

Electron paramagnetic resonance as a sensitive tool to assess the iron oxide content in cells for MRI cell labeling studies

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ABSTRACT: MRI cell tracking is a promising technique to track various cell types (stem cells, tumor cells, etc.) in living animals. Usually, cells are incubated with iron oxides (T_2 contrast agent) in order to take up the particles before being injected *in vivo*. Iron oxide quantification is important in such studies for validating the labeling protocols and assessing the dilution of the particles with cell proliferation. We here propose to implement electron paramagnetic resonance (EPR) as a very sensitive method to quantify iron oxide concentration in cells. Iron oxide particles exhibit a unique EPR spectrum, which directly reflects the number of particles in a sample. In order to compare EPR with existing methods (Perls's Prussian blue reaction, ICP-MS and fluorimetry), we labeled tumor cells (melanoma and renal adenocarcinoma cell lines) and fibroblasts with fluorescent iron oxide particles, and determined the limits of detection of the different techniques. We show that EPR is a very sensitive technique and is specific for iron oxide quantification as measurements are not affected by endogenous iron. As a consequence, EPR is well adapted to perform *ex vivo* analysis of tissues after cell tracking experiments in order to confirm MRI results. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: EPR; cell labeling; iron oxide particles

1. INTRODUCTION

MRI cell tracking using iron oxide particles is a promising technique for assessing the fate of various cell lines after injection in a living organism. Numerous applications, such as stem cell follow-up in cell-based therapy (1–4) or cancer cells (5–7) in fundamental research, have been described. Intracellular iron quantification assays are essential for evaluating the initial iron oxide loading, optimizing the yield of iron uptake (8–10), and measuring the dilution of the iron oxide particles with cell proliferation (11). All these steps are crucial and can seriously affect cellular MRI studies. For example, iron quantification studies have shown that B16F10 melanoma labeled with MPIO lose all their iron content after three days (12). This experiment allowed prediction of the time window for tracking cells of interest by MRI. Eventually, a quantification method able to discriminate exogenous iron oxide particles from endogenous iron should be particularly interesting for *ex vivo* analysis of organs after cellular MRI studies in order to confirm previous MRI results.

Until now, the two main techniques used for assessing intracellular iron oxide content are spectrophotometric techniques (especially based on Perls' Prussian blue reaction) and inductively coupled plasma mass spectroscopy (ICP-MS) (13). The first one has the advantage of being implementable at low cost but may suffer from a lack of sensitivity whereas ICP-MS is expensive but highly sensitive. Perls' reaction-based colorimetry and ICP-MS measure both endogenous and exogenous iron, meaning that these two methods are not suitable for *ex vivo* analysis as described above. Many other iron oxide

particles are also grafted with fluorophores (for example MPIO commercialized by Bangs or VSOP from Ferropharm). Fluorescence of such particles is mainly used in microscopy to address the localization of the particles in the cells or tissues (6,14), but can also be used for quantifying indirectly the iron content (using fluorimetry). All these techniques are summarized in Fig. 1.

Electron paramagnetic resonance (EPR) is a spectrometric technique which is used to study free radicals and (super) paramagnetic molecules. A very important characteristic of EPR is its high sensitivity (owing to the gyromagnetic ratio

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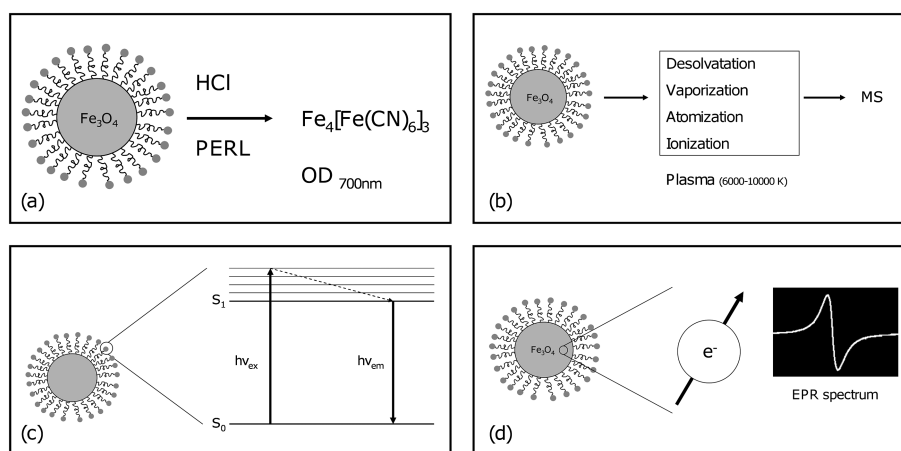


Figure 1. Methods used to quantify iron oxides in cell labeling. Perls' reaction-based colorimetry (a) and ICP-MS (b) measure the total iron content in the sample, while fluorimetry (c) requires the presence of a grafted fluorophore on the coating of the particle. Electron paramagnetic resonance is specific for the iron oxide core of the particles (d).

of the electronic spin which is much higher than that of proton). Iron oxides are superparamagnetic and present a typical EPR spectrum as represented in Fig. 2. Previously, EPR has already been described for studying iron oxide particles, mainly to characterize their physicochemical properties (15,16) or for SPIO distribution studies in tissues after systemic injection (17–19). This technique was also proposed by Wilhelm *et al.* for iron oxide quantification in macrophages (20). The goals of the present study were (i) to develop EPR for cell labeling experiments, and (ii) to compare the performances of EPR with the three classical methods (Perls' reaction-based colorimetry, ICP-MS and fluorimetry).

2. RESULTS AND DISCUSSION

2.1. Labeling Localization

Magnetic labeling using Molday ion rhodamine B (MIRB) of three cell lines (B16F10-luc melanoma, RENCA-luc renal adenocarcinoma and 3T3 fibroblasts) was assessed using fluorescence microscopy in order to confirm that further iron quantification studies correctly reflect intracellular iron content. Microscopy is essential to avoid any iron oxide quantification bias (particles adhering to plasma membranes or plates). All

cell lines incorporated MIRB particles efficiently (Fig. 3), with iron oxide particles localized in endosomes in the cytosol of the cells. Using microscopy, the labeling yield appeared to be higher for 3T3 cells than for RENCA-luc and B16F10-luc cells. The influence of serum deprivation was also evaluated. No clear effect of serum on the uptake yield was evidenced on the basis of microscopy observations, except for B16F10-luc, which incorporated fewer particles when the cells were incubated with MIRB particles in the presence compared with in the absence of serum.

2.2. Limit of Detection and Limit of Quantification

Several calibration curves were performed to determine the sensitivity of the different techniques (EPR and the pre-existing methods). The sensitivity of ICP-MS was not assessed since it is already well known that this technique is highly sensitive (detection limit lower than 1 ppm) (13). Limits of detection (LOD) and quantification (LOQ) were calculated using the following formulas: $LOD = 3SD/a$ and $LOQ = 10SD/a$ (where SD is the standard deviation and a is the slope of the calibration curves). As shown in Fig. 4, a linear relationship between optical density and iron concentration was observed for Perls' reaction-based colorimetry technique. However, the Lambert–Beer relationship was not preserved at lower concentration. This phenomenon has already been observed in a previous study (13) and was attributed to solubilization of the Prussian blue colloid at low concentration. For LOD and LOQ determination, a threshold value of $0.5 \mu\text{g Fe ml}^{-1}$ (beginning of linearity) was added to the previous formula. Therefore the LOD and LOQ for the Perls' reaction-based colorimetric technique were respectively 0.676 and $1.088 \mu\text{g Fe ml}^{-1}$.

Concerning fluorimetry, the emission of fluorescence from rhodamine B grafted to the MIRB particles was measured. The LOD and LOQ for this technique were 0.407 and $1.357 \mu\text{g Fe ml}^{-1}$. Finally, the EPR LOD and LOQ were, respectively, 91 and $304 \text{ ng Fe ml}^{-1}$. It is important to note that the volumes used for EPR experiments were low ($75 \mu\text{l}$), meaning that we were able to detect quantities of iron oxide as low as 6.8 ng. All LOD and LOQ of all techniques are summarized in Table 1.

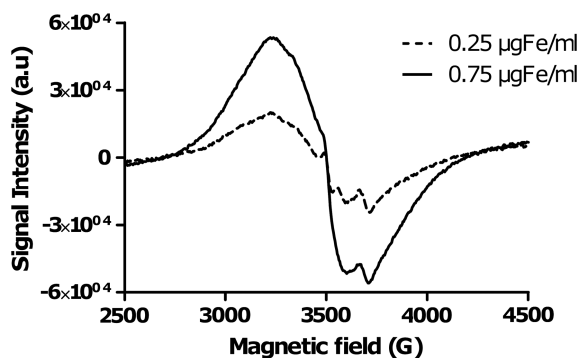


Figure 2. Typical electron paramagnetic resonance spectra of iron oxide (Molday ion rhodamine B particles) recorded at two different concentrations.

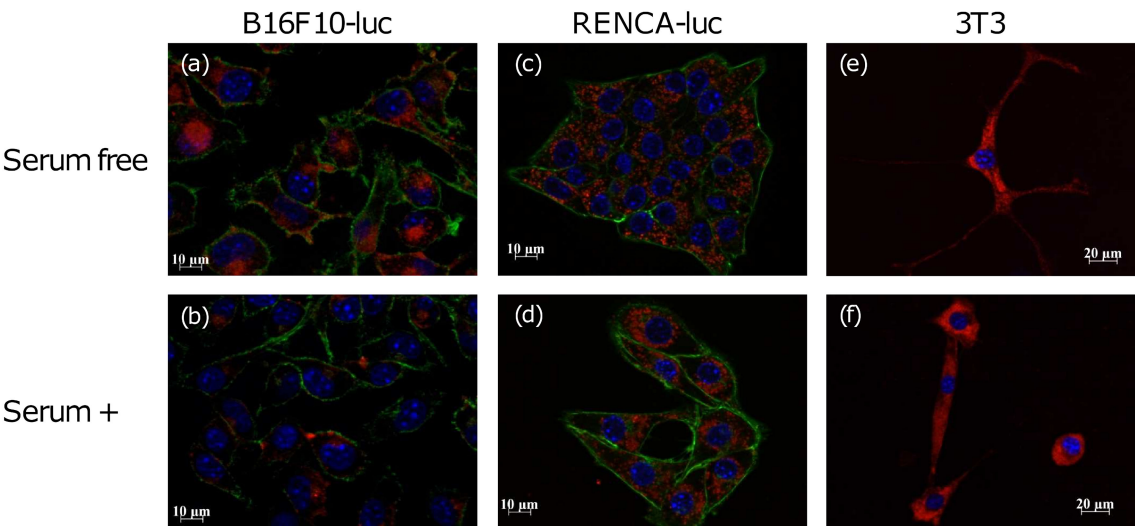


Figure 3. Microscopic images of cells labeled with Molday ion rhodamine B particles (MIRB; 100 $\mu\text{g Fe}/2\text{ ml}/9.6\text{ cm}^2$). The upper panels show the cells after overnight incubation in a serum-free medium and bottom panels after overnight incubation in the presence of serum. Three different cell lines were used: B16F10-luc melanoma cells (a, b), Renca-luc renal carcinoma cells (c, d), 3 T3 fibroblasts (e, f). Membranes are in green (phalloidin-FITC, not used for fibroblasts), nuclei in blue (Hoechst 33342), and MIRB particles (rhodamine B) in red.

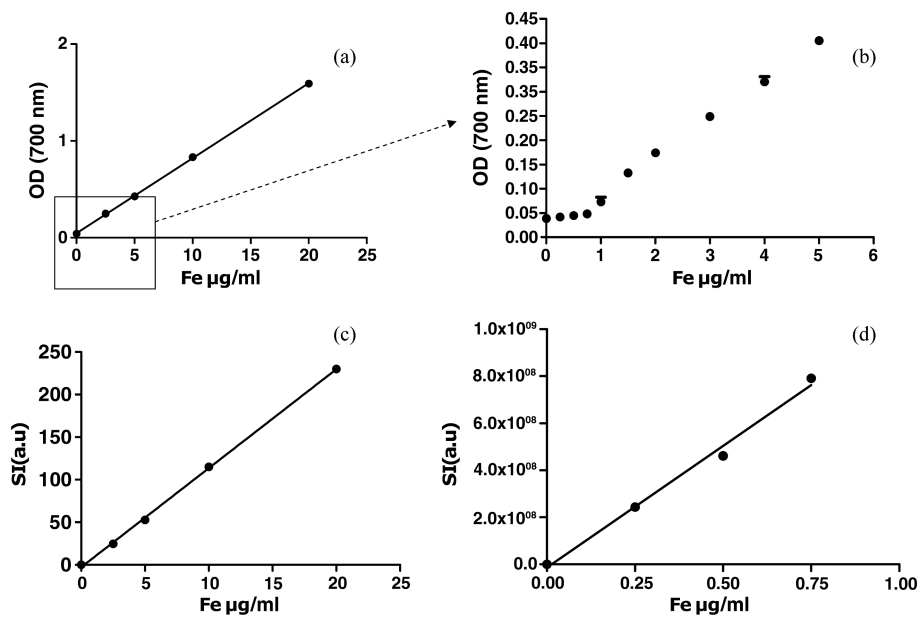


Figure 4. Calibration curves obtained for Perls' reaction-based colorimetry (a, b), fluorimetry (c) and electron paramagnetic resonance (EPR) (d). Note the differences of range in the x-axis (concentration), reflecting the difference in sensitivity of the methods. The highest sensitivity was found using EPR spectroscopy (c). Note also the absence of linearity at low concentrations using Perls' reaction-based colorimetry (b) (mean $\pm$ SEM, $n = 3$ for b).

Table 1. Limits of detection and quantification (LOD and LOQ) of the different techniques		
Method	LOD	LOQ
Electron paramagnetic resonance	91 ng Fe ml ⁻¹	304 ng Fe ml ⁻¹
Perls' reaction-based colorimetry	0.676 $\mu\text{g Fe ml}^{-1}$	1.088 $\mu\text{g Fe ml}^{-1}$
Fluorimetry	0.407 $\mu\text{g Fe ml}^{-1}$	1.357 $\mu\text{g Fe ml}^{-1}$ (!Auto-fluorescence)
ICP-MS	<1 ppm (not assessed) (13)	Not assessed

2.3. Comparison of Iron Quantification Methods

To determine the reliability of EPR as a quantitative tool for measuring the content of iron oxide incorporated into cells, we compared the results obtained using EPR vs other methods above the LOQ of all methods where endogenous iron cannot interfere with the measurement. For this purpose, 3T3 cells were incubated overnight with full medium in the presence of MIRB particles ($28.5 \mu\text{g Fe}/2 \text{ ml}/9.6 \text{ cm}^2$). The next day, labeled cells were collected and intracellular iron content was measured with the four methods. Figure 5 shows that the average iron content per cell did not differ with the method used. Note that fluorimetry tended to slightly underestimate the amount of particles in cells. This was probably due to auto-fluorescence of cell preparations, as the values presented are corrected for the fluorescence measured in the absence of the particles.

2.4. Illustrative Application: Kinetics of Iron Oxide Uptake by Different Cell Lines

Kinetic experiments of MIRB uptake were performed in order to check whether EPR could be a useful method for optimizing labeling protocols. As shown in Fig. 6, B16F10-luc cells incorporated up to 7 pg Fe per cell while RENCA-luc cells incorporated 10–13 pg Fe per cell. Two interesting features are also displayed in this figure: the presence of serum dramatically decreased the yield of labeling and a plateau was observed after 24 h. The influence of serum was not so clear with fluorescence microscopy results, and this observation highlights the relevance of using quantitative and sensitive techniques as EPR for setting up the correct parameters for cell labeling procedures. In this case, the absence of serum is important to improve the yield of labeling and an overnight incubation in the presence of MIRB particles was found sufficient to obtain a maximal incorporation of iron oxide particles.

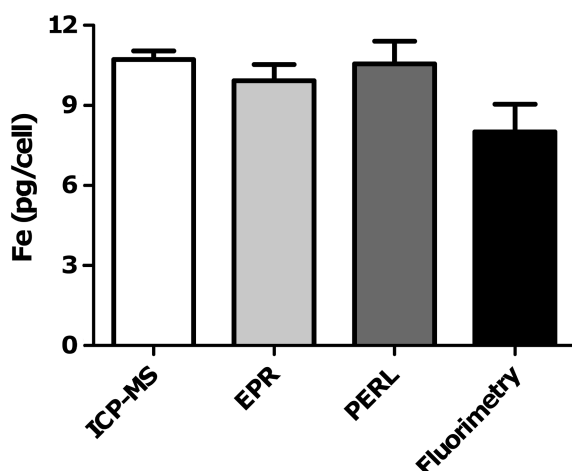


Figure 5. Comparison of techniques as tools for the quantification of iron oxide uptake in 3T3 cells. The techniques compared were ICP-MS, electron paramagnetic resonance, Perl's reaction-based colorimetry and fluorimetry. Bar graphs are expressed as picograms per cell (mean $\pm$ SEM, $n=3$ for a–c; $n=6$ for d). A Kruskal–Wallis test showed no statistical differences between the four methods ($p > 0.05$).

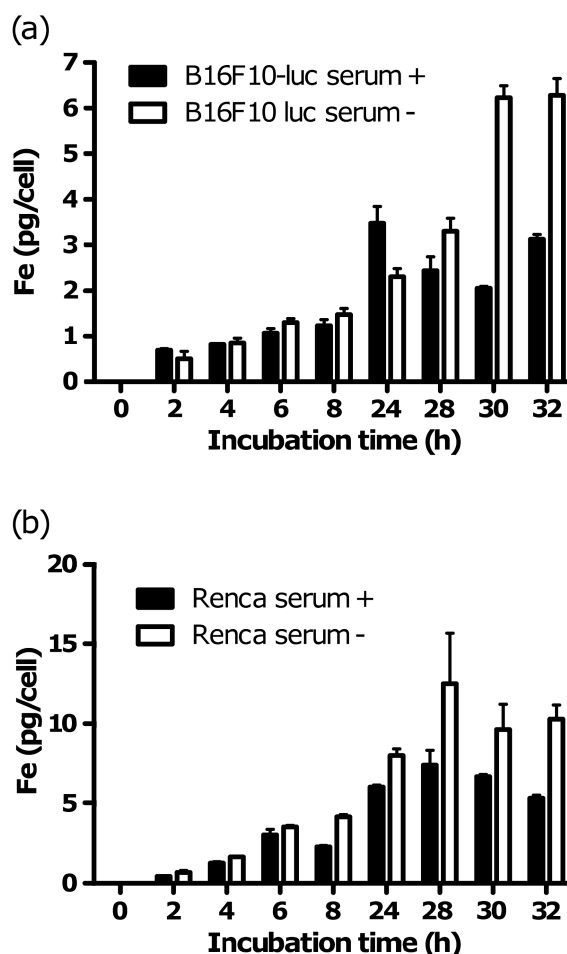


Figure 6. Kinetics of Molday ion rhodamine B uptake in B16F10-luc cells (a) and RENCA-luc cells (b). The experiments were carried out in serum or serum-free conditions. The iron oxide content of cells was estimated using electron paramagnetic resonance spectroscopy. Note the important influence of serum on particle uptake and on the plateau reached after 24 h of incubation (mean $\pm$ SEM, in triplicate).

3. CONCLUSIONS

We have evidenced in this present study that EPR is a highly sensitive technique for iron quantification for cell labeling studies when compared with all other available techniques: the limit of detection of EPR is about 7.5 times higher than that of the Perl's reaction-based colorimetry technique. This high sensitivity allows the quantification of cells labeled with iron oxides in very small volumes ($75 \mu\text{l}$). The EPR method also allows easy optimization of cell labeling protocols. Microscopy will of course remain essential to confirm that iron oxides are well localized in the cytosol and do not merely adhere to plasma membranes.

Although other techniques are also very sensitive (e.g. ICP-MS), EPR exhibits a major advantage, the intrinsic specificity for iron oxide compared with iron species contained in biofluids, which are not superparamagnetic. As a consequence, *ex vivo* measurements of tissue samples could be performed after MRI studies. Such experiments could be highly relevant for discriminating hypointense areas owing to artifacts (tissue interfaces, presence of air) and hypointense

regions owing to the labeled cells (13,21). Therefore, EPR could provide an important contribution to an unambiguous assessment of iron oxide content in tissues in MRI cell-tracking experiments.

4. EXPERIMENTAL

4.1. Cell Culture

Murine melanoma B16F10-luc were grown in Dulbecco's modified Eagle medium, with GlutaMAX™ I, 1000 mg l⁻¹ D-glucose, sodium pyruvate (Invitrogen, Merelbeke, Belgium), supplemented with 10% fetal bovine serum (Invitrogen, Merelbeke, Belgium) and 1% (v/v) penicillin–streptomycin (Invitrogen, Merelbeke, Belgium). Murine renal adenocarcinoma RENCA-luc were grown in RPMI-1640 medium with L-glutamine (Invitrogen, Merelbeke, Belgium), supplemented with additional L-glutamine 2 mM (Invitrogen, Merelbeke, Belgium), 10% (v/v) fetal bovine serum, nonessential amino acids 0.1 mM (Invitrogen, Merelbeke, Belgium), additional sodium pyruvate 1 mM (Invitrogen, Merelbeke, Belgium) and 1% (v/v) penicillin–streptomycin. Murine 3T3 fibroblasts were grown in Dulbecco's modified Eagle medium, with L-glutamine, 4500 mg l⁻¹ D-glucose and 25 mM HEPES buffer, without sodium pyruvate (Invitrogen, Merelbeke, Belgium), supplemented with 10% (v/v) heat-inactivated newborn calf serum (Invitrogen, Merelbeke, Belgium), sodium pyruvate 1 mM and 1% (v/v) penicillin–streptomycin.

4.2. MIRB Localization After Cell Labeling

3T3, B16F10-luc and Renca-luc cells were seeded at low concentration on sterile glass coverslips. Cells were then incubated with Molday ion rhodamine B particles (Biopal, Worcester, USA) at 100 µg Fe/2 ml/9.6 cm² in medium overnight. These particles are especially designed for cell labeling experiments and were chosen because of their good uptake by cells and their lack of toxicity (14). After the incubation, cells were rinsed three times with phosphate-buffered saline (PBS; Lonza, Braine-l'Alleud, Belgium), fixed with paraformaldehyde 4% (Sigma-Aldrich, Bornem, Belgium), and washed three times with PBS containing 0.1% Triton for membrane permeabilization. Actin was stained using phalloidin-fluorescein isothiocyanate (FITC) (0.5 mg ml⁻¹ in methanol, diluted 1:250 in PBS; Sigma-Aldrich, Bornem, Belgium). After three new washes with PBS, nuclei were stained using Hoechst 33342 (0.02 g in 10 ml distilled water, diluted 1:10 000; Sigma-Aldrich, Bornem, Belgium). Coverslips were then mounted using Vectashield H1000 (Vector Labs, Brussels, Belgium) and slides were analyzed using a fluorescence microscope Zeiss Axio Imager equipped with an ApoTome module (pseudo-confocal).

4.3. Sample Preparation for Iron Quantification

For the experiment comparing the methods, 3T3 cells were seeded one day before the experiment (10 000 cell cm⁻²) in six-well plates (three wells per method; Sarsedt, Essen, Belgium). At day 0, the medium was removed and replaced by a mix solution containing full medium and MIRB particles (28.5 µg Fe/2 ml/9.6 cm²) for an overnight incubation. Cells were washed four times with PBS the next day, gently harvested with 1 ml of trypsin EDTA 0.05% (Invitrogen, Merelbeke, Belgium) and neutralized with 1 ml of full medium. Cells were centrifuged at 180g for 5 min and resuspended in 500 µl cold PBS in a 1.5 ml Eppendorf cup. A 10 µl

aliquot of the cell suspension was collected and mixed with 10 µl of trypan blue (Sigma-Aldrich, Bornem, Belgium) for cell counting using a hemocytometer. In the mean time, the cell suspension was centrifuged at 160g for 3 min. The 3T3 fibroblasts were eventually resuspended in 200 µl PBS for EPR analysis, in 200 µl HCl 5 M for Perls' reaction-based colorimetry, in 1 ml PBS for fluorimetry and 500 µl PBS for ICP-MS.

For the kinetics of MIRB uptake, Renca-luc cells (15 000 cells cm⁻²) and B16F10-luc (10000 cells cm⁻²) were seeded the day prior to the experiment in 60 mm tissue dishes (Sarsedt, Essen, Belgium). The next day, the medium was removed and cells were washed with PBS to remove traces of serum; then 4 ml of a final solution containing full or serum free medium and MIRB particles (80 µg Fe/4 ml/28 cm²) was added. Cells were collected at each time point using the same protocol as the methods comparison protocol except that the cells were finally resuspended in 300 µl of PBS. EPR measurements were done in triplicate.

4.3.1. Perls' reaction-based colorimetry

The sample resuspended in 200 µl HCl was digested for 24 h at 37 °C in closed Eppendorfs with regular agitation. After that the solution was mixed with 100 µl of distilled water containing 5% potassium ferrocyanide (Sigma-Aldrich, Bornem, Belgium), the Perls' reagent. The optical density at 700 nm (OD₇₀₀) was measured 15 min after addition of the Perls reagent in a 96-well plate (200 µl per well) using a Molecular Devices SpectraMax 190 microplate reader. Calibration curves were performed with different MIRB dilutions (from 20 to 0 µg Fe ml⁻¹) in HCl 5 M mixed with ferrocyanide 5% solution (ratio 2:1).

4.3.2. Electron paramagnetic resonance

Cells resuspended in PBS were drawn into a 75 µl glass capillary tubes. Samples were analyzed at room temperature using a Bruker EMX EPR spectrometer operating at 9 GHz (Bruker Biospin GmbH, Germany) with the typical parameters: 3 mT modulation amplitude, 10.11 mW power, 325.1 mT center field, and field 400 mT sweep width. Signal intensity was recorded using a double integration of the iron oxides EPR spectra. Calibration curves were realized in the same conditions with MIRB solutions at different concentrations (from 20 to 0 µg Fe ml⁻¹).

4.3.3. Fluorimetry

Samples in 1 ml PBS were analyzed using PerkinElmer LS 55 with quartz cuvettes. MIRB emission spectra were recorded with the following parameters: 555 nm excitation, maximum emission at 578 nm and 9 nm slit. MIRB calibration curves (from 20 to 0 µg Fe ml⁻¹) were realized in the same conditions.

4.3.4. ICP-MS

The samples were analyzed by inductively coupled argon plasma with mass spectrometry using an Agilent 7500ce instrument. Briefly, samples (50 µl) were diluted quantitatively (1:100) with a HNO₃ 1%, HCl 0.5% solution containing all internal standards (Sc, Ge, Rh and Ir). Fe was quantified using

helium mode (collision cell) and its isotope at m/z 56 with Ge (at m/z 74) as internal standard.

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