the
148 uncoated SPIONs is at 26 ± 2 mV and slightly decreases to 20 ± 2 mV when the A-PVA-
149 coating is added. When transferred into FCS-free DMEM, the A-PVA-SPIONs hydrodynamic
150 diameter increases to 42 ± 2 nm, in the presence of FCS to 45 ± 2 nm. The addition of FCS to
151 the cell culture media results in a negative shift in the Zeta potential of A-PVA-SPIONs from
152 21 ± 5 mV to -25 ± 5 mV (summarized in **Table S1Table 1**). Both, the increased
153 hydrodynamic diameter and the negative zeta potential confirm the adsorption of proteins.^[51]
154 In conclusion, we now expect intracellular A-PVA-SPIONs with a diameter of 42 ± 2 nm to
155 45 ± 2 nm in the following experiments proving their internalization. In addition, the

156 determined size of 45 ± 2 nm will be the basis for calculating the A-PVA-SPIONs colloidal
157 behavior that is needed for establishing a dosimetry (details see **Table S1**).

158 We then investigated whether A-PVA-SPIONs are internalized by MSCs without any external
159 support such as transfection agents or magnetic fields. To confirm cellular internalization, we
160 used methods beyond Prussian Blue staining that allow resolution in the nanoscale:
161 transmission electron microscopy (TEM) and transmission X-ray microscopy (TXM). For this
162 purpose, MSCs were incubated for four hours with A-PVA-SPIONs ($100\mu\text{g}_{\text{Fe}}/\text{ml}$) under
163 serum-deprived conditions, which is known to be beneficial for efficient internalization.^[53]

164 The qualitative assessment of A-PVA-SPION internalization was facilitated by TEM and
165 TXM. TEM revealed that A-PVA-SPIONs are internalized by MSCs and stored in
166 intracellular vesicles (mean vesicle diameter: 357 ± 68.4 nm) (**Figure 2**). TXM supported
167 these findings (mean vesicle diameter: 387 ± 48.4 nm) and provided additional information
168 that A-PVA-SPIONs are also found in smaller high contrast spheres (mean sphere diameter:
169 52 ± 9.2 nm), clusters of irregular shape, and a micron-sized cluster (length: 2000 nm; width:
170 291 nm) in the cytoplasm (**Figure 3 and see also Video S1**).

171 After having proven that A-PVA-SPIONs are internalized by MSCs, we sought experimental
172 conditions to optimize their cellular dose and define the corresponding dosimetry. Reporting a
173 comprehensive dosimetry that consists of the administered, delivered and cellular dose is
174 crucial for the establishment of a correct dose-response relationship.^[53] The administered dose
175 itself only describes the amount of nanoparticles that was employed at the beginning of the
176 experiment. A more relevant metric is described by the delivered dose that also takes the
177 particles colloidal behavior and the exposure time into account and gives thus information
178 about the amount of particles that reaches the cell monolayer.^[34] Finally the cellular dose can
179 be determined experimentally and describes the amount of A-PVA-SPIONs internalized by
180 the cells. For this, MSCs were incubated with varying concentrations of A-PVA-SPIONs
181 (administered dose) and the corresponding cell-bound iron (cellular dose) was determined.

182 After four hours, the value for cell-bound iron reaches 5.9 ± 2.5 pg_{Fe}/cell at the lowest
183 administered dose (50µg Fe/ml), which does not further increase significantly at higher
184 administered doses. The TA Protamine had no beneficial effect (ANOVA, p=0.126) on this
185 pattern (**Figure 4A**). However, when incubation time was extended to 24 hours, an increase of
186 cell-bound iron was observed (ANOVA, p=0.014). After 24 hours, the cell-bound iron
187 increases to 8.2 ± 3.6 pg_{Fe}/cell at the lowest administered dose (50µg_{Fe}/ml), which is again not
188 affected by increasing the administered dose (**Figure 4B**). For accurate interpretation of the
189 results and comparability with other studies, a summary of the particle dosimetry results is
190 given in **Table 2**. In summary, we found that an optimized cellular dose in MSCs is reached at
191 A-PVA-SPION-labeling for 4h under serum-deprived conditions followed by 20h under
192 standard MSC culture conditions without the need of additional Protamine as TA.

193

194 **2.2. Non-toxic A-PVA-SPIONs stimulate MSCs migration**

195 When using A-PVA-SPIONs for MSC-labeling in cell-based therapy approaches,
196 compromising effects on MSC survival and function have to be avoided. We thus investigated
197 viability, multilineage differentiation and migration of MSCs after A-PVA-SPION-labeling
198 with four different administered doses ranging from 0 to 100µg_{Fe}/ml.

199 Viability and proliferation of A-PVA-SPION-labeled MSCs was assessed after four and eight
200 days and found to be unaffected compared to unlabeled MSCs (**Figure 5**). Notably, the
201 amount of cell bound iron is below the critical value that leads to interference with these
202 assays (**Figure S2 and Information S3**). Differentiation of A-PVA-SPION-labeled MSCs
203 towards the adipogenic, osteogenic, and chondrogenic phenotype was achieved without
204 differences to their respective controls (**Figure 6**). Migration was analyzed in a modified
205 wound scratch assay. A-PVA-SPION-labeled MSCs exhibit an increase in migration rate
206 compared to unlabeled controls (**Figure 7 and Video S2 and S3**). Quantitative analysis
207 revealed that this effect reaches statistical significance at the highest analyzed A-PVA-SPION

208 concentration ($50\mu\text{g}_{\text{Fe}}/\text{ml}$ vs. control: $p=0.069$; $100\mu\text{g}_{\text{Fe}}/\text{ml}$ vs. control: $p=0.001$; **Figure 7**).
209 Our results show that A-PVA-SPION-labeling does not affect differentiation, a key function
210 of MSCs as defined by The International Society for Cellular Therapy (ISCT), but rather
211 stimulates their migratory behavior.^[35]

212

213 **2.3. A-PVA-SPION-labeled MSCs can be visualized in MRI *in vitro* and *in vivo***

214 We found that labeling of MSCs with A-PVA-SPIONs had no negative effects on their
215 viability or regenerative and therefore we investigated whether the amount of cell-bound iron
216 was sufficient for visualization of A-PVA-SPION labeled MSCs using MRI *in vitro* and *in*
217 *vivo*. To this end, cell phantoms with different numbers of A-PVA-SPION-labeled MSCs
218 were prepared and scanned by MRI using T2 STIR and T1 VIBE sequences. A small effect
219 could be seen using both sequences where a signal loss due to the A-PVA-SPION labeled
220 MSCs was detected only at the highest cell concentration on the transverse plane of the MR
221 images (**Figure 8A**). Acquisition of the orthogonal plane showed that the cells were
222 concentrated at the bottom of the wells (data not shown). However, due to the small depth of
223 the gel, which was lower than the minimum slice thickness available, the meniscal ‘partial
224 volume’ effect precluded any quantification. Cell distribution was not homogenous enough to
225 determine a precise effect of cell number on T1 and T2 star relaxation times. Nonetheless, the
226 phantom results showed a trend in effect on T2 and indicated that MSC labeling was efficient
227 for MRI visualization with the sequences used.

228 *In vivo*, A-PVA-SPION induced signal loss was detectable on T1 weighted (VIBE) MR
229 images as a black region superior and anterior to the lower section of the femur 24 hours after
230 the injection of the A-PVA-SPION-labeled MSCs into the right naïve knee joint of Lewis rats
231 (**Figure 8B I, III**). No signal could be seen at the region on VIBE MR images of the left knee
232 joint where non-labeled MSCs were injected (**Figure 8C I and III**). These findings were
233 confirmed using corresponding dUTE MR images where A-PVA-SPION-labeled MSCs result

234 in positive MR enhancement and can be seen as a white region at the same position which is
235 absent in controls (**Figure 8B II, IV and 8C II, IV**). Post mortem histology of the animal's
236 knee joints confirmed the presence of A-PVA-SPION labeled MSCs (**Figure S3**).

237

238

239 3. Discussion

240 Our aim in this study was to develop an efficient labeling procedure for human MSCs with A-
241 PVA-SPIONs bypassing detrimental secondary effects on MSC viability and functions and
242 verifying the feasibility of visualizing A-PVA-SPION-labeled MSCs in MRI.

243

244 3.1. Development of an efficient labeling procedure

245 A major advantage of PVA is the fact that it is biocompatible and safe to use in humans as it
246 has been in medical use for several years, such as for cartilage replacements, wound packing
247 and contact lenses.^[54] A-PVA-SPIONs are already well characterized for their physiochemical
248 and magnetic properties.^[55] In contrast to dextran- or carboxydextran-SPIONs, they exhibit
249 excellent colloidal stability and dispersion in different cell culture media *in vitro* even in the
250 presence of fetal calf serum (FCS).^[52] The amine-functionalization promotes A-PVA-SPION
251 internalization by non-phagocytic cells without the need for compromising TAs, which also
252 applies for primary human cells such as MSCs as proven in our study.^[38, 56, 57]

253 Evidence of the internalization of A-PVA-SPIONs by MSCs and their subcellular location
254 was provided by both TEM and TXM approaches. The advantages of the TXM approach over
255 methods used in other studies are artifact-free sample preparation of the MSCs, visualization
256 in the nanometer range and 3D spatial information, i.e. conclusive evidence of cellular
257 internalization. The TEM approach showed A-PVA-SPIONs as high contrast particles that
258 accumulate in intracellular vesicles. The TXM data confirmed this result, but also
259 demonstrated that smaller high contrast spheres and irregular shaped clusters can be found.
260 The size of these small high contrast spheres analyzed by TXM is similar to the size of A-
261 PVA-SPIONs in DMEM + FCS determined by PCS (TXM: 52.9 ± 9 nm vs. PCS: 45 ± 2 nm).
262 We thus assume that single A-PVA-SPIONs are either internalized individually or are a result
263 of endosomal escapes. So far, we cannot distinguish whether the single A-PVA-SPIONs are
264 either vesicle-bound or freely dispersed in the cytosol. Vesicle-bound single particles would

265 indicate that A-PVA-SPIONs enter via a typical endocytosis-exocytosis route by being
266 internalized as individual nanoparticles and further sorted into bigger vesicles like lyso- or
267 exosomes.^[58] Freely dispersed A-PVA-SPIONs could directly interact with constituents of the
268 cytosol, i.e. proteins, mRNA, and cellular organelles, which may be other avenues of A-PVA-
269 SPION-induced functional changes. However, further research is needed to provide
270 conclusive evidence for one of those assumptions. Quantitative assessment of the cellular
271 dose revealed that a higher amount of cell-bound iron can be achieved by prolonging the
272 incubation time, but not by increasing the administered dose above $50\mu\text{g}_{\text{Fe}}/\text{ml}$. Similar results
273 were already observed for the internalization of PVA-SPIONs by non-phagocytic cell lines.^{[38,}
274 ^{57]} These results point towards an active uptake mechanism, which is energy dependent as
275 recently suggested.^[52] A more detailed discussion of the dosimetry can be found in

276 **Information S4.**

277

278 **3.2. Analysis of possible secondary effects**

279 For MSC tracking approaches *in vivo*, it is important that those A-PVA-SPIONs are not only
280 non-toxic, but also do not interfere with the cells' regenerative functions. Therefore, we first
281 focused on proliferation and multi-lineage differentiation both are key functions of MSCs as
282 defined by The International Society for Cellular Therapy (ISCT).^[35]

283 In our study, we observed no signs of A-PVA-SPION-induced toxicity as proliferation and
284 mitochondrial activity were unchanged similar to results observed for other cells.^[52, 57] Next,
285 the MSCs' ability to differentiate into the adipogenic, osteogenic and chondrogenic lineage
286 was investigated and was found to be unchanged. These positive results are noteworthy since
287 a number of studies reported impaired chondrogenesis after SPION-application.^[18, 19, 25, 59]

288 Only two of these publications report the corresponding cellular doses that were higher than
289 the one determined in our study; $25.7 \pm 0.96 \text{ pg}_{\text{Fe}}/\text{cell}$ and $13 - 16 \text{ pg}_{\text{Fe}}/\text{cell}$.^[19, 25] The
290 impairment of chondrogenesis might thus be caused by a high intracellular iron load as

291 already hypothesized.^[18, 25] It can be thus assumed that the cellular dose of 8.2 ± 3.6 pg/cell in
292 our study is below a critical threshold that leads to impaired chondrogenesis.

293 A number of *in vivo* studies provide evidence that exogenously delivered MSCs migrate and
294 target specific tissues via an active mechanism. For example, when injected into femurs
295 MSCs were later detected in the contralateral bone or MSCs implanted into the tibial bone
296 marrow cavity were detected in the callus of the ulnar fracture site after three weeks.^[60, 61]
297 Three days after injection into the tail-vein MSCs were detected at the fracture site.^[62]
298 Interestingly, in our study, migration of MSCs is increased after labeling with A-PVA-
299 SPIONs. This effect could be advantageous in the context of cell-based therapies as
300 exogenously delivered MSCs might migrate better *in vivo*. On the other hand, this effect could
301 also be disadvantageous as it indicates cellular changes by A-PVA-SPIONs in MSCs that
302 could influence so-far unknown parameters beyond migration. Future studies are needed to
303 determine if the change in migration upon A-PVA-SPION-labeling has consequences for the
304 outcome of MSC-based therapies.

305

306

307

308 **4. Conclusion**

309 SPION-labeling in combination with MRI is still the most promising approach for *in vivo*
310 visualization of exogenously delivered cells and has gained high interest in cell-based
311 therapies using MSCs. In the current study, we characterized the physiochemical properties of
312 A-PVA-SPIONs, investigated their interference with viability assays and their internalization
313 by human MSCs, report a correct dosimetry, found no impact on MSC viability and
314 differentiation, but enhanced migration, and finally provided the proof of principle for MRI
315 visualization of A-PVA-SPION-labeled MSCs *in vitro* and *in vivo*. The current study thus
316 provides comprehensive information about the impact of A-PVA-SPIONs on MSCs and the
317 feasibility of MRI visualization. In summary, the A-PVA/PVA copolymer has proven to be a
318 suitable SPION-coating used for MSC labeling. What remains unknown is the particles' long-
319 term fate **with respect to MRI visualization of A-PVA-SPION labeled MSCs**. For example,
320 the accuracy of MRI data *in vivo* is compromised by the inability to distinguish signals (1)
321 from viable and dead cells, (2) from internalized and excreted SPIONs and (3) from SPIONs
322 and MSCs engulfed by macrophages. **Another concern is the A-PVA-SPIONs metabolism**
323 **within the body that is determined by its stability *in vivo***. Future work should therefore focus
324 on research addressing **1) the A-PVA-SPIONs' retention time in the cell and elucidation of**
325 **the involved endo- and exocytosis mechanisms and 2) whether the A-PVA-coating separates**
326 **from the iron core resulting in renal excretion of A-PVA and integration of the SPION's iron**
327 **in the body's iron metabolism**. Taken together, these data help to develop A-PVA-SPION-
328 based MRI-tracking of MSCs towards a reliable research tool where non-invasiveness, deep
329 penetration, and high spatial resolution are needed. Thereby, it might be possible to gain
330 further insight into the spatial and temporal distribution of transplanted MSCs in tissue repair
331 and thus to optimize cell-based therapies.

332

333

335 5. Experimental Section

336

337 *SPION synthesis and A-PVA surface modification: A-PVA-SPION.* SPIONs were synthesized
338 following a co-precipitation protocol.^[36, 37] Briefly 0.064 moles of iron II from FeCl₂ and
339 0.128 moles of iron III from FeCl₃ were solved in 1.5 L deionized (DI) water and mixed with
340 120 mL of an NH₄OH solution (25%). After 10 min the suspension was sedimented under a
341 magnetic field and washed with DI water until pH 7. SPIONs were redispersed in 400 mL and
342 oxidized with 160 mL HNO₃ (2M) and 240 mL Fe(NO₃)₃ (0.35M) under reflux for 1.5h to
343 achieve maghemite (γ -Fe₂O₃). The suspension was washed again with DI water and was
344 dialyzed (with MWCO 12-14 kDa cellulose membrane dialysis tubing) against HNO₃
345 (10mM) for 3 days by changing the solution every 12h. The suspension was finally
346 centrifuged at 30000 g for 15 min and the supernatant was kept. The final suspension of
347 SPION had a concentration of 10 mg_{Fe}/mL and a pH of approximately 2. Surface modification
348 of the SPION with PVA was done following a protocol described previously.^[36, 38, 39] PVA-
349 OH (10 wt%; Mowiol 3-85, Kuraray Europe GmbH) and A-PVA (2 wt %; M12, Erkol.)
350 solutions were prepared by dissolving dry PVA in ultrapure DI water and the solutions were
351 rapidly heated for 1 hour at 90°C, cooled down, filtered at 0.45 μ m with a PTFE filter syringe
352 and stored at 4°C. 10 volumes of naked SPION were mixed with 9 volumes of PVA-OH
353 solution (100 mg PVA OH/mL) and 1 volume of A-PVA solution (20 mg A-PVA/mL). The
354 final A-PVA-SPION suspension (5 mg_{Fe}/mL, pH 3) was stored at least 1 week at 4°C before
355 further use.

356

357 *A-PVA-SPION characterization:* Crystallite's size was measured by counting of 400
358 crystallites sizes on Transmission Electron Microscopy (TEM CM12; FEI Co. Philips
359 Electron Optics, Zürich, Switzerland) pictures. Hydrodynamic diameters and Zeta potential of
360 A-PVA-SPIONs were measured on a Photon Correlation Spectroscopy apparatus (PCS

361 ZetaPals from Brookhaven: Laborchemie GES.M.B.H., Vienna, Austria). Uncoated SPIONs
362 and A-PVA-SPION suspension were investigated by Fourier Transformation InfraRed
363 spectroscopy (FTIR) showing the characteristic vibration bands for γ -Fe₂O₃ and A-PVA
364 (Figure S1 and Information S2).

365
366 *Human MSC isolation, cultivation and functional analysis:* This study was approved by the
367 local ethical committee; all donors gave informed written consent. Primary human MSCs
368 were isolated from bone marrow of human donors (8 male, mean age: 59 ± 9.1 years; 7
369 female, mean age: 60 ± 16.6 years) undergoing hip surgery as described previously.^[40] The
370 “culture medium” was Dulbecco’s modified Eagle’s medium (DMEM; Low Glucose, Gibco,
371 Grand Island, NY) with 10% fetal calf serum (FCS; Biochrom AG, Berlin, Germany), 5mM
372 L-alanyl-L-glutamine (Gibco, Grand Island, NY), 100 U/mL penicillin plus 100 μ g/mL
373 streptomycin. Cells were counted by using CasyTT for standard cell culture (Schärfe Systems,
374 Reutlingen, Germany) or a Neubauer chamber (C-Chip, Peqlab, Erlangen, Germany) when
375 only small volumes of cells were available. All experiments described in this section were
376 performed with cells from n=5 individual donors.

377 Proliferation rates were assessed by using a CyquantNF® Cell Proliferation assay kit (Life
378 Technologies, Carlsbad, CA, United States). Cell viability was assessed using PrestoBlue®
379 (Life Technologies, Carlsbad, CA, United States) as described earlier.^[41] Briefly, 2000
380 MSCs/cm² were seeded into 48-well plates and measured after one (d1), four (d4) and eight
381 (d8) days of culture. CyquantNF® values from d4 and d8 were normalized to d1.
382 PrestoBlue® values were normalized to cell number determined by CyquantNF®. All
383 measurements were performed in triplicates using a multimode microplate reader (m200 pro,
384 Tecan).

385 Osteogenic differentiation of 2.4×10^4 MSCs per 24-well was induced by supplementing
386 culture media with 200 μ M ascorbic acid, 7 mM β -glycerol phosphate, 0.01 μ M

387 dexamethasone for 13 d. The calcified matrix was visualized by Alizarin Red S (AR) and
388 quantified photometrical by dissolving AR in 10% cetylpyridinium chloride (readout
389 wavelength at $\lambda=562\text{nm}$). Adipogenic differentiation was induced by supplementing culture
390 media with 1 μM dexamethasone, 2 μM insulin, 200 μM indomethacin, 500 μM isobutyl-
391 methyl-xanthin for 14 d. Fatty acids were detected by OilRed O staining and quantified
392 photometrical by dissolving in 100% isopropanol (readout wavelength at $\lambda=500\text{nm}$). Each
393 experiment was conducted in triplicate. Chondrogenesis was induced by stimulating a pellet
394 culture (3×10^5 cells/pellet) with FCS-free culture media plus 10 ng/mL TGF- β 1, 10^{-7} M
395 dexamethasone, 50 $\mu\text{g/mL}$ ascorbic acid, 40 $\mu\text{g/mL}$ proline, 100 $\mu\text{g/mL}$ pyruvate, 6.25 $\mu\text{g/mL}$
396 ITS, 1.25 mg/mL BSA, 5.35 mg/mL linoleic acid) for 21 d and detected by Alcian Blue
397 staining and quantification of proteoglycan as described elsewhere with the modification for
398 pellet cultures and optimized read out wavelength ($\lambda= 516\text{nm}$).^[42]

399 Migration was analyzed in culture inserts for self-insertion (IBIDI, Munich, Germany) in
400 duplicate. 8×10^3 cells were allowed to attach for 5h in each cavity of the insert prior to insert
401 removal and addition of culture media with 5 $\mu\text{g/ml}$ Mitomycin C (Sigma-Aldrich, St. Louis,
402 USA). Migration into the defined cell free gap (500 μm) was observed for 24h under an
403 inverted microscope (DMI6000B, Leica, Germany) with a live cell imaging system, taking
404 images every 20min. Assay analysis (area covered by cells) was performed with Tscratch.^[43]

405
406 *A-PVA-SPION-labeling of MSCs and A-PVA-SPION dosimetry:* Prior use, A-PVA-SPIONs
407 underwent sonication for 1 min and pH-adjustment to neutral range (7.2 - 7.6). Adherent
408 MSCs (80-90% confluence) were washed with PBS and incubated with A-PVA-SPION
409 containing FCS-free culture media for 4h (n=4) with fixed media height (1.3 mm) throughout
410 different culture vessels to prevent variations in the administered dose. Protamine was used at
411 a final concentration of 5 $\mu\text{g/ml}$ (n=2). If MSCs were labeled for 24h (n=2), 10% FCS was
412 added after 4h for sufficient cell nutrition. Finally, A-PVA-labeled MSCs were washed 6x

413 with PBS before further use. For dosimetry calculations, we used a simplified model based on
414 *In vitro* Sedimentation, Diffusion and Dosimetry model (ISDD) developed by Hinterliter et al.,
415 taking additive transport by diffusion and sedimentation into account.^[44] The error compared
416 to the ISDD model is reasonably small compared to all the uncertainty arising from the *in*
417 *vitro* agglomeration and formation of the protein corona, both influencing the diameter and
418 density of the particles. The characteristic properties of the particles used for the calculation
419 of the dose delivered to the cell surface are summarized in Table S2.

420
421 *Determination of cell-bound iron:* After centrifugation at 400xg, the cell pellet was dried
422 overnight at 50°C, re-suspended in 125µl 6N HCL followed by a second overnight incubation
423 step at 50°C. 25µl sample was then mixed with 25µl 6N HCL followed by adding 50µl of 5%
424 K₄[Fe(CN)₆] (Merck, Darmstadt, Germany). After 20min, absorbance was read at 690nm
425 (m200 pro, Tecan, Männedorf, Switzerland) against a standard curve using FeCl₃. Each
426 measurement was carried out in quadruplicate. To obtain cell bound iron, iron (pg_{Fe}/cell) was
427 normalized to total cell number (average of 2 x 10⁶ cells).

428
429 *Transmission electron microscopy (TEM) and Transmission X-ray microscopy (TXM):*. For
430 TEM, 5x10⁵ MSCs were trypsinized and further processed as described previously^[45]. After
431 fixation and prior embedding, fixed cells were centrifuged for pellet formation. The mean
432 vesicle size was determined by measuring the diameter of n=4 vesicles from one
433 representative TEM micrograph using ImageJ Software.^[46]

434 For TXM, MSC were cultivated for 24 h on gold grids (type HZB-2, Gilder Grids, Grantham,
435 UK) coated with a perforated carbon film (Quantifoil Micro Tools GmbH, Jena, Germany)
436 prior to A-PVA-SPION-labeling. Samples were then plunge frozen in liquid ethane and
437 transferred into liquid nitrogen. Data acquisition using the HZB TXM at the undulator
438 beamline U41-FSGM, electron storage ring BESSY II, Berlin, was performed as described

439 previously.^[47] For imaging, a zone plate objective with 25 nm outermost zone width was used.
440 The tilt range of the sample was from -60° to $+60^\circ$. For tomographic reconstruction of the
441 acquired Tilt series eTomo was used and visualized using CTvox (CTvox 2.6, Bruker CT,
442 Kontich, Belgium) for 3D remodeling of the volumetric data.^[48] The mean diameter of
443 vesicles and high contrast spheres was determined from seven representative images of the
444 tomograms z-stack using ImageJ Software (vesicles measured: n=9; high contrast spheres
445 measured: n=80).

446
447 *Visualization by MRI in vitro (phantoms) and in vivo (animals):* Female Lewis rats were
448 obtained from Janvier Labs (Cedex, France). The rats weighed between 150 and 175g and
449 were 6-8 weeks old on arrival. They were housed in the animal facility at the University of
450 Geneva under pathogen-free conditions in standard cages and were fed standard diet and
451 water ad libitum. Animal handling was in accordance with guidelines of the Swiss Committee
452 of Animal Experiments. The experimental protocol was approved by the Animal Care
453 Committee at the University of Geneva (authorization no.1049/3580/3). MSC labeling was
454 performed as described. A-PVA-SPION-labeled ($100\mu\text{g}_{\text{Fe}}/\text{ml}$) MSCs were trypsinized,
455 counted, and fixed in 4% formaldehyde (Roth, Karlsruhe, Germany). Increasing numbers of
456 fixed cells were spun down and embedded in 200 μl 3% (w/v) gelatine on a 48-well plate. The
457 cell phantoms were scanned using the same sequences as optimized for *in vivo* imaging and a
458 15cm surface for homogeneous signal response. The experiment was carried out on 1.5T
459 scanner. Longer scan times (10 signal averages) were needed to regain the SNR lost going to
460 a larger coil and lower field.

461 The MR imaging parameters for the phantoms are as followed: A 'T2-weighted' 2D
462 acquisition with TR/TE/TI 8640/44/160ms, Flip angle 160° , Resolution 0.26 mm, FOV
463 200*100 mm and slice thickness 1mm. The 'T1-weighted' gradient echo is a 3D acquisition

464 with resolution of 0.35mm and slice thickness 0.2mm, TR/TE 22/9.5ms, Flip angle 10° and
465 FOV 160*80mm.

466 For *in vivo* visualization of labelled cells, the A-PVA-SPION-labeled MSCs and the non-
467 labelled MSCs were injected intra-articularly into the right and left naïve knee joints of Lewis
468 rats (Janvier Labs, Cedex, France) respectively. The following day, MR imaging of rat knee
469 joints *in vivo* was conducted using a Siemens Magnetom® Trio 3T clinical scanner. A
470 standard 4cm loop coil and respiratory monitoring with a pressure pad were used during the
471 imaging session. The imaging protocol begins with a standard low-resolution localization
472 sequence and the isotropic resolution 3D Ultra-short Echo time (UTE) double echo MR
473 sequence fixed orthogonal and at the magnet centre. This was subsequently used to localize
474 the correct plane for the 2D or thinner slab 3D images as well as for quantitative analysis. The
475 protocol parameters of the sequences used were as follows:

476 3D T1 gradient echo was used to detect and visualize A-PVA-SPIONs by signal loss.
477 Parameters: TR/TE 14.3/5.9ms, flip angle 12°, fat suppression, isotropic resolution 0.31mm,
478 and FOV 100mm, acquisition time 4 minutes 54 seconds.

479 Difference Ultra-short Echo time imaging (dUTE) was used for A-PVA-SPION positive
480 contrast detection and quantification. Parameters: 3D isotropic matrix 448 and 80mm FOV,
481 giving 180 µm in all three dimensions, 50000 radial projections, UTE/TE(2) 0.07ms/2.46ms
482 (for in-phase fat/water image), TR 9.6ms (in vivo 100 segments), flip angle 10° , acquisition
483 time 16 minutes 54 seconds.

484
485 *Statistics:* When performing multiple pair-wise comparisons, one-way or two-way analysis of
486 variance (ANOVA) were performed, and p-values were adjusted using Bonferroni's p-value
487 adjustment multiple comparison procedure. Results are presented as mean ± standard
488 deviation (SD). P-values < 0.05 were considered statistically significant.

489

490 **Supporting Information**

491 Supporting Information is available from the Wiley Online Library or from the author.

492

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494

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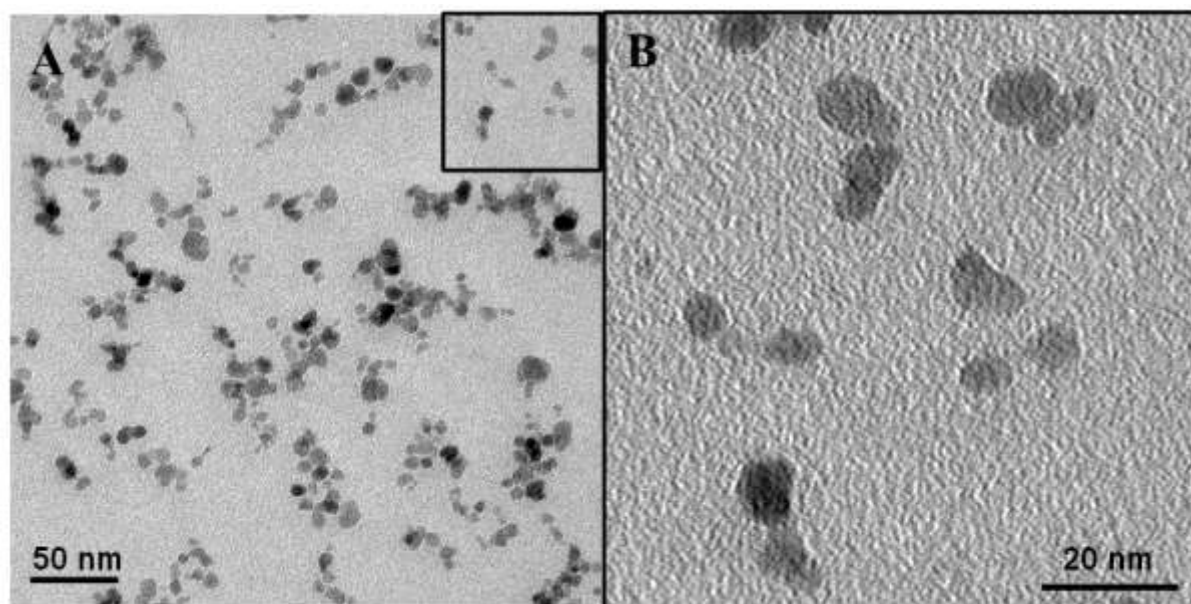
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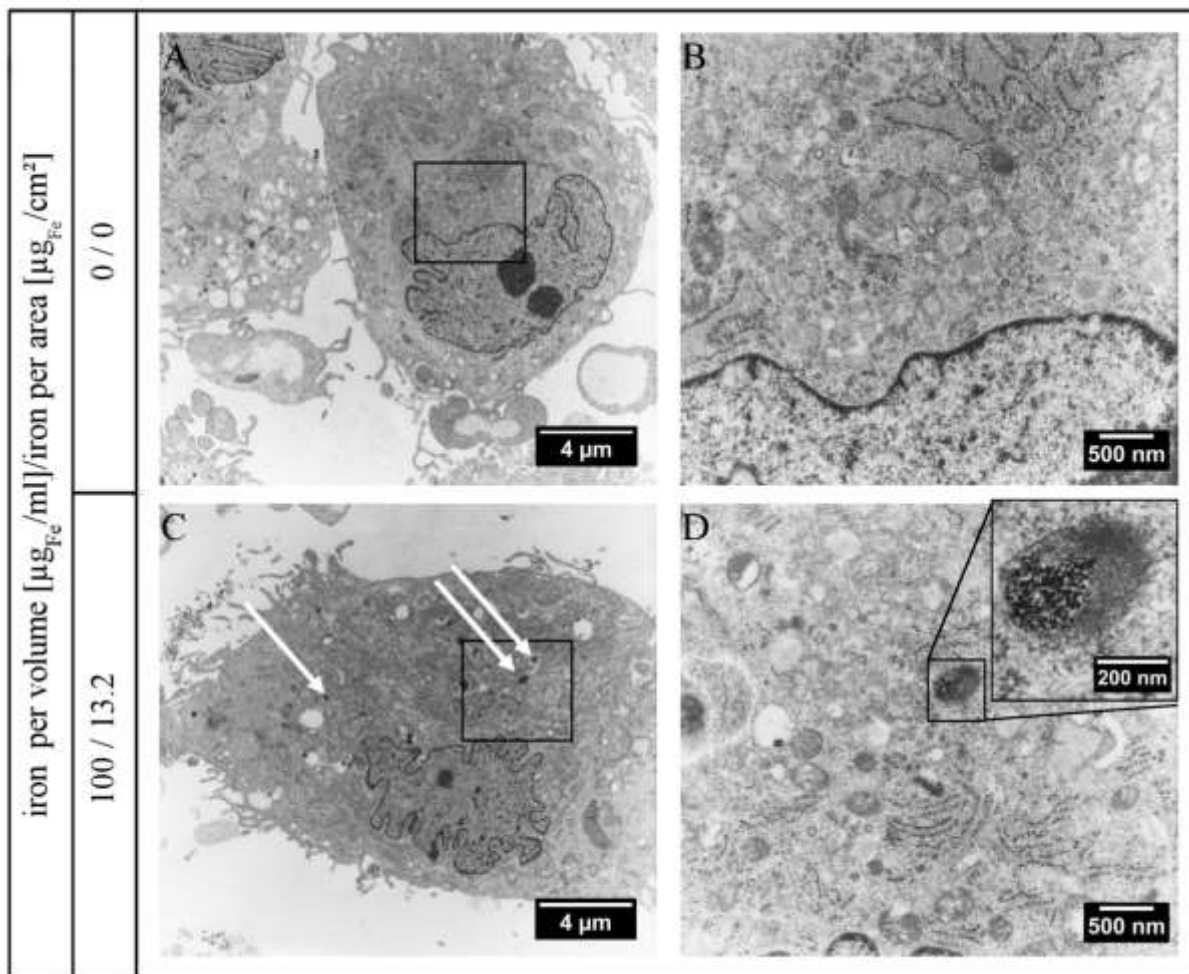
652

653 **Figure 1. TEM pictures of A-PVA-SPIONs.** (A+B) TEM micrographs show iron oxide

654 cores from A-PVA-SPIONs and were used to determine the mean average size of the γ Fe_2O_3

655 crystals

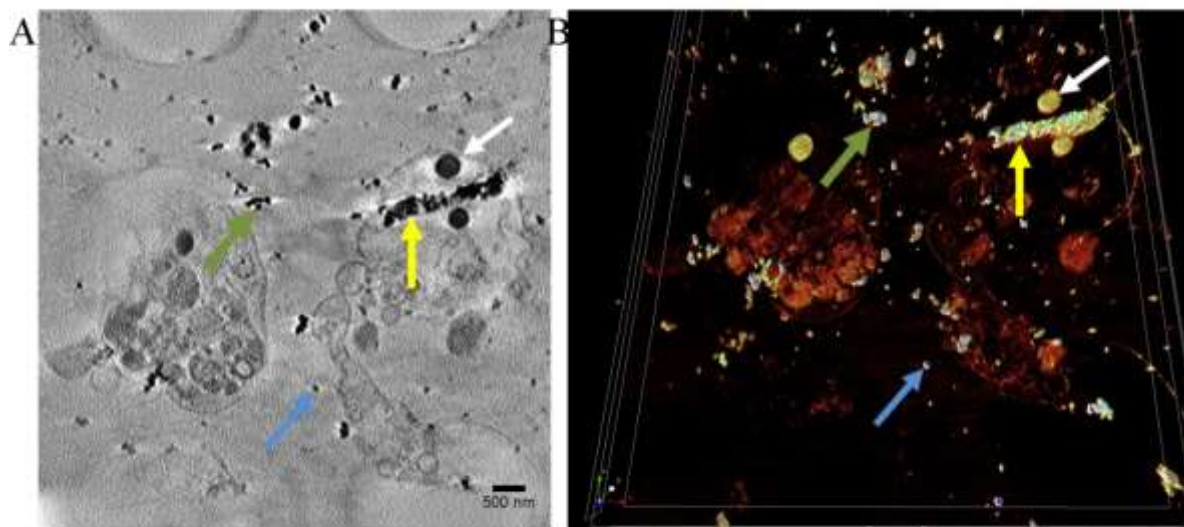
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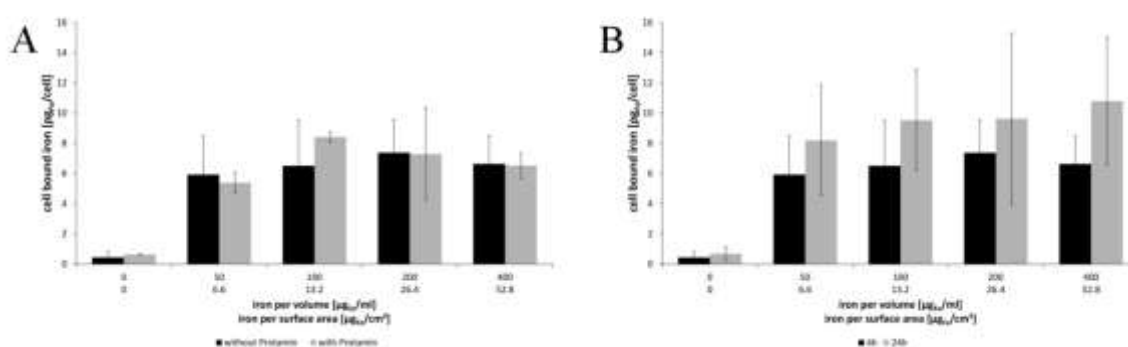
657

658 **Figure 2. A-PVA-SPIONs are internalized by MSCs that store them in intracellular**
659 **vesicles.** Shown are two representative pictures of (A, B) non-labeled and (C, D) A-PVA-
660 labeled MSCs at different magnification detection by TEM. A-PVA-SPIONs are visible as
661 intra-vesicular colloids (white arrows) in labeled MSCs that are absent in unlabeled control
662 cells.

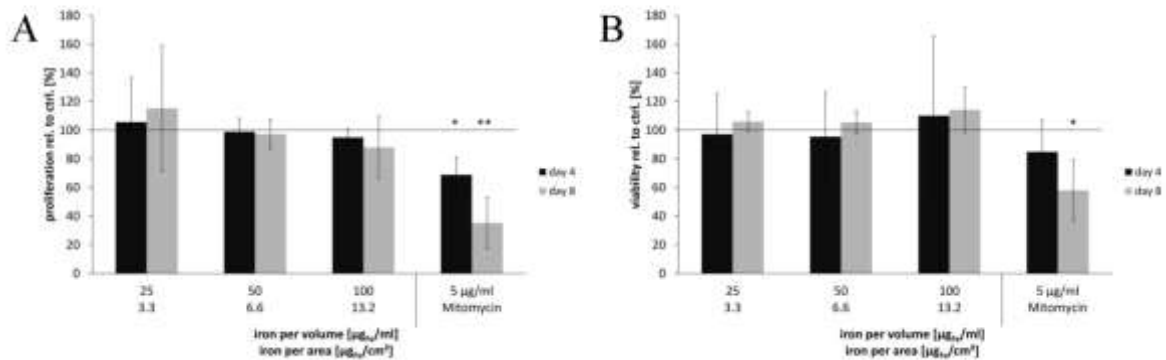
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664
 665 **Figure 3. Internalized A-PVA-SPIONs are differently distributed in cytoplasm.** The
 666 acquired tilt series of A-PVA-SPION-labeled MSCs by TXM allowed tomographic
 667 reconstruction. Shown are (A) one slice from z-stack and (B) subsequent 3D modeling. A-
 668 PVA-SPIONs are visible not only as intravesicular colloids (white arrows), but also as smaller
 669 high contrast spheres (blue arrows), clusters of irregular shape (green arrows), and as a
 670 micron-sized cluster (yellow arrow) in the cytoplasm.



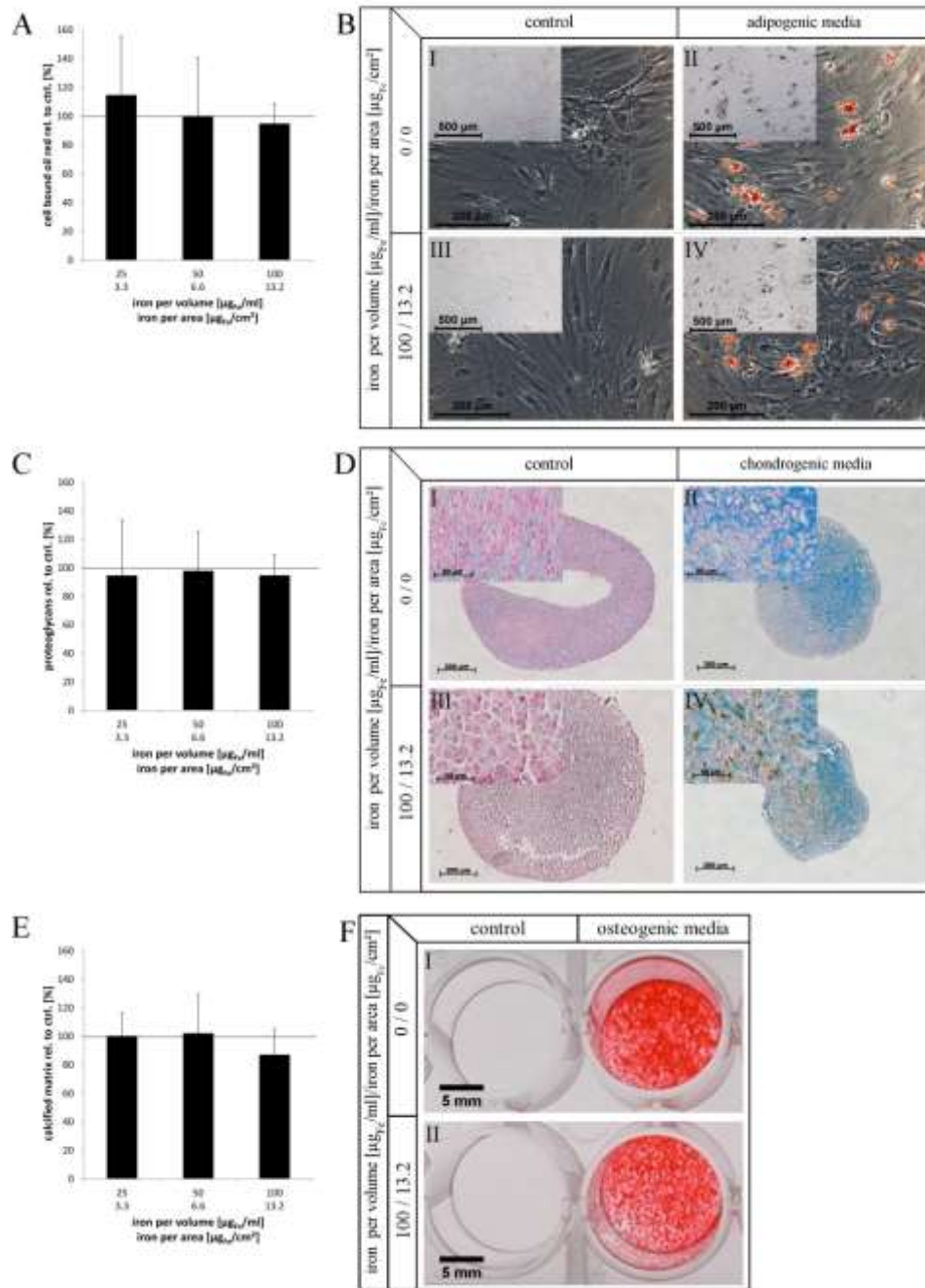
671
 672 **Figure 4. Efficient labeling of MSCs with A-PVA-SPIONs can be achieved at low**
 673 **administered doses and does not require Protamine.** MSCs were incubated with A-PVA-
 674 SPIONs (A) for 4h with and without Protamine under serum-free conditions and (B) for 24h
 675 in the absence of Protamine.



676

677 **Figure 5. Proliferation and viability of MSCs are not affected by A-PVA-SPION-**678 **labeling.** (A) Proliferation and (B) viability of A-PVA-SPION-labeled MSCs was assessed

679 after four and eight days.

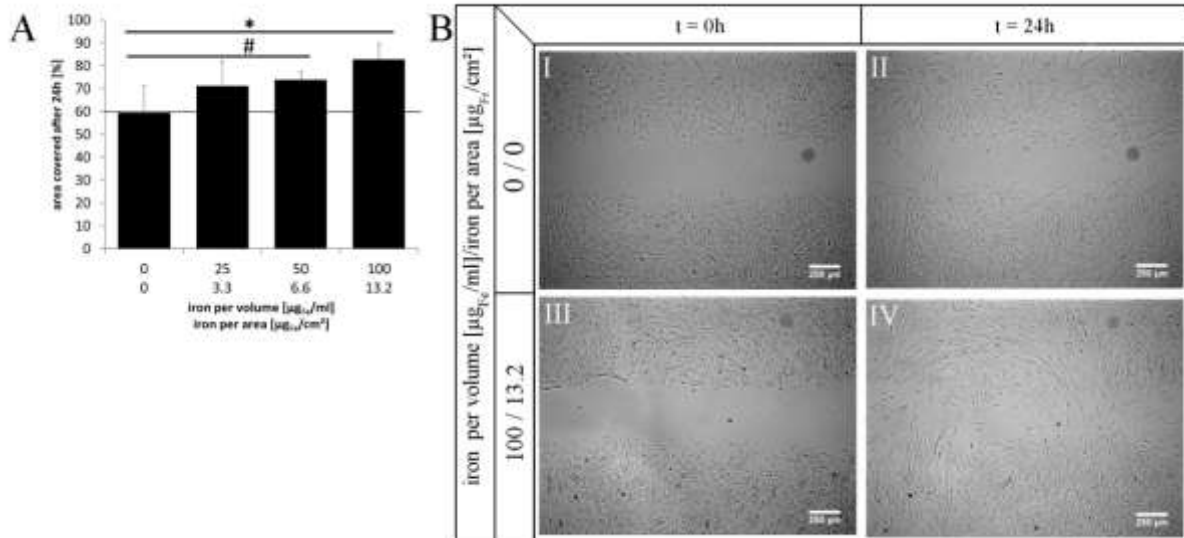


680

681 **Figure 6. Differentiation capacity of MSCs is not influenced by A-PVA-**
 682 **SPION-labeled MSCs were (A+C+E) quantitatively and (B+D+F) qualitatively investigated**
 683 **towards (A+B) adipogenic differentiation by Oil red staining(C+D) chondrogenic**
 684 **differentiation by proteoglycan assay and Alcian blue staining, and (E+F) osteogenic**
 685 **differentiation by Alizarin red staining.**

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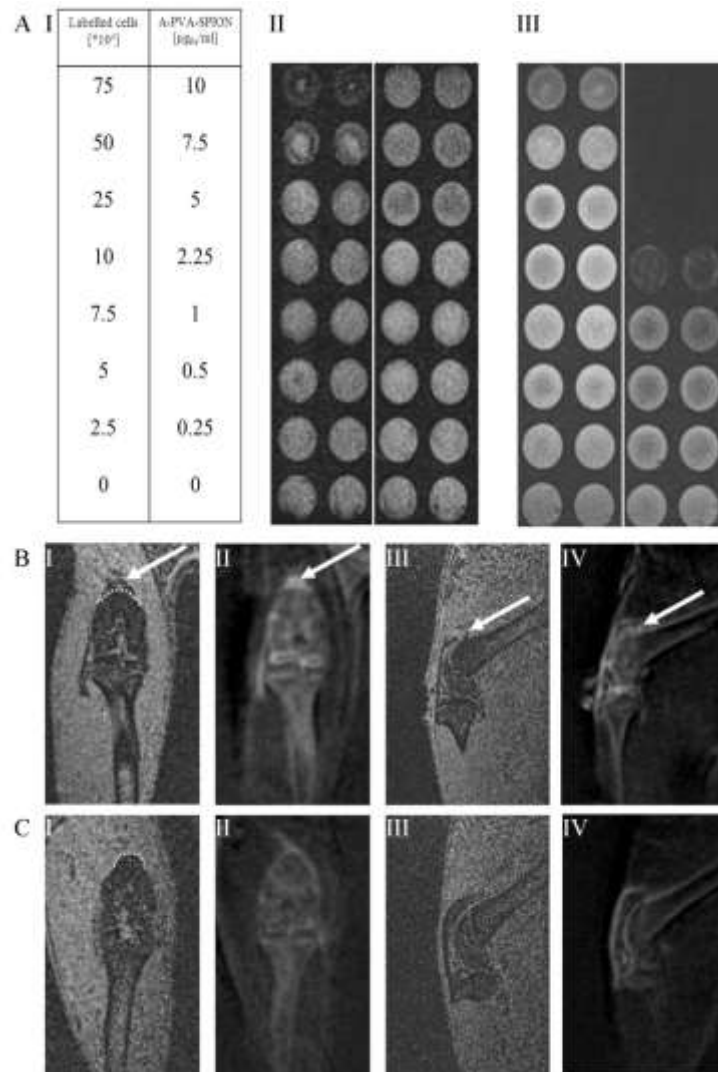


688

689 **Figure 7. Migration of MSCs is stimulated by A-PVA-SPION-labeling.** (A) Migration of
 690 A-PVA-SPION-labeled MSCs was investigated in a wound healing assay for 24h (ANOVA,
 691 Post Hoc Bonferoni; *, $p = 0.001$; #, $p = 0.069$). (B) Representative images of the wound
 692 healing gap from one donor are shown.

693

694



695

696 **Figure 8. Visualization of A-PVA-labeled MSCs in MRI *in vitro* and *in vivo*.** (A) A
 697 diagrammatic representation showing the layout and the different concentrations of A-PVA-
 698 SPIION labeled MSCs and SPIION alone used in the 24-well plate gel phantom study (I). T1
 699 weighted gradient echo MR images of the gel phantom (II) and T2 weighted (STIR) MR
 700 images of the same gel phantom (III). (B) Coronal (I, II) and sagittal (III, IV) views of rat
 701 knee joint injected with A-PVA-SPIION labeled MSCs and scanned *in vivo*. (C) Coronal (I, II)
 702 and sagittal (III, IV) views of rat knee joint injected with non labeled MSCs and scanned *in*
 703 *vivo*. Phantom was scanned using 1.5T MRI, rat knees were scanned *in vivo* using a 3T MRI.
 704 White arrow: A-PVA-SPIION labeled MSCs. Dotted white line: contour of the femoral
 705 diaphysis.

706

707 **Table 1. Physicochemical properties of A-PVA-SPIONs in different solvents**

Particles	Medium	Concentration (mg _{Fe} /mL)	γ Fe ₂ O ₃ crystal (nm)	Hydrodynamic diameter (nm)	Zeta potential (mV)	PVA/Fe ratio (mg _{PVA} /mg _{Fe})
SPION	HNO ₃ 10mM	10	7.2 ± 2.5	14 ± 2	+26 ± 2	0
PVA-SPION	HNO ₃ 10mM	5	7.2 ± 2.5	25 ± 3	+20 ± 2	9
PVA-SPION	DMEM	5	7.2 ± 2.5	42 ± 2	+21 ± 5	9
PVA-SPION	DMEM+FCS	5	7.2 ± 2.5	45 ± 2	-25 ± 5	9

708

709 **Table 2. Dosimetry**

administered dose							
iron per volume [μg _{Fe} /ml]			0	50	100	200	400
iron per area [μg _{Fe} /cm ²]			0	6.6	13.2	26.4	52.8
delivered dose							
iron per area [μg _{Fe} /cm ²]	4h		0	0.9	1.6	3.1	6.2
	24h		0	2.3	4.1	7.9	15.3
iron per cell [pg _{Fe} /cell]	4h		0	3	6	12	24
	24h		0	7.5	15	30	60
cellular dose							
iron per cell [pg _{Fe} /cell]	4h	0.4 ± 0.3	5.9 ± 2.5	6.5 ± 3.0	7.4 ± 2.2	6.6 ± 1.9	
	24h	0.7 ± 0.5	8.2 ± 3.6	9.5 ± 3.3	9.6 ± 5.7	10.8 ± 4.2	

710

711

712 Table of content

713

714 **Amino-polyvinyl alcohol coated superparamagnetic iron oxide nanoparticles (A-PVA-**
715 **SPIONs)** were used to label mesenchymal stromal cells (MSCs) for visualization in magnetic
716 resonance imaging. The A-PVA-SPIONs were non-toxic to MSCs and did not change their
717 differentiation potential. However, an increase in MSCs migration was observed. In
718 conclusion, labeling MSCs using A-PVA-SPIONs is feasible.

719

720 **Keyword:** mesenchymal stromal cells, superparamagnetic iron oxide nanoparticles,
721 polyvinylalcohol, magnetic resonance imaging, cell based therapies

722

723 Frank Schulze, Anke Dienelt, Sven Geissler, Paul Zaslansky, Janosch Schoon, Katja Henzler,
724 Peter Guttmann, Azza Gramoun, Lindsey A. Crowe, Lionel Maurizi, Jean-Paul Vallée,
725 Heinrich Hofmann, Georg N. Duda*, Andrea Ode

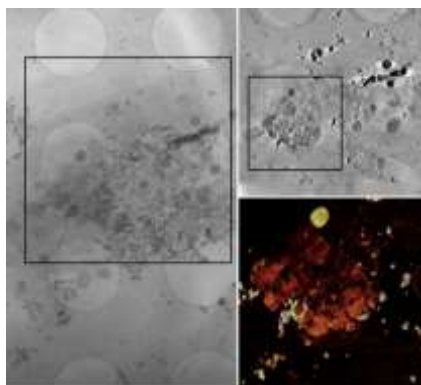
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727 **Title**

728 Amino-polyvinyl alcohol coated superparamagnetic iron oxide nanoparticles are suitable for
729 monitoring of human mesenchymal stromal cells *in vivo*

730

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732 high. Please do not use any other dimensions))



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