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Quantitative ferromagnetic resonance analysis of *CD*133 stem cells labeled with iron oxide nanoparticles

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Abstract

The aim of this work is to provide a quantitative method for analysis of the concentration of superparamagnetic iron oxide nanoparticles (SPION), determined by means of ferromagnetic resonance (FMR), with the nanoparticles coupled to a specific antibody (*AC*133), and thus to express the antigenic labeling evidence for the stem cells $CD133^+$. The FMR efficiency and sensitivity were proven adequate for detecting and quantifying the low amounts of iron content in the $CD133^+$ cells ($\sim 6.16 \times 10^5$ pg in the volume of 2 μl containing 4.5×10^{11} SPION). The quantitative method led to the result of 1.70×10^{-13} mol of Fe (9.5 pg), or 7.0×10^6 nanoparticles per cell. For the quantification analysis via the FMR technique it was necessary to carry out a preliminary quantitative visualization of iron oxide-labeled cells in order to ensure that the nanoparticles coupled to the antibodies are indeed tied to the antigen at the stem cell surface and that the cellular morphology was conserved, as proof of the validity of this method. The quantitative analysis by means of FMR is necessary for determining the signal intensity for the study of molecular imaging by means of magnetic resonance imaging (MRI).

1. Introduction

The use of superparamagnetic iron oxide nanoparticles (SPION), coupled to a specific antibody, has been successfully applied to label antigenic probes in molecular imaging, especially magnetic resonance imaging (MRI) [1–3].

The cell in culture labeling with SPION, together with MRI, provides a non-invasive method for study of the destinations of cells transplanted *in vivo* [4, 5]. The cells may be labeled using contrast agents on the basis on nanoparticles, during their incubation in a culture, before their transplantation into tissue.

Superparamagnetic (magnetic fluid) contrast agents with a superparamagnetic core consisted of crystalline structures based on iron oxide, described by the general formula $Fe_2^{3+}O_3M^{2+}O$, where M^{2+} is a divalent metal ion such as iron, manganese, nickel, cobalt or magnesium. For the synthesis of the contrast agents, small $Fe_2^{3+}O_3Fe^{2+}O$ (magnetite) crystals are mainly employed [6]. Preparatory processes are of paramount importance—before the acquisition of molecular images based on MRI of the stem cells labeled with SPION. The process can be divided into the following parts: quantitative evaluation and qualitative expression of the antigens [3, 7], labeling efficiency [3, 8, 9], SPION toxicity [8–10], cell viability [3, 9], proliferation [3, 8, 9] and differentiation assays [8], qualitative visualization of iron oxide-labeled cells [3], iron content quantitative analysis [11, 12], among others.

There are several techniques for performing iron content quantitative analyses [11, 12], such as that using ferromagnetic resonance (FMR) which is the electron paramagnetic resonance (EPR) of small ferromagnetic particles. The only difference is that the electron spins interact among themselves in the lattice. This leads to a ferromagnetic or ferrimagnetic order in the nanoparticles, assumed to be composed of magnetic monodomains and having nearly spherical shape. Thus, the total magnetic momentum of each nanoparticle is precessing over the direction of the total static field, which is the sum of the external static field, the internal contribution of the domain magnetization and the anisotropic magnetic field of the local lattice [13]. The magnetic fluids based on SPION present physical mechanisms that are essentially the same for ferromagnetic solids and magnetic suspensions. However, in the ferrofluids the FMR is affected considerably by two specific characteristics. The first one stems from the smallness of the particles, and imparts a fluctuational component to the magnetic moment motion. The second originates from mechanical mobility of the particles and results in a change of their anisotropy axis distribution under the influence of the external fields [14].

For the molecular imaging purposes, a great deal of interest has been focused on $CD133$ stem cell labeling. This antigen may be expressed in a variety of tissues including the kidney, pancreas, placenta, fetal liver [15], skeletal muscles and human neural tissue. This vast number of tissues suggests an equal number of possible clinical applications, including, among other interesting possibilities, the utilization of the progenitor stem cells $CD133^+$ in tissue engineering. The antigen $CD133$ is an integral glycoprotein of a 97 kDa membrane that belongs to a molecular family of proteins 5-TM [15, 16]. In the human body, the monoclonal antibodies $AC133$ may be bonded together in different epitopes, but they were originally demonstrated to react with a cellular surface antigen expressed in human stem cells and in various cellular progenitors, including those derived from the hematopoietic system [15].

The aim of the present work is the quantitative analysis of the SPION (Fe_3O_4) concentration by means of FMR, where the nanoparticles are coupled to a specific monoclonal antibody ($AC133$) expressing the antigenic labeling evidence of the stem cells $CD133^+$ of the human blood and umbilical cord. The study is completed using the techniques of flow cytometry and transmission electron microscopy (TEM).

We first carried out a study to determine whether the cells were actually expressing the trans-membrane glycoprotein antigen $CD133$, selected by affinity chromatography. The second point was to establish the efficiency of the selection procedure. The TEM analysis was used to detect the presence of antibodies coupled with SPION attached on the cellular membrane.

2. Materials and methods

The $CD133$ cell labeling was achieved by an *in vitro* protocol using the monoclonal antibody anti-$CD133$ coupled to magnetic beads composed of SPION—Fe_3O_4 (Miltenyi Biotec). These nanoparticles (average diameter of 9.0 ± 0.3 nm) are found in a colloidal suspension of a ferrofluid, or magnetic fluid with the iron content of 200 μg ml^{-1}. Umbilical cord blood was obtained from volunteer donors ($n = 5$) after the registering of their written consent (CEP-IEPAE No. 105/02). Mononuclear cells were purified by density gradient centrifugation (Ficoll-Paque™ Plus (GE Healthcare)), according to a modified method published previously [17]. The $CD133^+$ cell population was purified using anti-$CD133$ mAb-coupled magnetic beads (Miltenyi Biotec) according to the manufacture's instructions.

After $CD133^+$ cell separation, the cell population was characterized by flow cytometry using the following monoclonal antibodies (Becton Dickinson, San Jose, CA, and Miltenyi Biotec): $CD34$ (clone: 581) FITC-conjugated, $CD45$ (clone: 2D1) PerCP Cy-5.5-conjugated and $CD133/2$ (clone: $AC141$) APC-conjugated and the respective isotype controls $IgG1$ FITC-conjugated, $IgG1$ PerCP Cy-5.5-conjugated and $IgG1$ APC-conjugated.

Cells were incubated with antibodies at 4 °C, in the dark for 30 min, and then washed with PBS and fixed with 1% paraformaldehyde. A total of 105 fluorescent cellular events were acquired in the FACSARIA flow cytometry (BD Bioscience) and analyzed using FACSDIVA software. Briefly, the analysis was performed by gating the cell population for forward scatter (FSC) versus side scatter (SSC) followed by gating only the $CD45^+$ cells. Within the $CD45^+$ cell population, cells were analyzed for expression of $CD34$ and $CD133$ markers.

After $CD133$ cell separation, the cell population was fixed in 2.5% glutaraldehyde in 0.2 M cacodylate buffer for 2 h at 4 °C. Later the routine procedure for TEM was carried out, including washes, post-fixation, contrasting, dehydration and inclusion in pure resin until complete polymerization was achieved. Semithin and ultrathin sections were obtained with the aid of a Porter Blum ultramicrotome. The ultrathin sections were placed on copper grids and photographed using a transmission electron microscope, PHILIPS $CM100$.

The quantification of the average iron content per cell, expressed as the average number of SPION per cell, was attained by means of the technique of FMR. The characteristic FMR of a ferrofluid compound, containing magnetite particles, is observed as a broad line at about $g = 2.1$. Since the resonance spectrum is recorded as the derivative of absorption, the number of resonant spins is proportional to the double integral of the signal, yielding the area under the absorption curve, measured over increasing values of the applied magnetic field, sweeping the complete interval where the resonance occurs. The constant of proportionality is determined using the calibration curve, constructed by weighing known amounts of ferrofluid which are directly correlated with the FMR signal intensities equal to the areas under the absorption curves, expressed in arbitrary units.

The calibration curve shown in figure 1 was constructed using different concentrations of the commercial ferrofluid Endorem (Endorem™—Guerbert; earlier trade name AMI-25, Laboratoire Guerbert, France). The concentrations covered a range of 2.6 μM–0.6 mM contained in the volume of 2 μl.

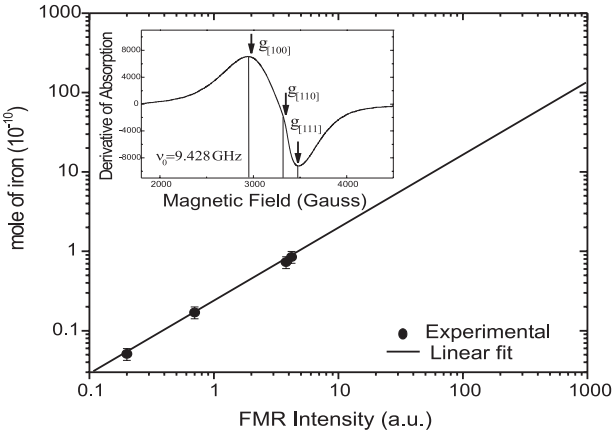

Figure 1. Calibration curves of the FMR of the nanoparticle concentration related to the area under the $g = 2.1$ resonance absorption curve. The inset shows a typical FMR spectrum of the $g = 2.1$ line of magnetite contained in the ferrofluid Endorem™ used in the data acquisition for the building of the curve calibration.

A typical FMR spectrum of the commercial ferrofluid is shown in the inset of figure 1.

The FMR line intensity was determined from the area under the absorption line (double integral of the resonance at $g = 2.1$), which is proportional to the iron particle concentration per mm^3. This calibration curve is shown in figure 1 (solid line). The FMR derivative of the absorption spectrum is used to calculate the calibration curve, obtained at room temperature (inset in figure 1). The absorption lineshape is typical of a convoluted cubic crystal powder pattern of the FMR of fine-grained precipitates of ferromagnetic or ferrimagnetic single domains [18], also seen when iron ion (Fe^{3+} and Fe^{2+}) dimers and/or clusters are precipitated in glasses [19]. From this curve a positive first-order magnetocrystalline anisotropy constant $K_1 = (1.2 \pm 0.2) \times 10^5$ erg cm^{-3} was determined from the relation $H_a = 2K_1/M_s$, taking into account that the nanoparticles are of magnetite. M_s is the maximum saturation magnetization of the crystal, equal to $H_s/(4\pi/3)$ erg G^{-1} cm^{-3} and $H_s \approx 2$ kG for magnetite. The nanoparticles were assumed to be monodomains of spherical shape. From the maximum (H_{max}), minimum (H_{min}) wings and maximum negative slope (H_{ms}) of the FMR spectrum, respectively, the effective g-values are $g_{[100]} = 2.28 \pm 0.01$, $g_{[110]} = 2.02 \pm 0.02$ and $g_{[111]} = 1.94 \pm 0.01$. The value of H_a is obtained from the field separation between the positions of H_{max} and H_{min} wings (equal to $(5/3)H_a$). From $H_0 = H_{max} - (2/3)H_a$ the value of $g_0 = (h\nu/(\beta H_0)) = 2.12 \pm 0.02$ is obtained, where $\nu = 9.428$ GHz, h is Planck's constant, ν is the spectrometer frequency and β is the Bohr magnetron.

The derivative of the absorption of the FMR was obtained using a Bruker EMX homodyne spectrometer, operating in the X band, at the frequency of 9.428 GHz, using a rectangular TE_{102} cavity with 100 kHz modulation.

3. Results and discussion

In our sample 98% of the cells were expressed by $CD45$, which characterizes their hematopoietic source (data not shown). After the selection of $CD45^+$ events, the expression of $CD34$ (progenitor marker) was analyzed; it was found that 82% of all $CD133^+$ selected cells also express $CD34$ (figure 2(A)) indicating their progenitor phenotype. The specificity of the test was considered adequate from the negative result found in the isotype control (figure 2(B)). The efficiency of $CD133$ selection after flow cytometry analyses was 83.6%.

The ultrastructural analysis highlighted the presence of electron dense granules in the cell surface. This demonstrates the presence of $AC133$ monoclonal antibodies bound to SPION expressed in the cell membrane ($CD133^+$; figures 3(A), (B), (E)) which does not occur in the cells of the control group (figure 3(G)), since these cells do not express the antigen $CD133$ ($CD133^-$).

The $CD133^+$ cells have a round morphology and an active nucleus that occupies almost the whole cell (figures 3(A) and (B)).

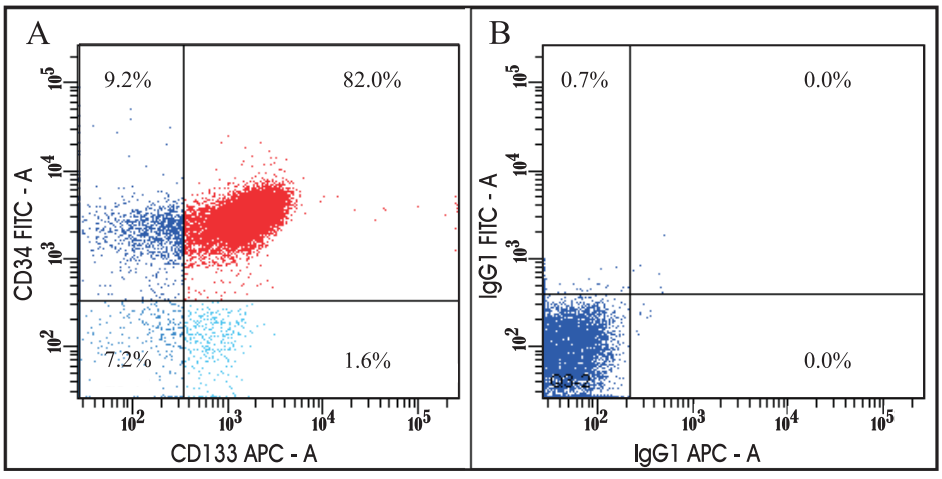

Figure 2. (A) The flow cytometry graph shows $CD133^+/CD34^+$ (82.0%), $CD133^-/CD34^+$ (1.6%) and $CD34^+CD133^-$ (9.2%). (B) The isotype control graph shows that there was no non-specific staining.
(This figure is in colour only in the electronic version)

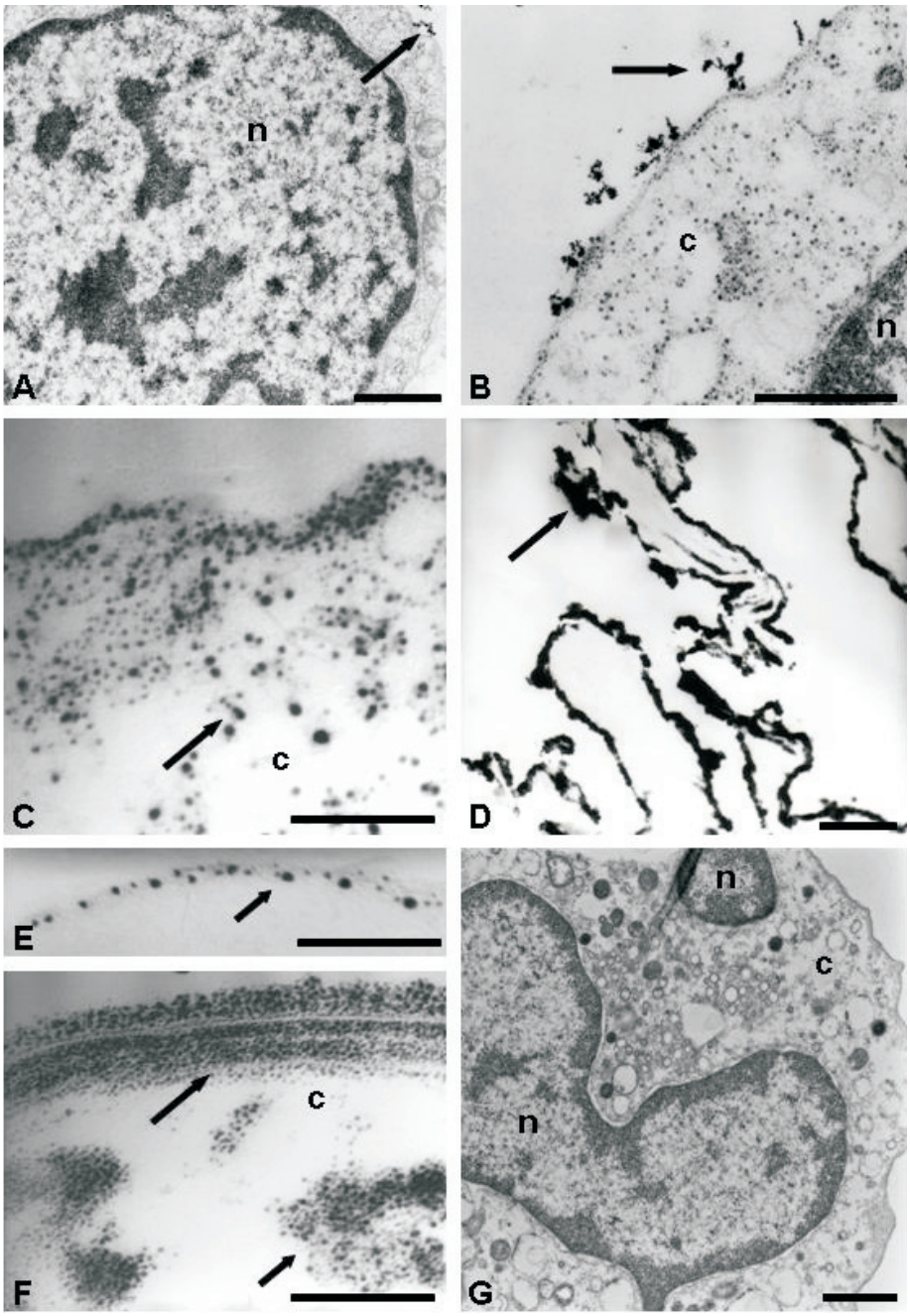

Figure 3. ((A)–(F)) TEM of $CD133^{+}$ stem cells. (G) TEM of $CD133^{-}$ stem cells (control). n = nucleus, c = cytoplasm, arrow = electron dense granules. Scale: ((A), (G)) 1 μm; ((B), (C), (E), (F)) 0.25 μm; (D) 0.5 μm.

The SPION were also observed in the cell cytoplasm (figure 3(C)). These nanoparticles bound to the antibodies expressed in the cellular membrane were more probably incorporated into the cell through the endocytosis process (pinocytosis). They can be visualized using TEM as electron dense particles (figure 3(C)).

Preliminary experiments performed by our group showed a strong marking, electron dense in the periphery of the cellular content, but the morphology of cell $CD133^{+}$ was not preserved (figure 3(D)), probably due to insufficient fixation of the material for 2.5% glutaraldehyde in 0.2 M cacodylate buffer.

The TEM also revealed an excess of nanoparticles electron dense in the cellular membrane, as well as in the cytoplasm (figures 3(C), (F)).

First, we obtained the FMR spectrum only for the magnetic beads (see figure 4) of the antigenic label, i.e., monoclonal antibody anti-$CD133$, coupled to the SPION (Miltenyi Biotec). The resonance was observed at $g = 2.1$, indicating the presence of multiple Fe^{3+} spins interacting and showing a superparamagnetic behavior [20, 21], which characterizes the presence of agglomerates. This signal consists of a strong absorption, broadened by the exchange interaction between the Fe^{3+} spins. A control sample with

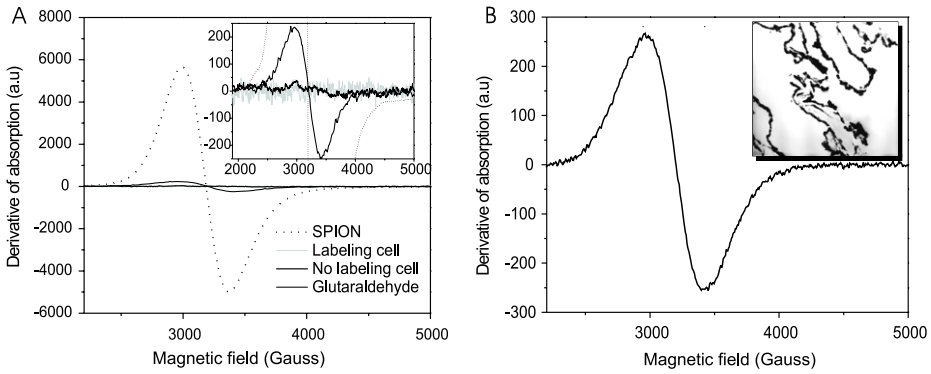

Figure 4. (A) FMR spectra, showing the derivative of the absorption curve of the isolated label, labeled cells, non-labeled control cells and the glutaraldehyde. In the inset the resonance is compared with the enhanced spectrum of a control sample. (B) FMR derivative of the absorption spectrum of SPION attached to the $CD133^+$ cells that exhibit high electron densities in the cell periphery, as shown in the TEM image of the inset.

$CD133$ control cells with no label was measured by means of FMR and no resonance could be observed (see figure 4(A)).

Subsequently an FMR spectrum of the labeled $CD133^+$ cells (0.64×10^5 labeled cells contained in the volume of 2 μl) showed the resonance at $g = 2.1$ (see figure 4(A)). This again demonstrated the presence of SPION in the cells immersed in the fixer glutaraldehyde 2.5%, attached to the cells as shown in figure 4(A).

In order to be sure that the nanoparticles were not present in the fixer, the latter was separated from the labeled cells by 1200 rpm centrifugation, for 10 min, and the floating substance was carefully removed. The FMR spectrum of the fixer in the glutaraldehyde was taken and the absence of SPION was confirmed (inset of figure 4(A)). This result was already expected from the TEM morphological study, where it was observed that the antibody coupled to the nanoparticles was found tied to the $CD133^+$ cells.

The quantification of the average iron content per cell was determined from the area under the FMR absorption curves of the labeled cells (figure 4(A)), by interpolation of the calibration curve of figure 1. The iron content per cell was 1.70×10^{-13} mol (9.5 pg) or 7.0×10^6 nanoparticles per cell.

For the quantification by means of FMR of the number of nanoparticles per cell, it was necessary to carry out a qualitative visualization of iron oxide-labeled cells. This was necessary in order to ensure that the nanoparticles coupled to antibodies were attached to the surface antigen of the stem cell, providing confirmatory information.

The FMR spectrum (figure 4(B)) of the $CD133^+$ sample confirmed a strong labeling of dense electron regions in the periphery of the cell (see the inset of figure 4(B)). Moreover, we have observed that the morphology was not conserved, as explained previously. For all cells the FMR signal of SPION was due to the nanoparticles coupled to the antibodies which in turn are bonded into the cells. The analysis of the signal obtained for the quantification gives the result of 1.64×10^{-13} mol of iron (9.4 pg) or 6.8×10^6 nanoparticles per cell, in good agreement with the values obtained with well labeled cells, for example, those of figures 3(B) and (G). It is then necessary to perform a morphological analysis before performing the quantitative FMR measurements.

4. Conclusion

The FMR method is an efficient technique for the quantification of the iron concentration in stem cells. In this work, FMR has allowed us to quantify the SPION concentration in the $CD133^+$ cells of small samples (volume of the order of μl). In practice, for ferromagnetic particles, an area unit can be accurately measured, being equal to the number of 4.5×10^{11} nanoparticles contained in the volume of 2 μl or about 6.16×10^5 pg of iron.

The information obtained from the FMR spectra of the $CD133^+$ stem cells is similar to that obtained from the cells where the morphology was not conserved. Thus, in order for the FMR quantification method be valid, it is necessary to perform a preliminary test of a qualitative visualization of iron oxide-labeled cells (TEM), in order to make sure that the nanoparticles coupled to the antibodies are effectively tied to the antigen located at the surface of the stem cell, and that the morphology of the cell was preserved.

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