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ABSTRACT

Cells are able to precisely control the amount of iron they acquire in the form of transferrin (TF)-bound iron by modulating the synthesis of the transferrin receptor 1 (TfR1). In tumor cells, elevated TfR1 seems to be related to poorer outcome for patients. Thus, the direct measurement of this biomarker in breast cancer tissues and cells might serve as a prognosis biomarker. In this work, we have used Nd-labelled antibodies to tag the TfR1 present on the cell surface of two cell models of breast cancer with different malignancy (MCF7 and MDA-MB 231). For this aim, the monoclonal antibody anti-TfR1 is first labelled with a polymeric chelator (MAXPAR®) with subsequent incorporation of several isotopic ^{143}Nd atoms. The characterization of the labelled antibody revealed a stoichiometry of 20 Nd atoms per antibody molecule that can be used for further quantification experiments. This antibody is used for cell tagging followed by single cell analysis using inductively coupled plasma mass spectrometric (ICP-MS) detection. In this case, cell introduction is conducted using a high efficiency nebulizer and spray chamber

to achieve transport efficiencies up to 55% for cells. Quantitative results revealed a number of receptors per cell significantly higher in the case of the most malignant phenotype (MDA-MB-231). Absolute and relative TfR1 concentration values are obtained in individual cells for the first time using the proposed system.

Key-words: transferrin receptor 1, single cell analysis, ICP-MS, antibody labelling, breast cancer.

Introduction

Iron is an essential element for cellular development. The main entrance mechanism of Fe in cells occurs through its association to transferrin (Tf) that is further bound to transferrin receptor 1 (TfR1) present in the cell surface. The Tf/TfR1 assembly is internalized into the cell cytosol, rapidly matures and turns into a proton-pumping endosome. The pH lowering in the endosome (pH close to 5.6 depending on cell type) allows iron to be released from Tf and be either stored (into ferritin) or managed in the different intracellular processes[1, 2]. Iron-depleted Tf is then returned to the cell surface where, encountering a pH of 7.4, the protein is released for another cycle of iron transport. Therefore, TfR1 plays a critical role in iron homeostasis regulating this metal uptake in the form of Fe_2-Tf complex. In this vein, fast proliferating cells with high iron demand, such as cancer cells, constitutively express TfR1 at very high levels and can further upregulate TfR1 expression in response to iron deficiency[3]. Recent publications showed also that elevated TfR1 expression is related to poorer outcome for patients for many types of cancer[4, 5]. Thus, TfR1 can be considered as an important biomarker for prognosis of some types of cancerous processes such as breast cancer since it affects

cell proliferation, migration, invasion and apoptosis[6, 7]. But it can be also a suitable biomarker for diagnosis and treatment of breast cancer patients at the early stage[8].

Structurally, mammalian TfR1 comprises 760 residue subunits that can be divided into three differential parts: a globular extracellular, a hydrophobic intramembranous and cytoplasmic region[9]. Consisting of two monomers, TfR1 is linked by two disulfide bridges, forming a 190 kDa molecule. The analysis of TfR1 in biological samples like cells or tissues has been traditionally conducted by immunohistochemistry (IHC) which provides qualitative or semiquantitative information[10]. As quantitative alternatives, conventional enzyme-linked immunosorbent assays (ELISA) or reverse-phase protein arrays (RPPA) have been applied to the determination of TfR1 and other iron homeostasis biomarkers in biological fluids[11]. As a major advantage, RPPA allows to assess target protein expression quantitatively in large sample sets, while requiring only a very low amount of biological sample making this platform attractive for the analysis of clinical materials[12]. However, in both cases, the applied samples are bio-fluids (serum, plasma) or cell lysates. Therefore, these methods provide information of the total TfR1 concentration. This value corresponds to the fraction present on the cell membrane and the fraction forming the Tf/TfR1 assembly within the cell cytosol after entering by endocytosis.

In order to tackle chemical compositions and biological variations in individual cells, strategies on single cell analysis have become increasingly important[13]. Among various mass spectrometric techniques, single cell ICP-MS (SC-ICP-MS) allows the determination of elemental compositions in individual cells since the first principal work has appeared in 2005[14]. Nowadays, this technique has proven to be a versatile tool for studies on the

determination of constitutive elements[15], the cellular uptake of metal-containing drugs[16, 17, 18], and the incorporation of metallic nanoparticles[19]. In combination with labelling strategies, further studies has shown the versatility of this technique to characterize individual cells or bacteria[20, 21].

In this work, we have tried to develop an analytical strategy that permits the quantification of TfR1 present uniquely in the cell membrane. Its concentration should provide the most relevant information regarding the intracellular status upon iron uptake and thus, the malignancy of a cell line or tissue. For this aim, we describe the tagging of TfR1 present on the cell surface by means of lanthanide-labelled antibodies in combination with the introduction of individual cells into the inductively coupled plasma mass spectrometer (ICP-MS) [16, 22].

For this aim, we have used the combination of a high-efficiency modified microconcentric nebulizer with a spray chamber including a sheath argon flow for maximum transport efficiency of the cells. The labelling of the antibody anti-TfR1 using a polymeric chelator (MAXPAR®) with incorporated lanthanide ions serves as strategy for cell tagging. The functionality of the labelled antibody is carefully assessed and the number of incorporated lanthanides ions is determined. The developed methodology is conducted using two breast cancer cell models of different malignancy, in which the expression of TfR1 is expected to be different. The quantitative results are critically compared with those obtained by commercial ELISA.

MATERIALS AND METHODS

Reagents and materials.- All solutions were prepared using 18 MΩ·cm^{-1} de-ionized water obtained from a Milli-Q system (Millipore, Bedford, MA, USA). Mouse anti-human

TfR1 monoclonal antibody was purchased from R&D Systems (Minneapolis, MN, USA). Characterization of the antibody was conducted by size exclusion chromatography (SEC) using 50 mmol L^{-1} ammonium acetate pH 7.0 (Merck Millipore, Darmstadt, Germany) as mobile phase at a flow rate of 0.7 mL min^{-1} in a Superdex 200 10/300 GL column (300 mm x 100 mm i.d., GE Healthcare, Uppsala, Sweden). The column was previously calibrated using protein standards (thyroglobulin 660 kDa, ferritin 450 kDa, immunoglobulin G 150 kDa, albumin 66 kDa and alfa-lactalbumin 15 kDa) obtained from Sigma-Aldrich (Madrid, Spain).

The antibody was labelled using a Maxpar X8 Antibody Labelling Kit (Fluidigm, San Francisco, CA, USA), following the instructions of the manufacturer. For the reduction of the antibody, tris(2-carboxyethyl)phosphine (TCEP) was purchased from Sigma-Aldrich. For the purification steps, centrifugal filter units of 3 kDa and 50 kDa were used (Amicon Ultra 0.5 mL, Merck Millipore). The purified conjugate was quantified by measuring the absorbance at 280 nm using a NanoDrop 2000c (Thermo Fisher Scientific, Bremen, Germany).

For cell fixation, a buffered aqueous solution of formaldehyde 4% (VWR Chemicals, Pennsylvania, USA) was used as a fixative. Phosphate buffered saline (PBS), tris buffered saline (TBS) and bovine serum albumin were obtained from Sigma Aldrich.

For single cell analysis, the transport efficiency was estimated using CyTOF® Calibration Beads containing naturally abundant europium (Fluidigm). The transport efficiency of liquid standards was calculated using the Reference Material 8012 of 30 nm gold nanoparticles standard from NIST (Gaithersburg, MD, USA).

Instrumentation.- All ICP-MS experiments during this study were performed using the triple quadrupole instrument iCAP TQ ICP-MS (Thermo Fisher Scientific, Bremen, Germany) using the oxygen-TQ mode for the measurement of phosphorous (mass shift from $^{31}P^{+}$ to $^{31}P^{16}O^{+}$ after reaction with oxygen in the reaction cell) and SQ-mode (single quadrupole-mode) for $^{142}Nd^{+}$ and $^{153}Eu^{+}$ monitoring. For the single cell experiment, the ICP-MS instrument was fitted with a high performance concentric nebulizer (HPCN) and a small-volume on-axis spray chamber using a sheath gas flow (AIST, Tsukuba, Japan, see Figure S1). A similar system has been described elsewhere[23]. The cells were pumped using a microflow syringe pump SP101i (Florida, USA) fitted with a 1 mL Hamilton syringe (Nevada, USA) at 10 µL min^{-1}. The data were recorded in time-resolved analysis mode during 3 min per analysis using a dwell time of 5 ms. Under these conditions, only a single isotope could be measured in one run due to the sequential nature of the measurements in a quadrupole system. The ICP-MS paprameter are summarized in Table S1.

For cell counting, a Flow Cytometer Cytoflex S Beckman Coulter (California, USA) was used. The cell number was determined by absolute counting. For this aim, the peristaltic pump of the flow cytometer was calibrated at 60 µL min^{-1}. Forward and scattered light from the blue laser (488 nm) was registered in order to determine and count the intact cells according to their size and morphology.

The characterization studies of the labelled antibody were carried out by SEC using an HPLC system Agilent 1260 (Agilent Technologies, Tokyo, Japan). The column was a Superdex 200 10/300 GL separation column (300 mm x 10 mm i.d., GE Healthcare Bio-Sciences) having a fractionation range from 10 to 600 kDa. Detection was performed on-

line using both the integrated UV/VIS multiple wavelength detector and the iCAP TQ ICP-MS.

Cell cultures.- Human breast cancer cell lines MCF7 and MDA-MB-231 were kindly provided by J. M. Pérez Freije (Dept. of Biochemistry and Molecular Biology, University of Oviedo). Cells were grown in T-25 flasks with Dulbecco's Modified Eagle Medium (DMEM, LabClinics, Barcelona, Spain) and supplemented with 10% Fetal Bovine Serum (Gibco, Life technologies, Madrid, Spain) and 5 μg mL^{-1} Plasmocin prophylactic (InvivoGen, Nucliber, Madrid, Spain) at 37°C in a 5% CO_2 atmosphere. Then, the cells were washed with PBS (three times) and collected by trypsination.

Analysis by commercial ELISA.- Human TfR 1 was quantified by a commercial ELISA for comparison using the Human Transferrin Receptor SimpleStep ELISA Kit from Abcam (Cambridge, UK). This is a sandwich assay that uses an affinity tag-labelled capture antibody and a reporter horseradish peroxidase (HRP)-conjugated detector antibody. The capture of the analyte (TfR1) is performed in solution, and the entire complex is then immobilized via immunoaffinity by an anti-tag antibody coating the well. Then, a substrate solution (3,3',5,5'-tetramethylbenzidine, TMB) is added, which reacts with the HRP to produce a blue coloration. The intensity of this signal is measured spectrophotometrically at 450 nm and is proportional to the TfR 1 concentration.

For the ELISA, the cells were lysed using the Cell Extraction Buffer included in the kit. The cell pellet was solubilized in the buffer, incubated on ice for 20 min and centrifuged at 18,000 g for 20 min at 4°C.

Cell fixation and tagging.- After collecting the cells by trypsination, the cell number was adjusted to 10^6 cells per aliquot. The pellet was resuspended in 500 μL buffered

formaldehyde 4% and incubated for 15 min at room temperature to fix the cellular structure during the labelling procedure. Afterwards, the cell pellet was washed with 3% BSA in PBS by centrifugation for 5 min at 300 g and the pellet was resuspended in 200 μL of the antibody solution in 3% BSA in PBS. This suspension was incubated for 30 min at room temperature and washed 3 times with 500 μL PBS (up to a total volume of 1500 μL) and 3 more times with 500 μL TBS (up to a total volume of 1500 μL) to minimize the phosphorus background.

Data treatment.- For the data treatment of single cell suspension measurements, an established iterative procedure was followed, based on averaging the entire data set and collecting all data points that are three standard deviations (3σ) above the mean. The resulting data set after removing the selected events is rearranged and the procedure is repeated until no new data points are above the 3σ threshold. After the selection of the single cell signals, those higher than 3σ above their mean were discarded, as reported previously[18], in order to eliminate multiple-cell events.

RESULTS AND DISCUSSION

Studies on the antibody labelling reaction.- The monoclonal antibody anti-TfR1 was labelled using the sequential steps detailed in the MAXPAR® manufacturer´s kit[24]. To conduct quantitative analysis of the number of receptors per cell it is necessary to characterize the obtained product in terms of lanthanide ions per molecule of antibody. This was conducted using complementary SEC-UV and SEC-ICP-MS. According to the manufacturer´s procedure, the first step involves the partial antibody reduction using TCEP at 37°C for 30 min. The effectiveness of the reduction under these conditions can be seen in the chromatogram of Figure 1A where the solid black trace corresponds to

the intact antibody and dotted trace to its reduced form. The unchanged retention time after reduction (about 21 min) confirms the partial reduction of the antibody, with no cleavage of the two antibody domains and, therefore, no change on its total molecular mass. The high-intensity peak obtained at 26.5 min for the reduced antibody corresponds to the EDTA present in the reduction buffer.

Figure 1A shows also a grey trace corresponding to the antibody after labelling. As can be seen, there is a significant shift in the retention time towards shorter time, thus higher molecular mass. According to the literature, the labelling polymer could contain an average of 22 DTPA (diethylenetriaminepentaacetic acid) individual chelators that could allocate up to 22 Nd ions providing a whole mass tag of approximately 23 kDa[22, 25]. To confirm the presence of Nd in the labelled antibody, SEC-ICP-MS was also used. In Figure 1B, it is possible to observe a maximum in the $^{142}Nd^{+}$ signal at about 16 min matching the UV trace and ascribed to the labelled antibody. The second peak at about 20 min could correspond to the excess of unreacted polymeric tag. The combined information of the UV and ICP-MS traces confirm that the antibody has been totally labelled (no signal of unlabeled species can be seen) and a low carryover of unreacted polymer. In any case, the unreacted polymeric tag will be further removed during the washing steps of the labelling protocol.

To obtain the number of Nd ions labelling each antibody, the commercial heterogeneous sandwich ELISA kit used for the determination of TfR1 was modified by changing the detection antibody by 1 $\mu g \cdot mL^{-1}$ of the Nd-labelled antibody. The rest of the protocol was maintained as recommended by the manufacturer. Three different antigen (TfR1) concentrations were tested (0.01, 0.04 and 0.08 nmol L^{-1}). After the last wash, instead

of adding the TMB substrate for HRP detection in the commercial ELISA, 100 μL of 2% HNO_3 were added to each well and incubated for 10 min at room temperature. Neodymium in this solution was quantified by flow-injection analysis using ICP-MS detection (FIA-ICP-MS). By applying adequate conversions, the area of each FIA peak can be transformed into the absolute amount of Nd for each of the analyzed TfR1 standards. In Figure 2, the correlation between the moles of Nd and those of TfR1 is depicted where the slope of the adjusted curve provides the Nd:antibody stoichiometry[26]. As can be seen, the linear regression (*$y=21.23\ x + 0.44$, $R^2=0.9920$*) provides a stoichiometry of approximately 21 moles of neodymium per mole of anti-TfR1 antibody. This result is in good agreement with the expected 22 DTPA chelator molecules in each Maxpar® polymer tag, meaning that only one polymer molecule attaches to the antibody.

Recognition capabilities of the Nd-labelled antibody in a modified ELISA. - One of the methods to quantitatively address the presence of TfR1 in biological fluids is an ELISA assay. The selected one is based on a sandwich immune assay, including an anti-tag pre-coated well plate where the affinity tag-labelled capture antibody is immobilized after the sandwich formation with the HRP-labelled secondary antibody. This assay, which is commercially available, allows the determination of TfR1 in liquid samples coming from biological fluids or cell lysates. In the latter case, the method implies the cell lysis with specific reagents (e.g. detergents) that permit to stabilize the TfR1 in solution before measurement. The TfR1 concentration results provided in this case include the cell surface as well as the internalized receptors.

To ensure that the recognition capabilities of the antibody are kept after the labelling with the Nd-loaded polymer, a set of cell culture samples were analyzed using both, the

commercial ELISA with spectrophotometric detection and the ICP-MS based assay with the Nd-labelled antibody. For this purpose, independent triplicates of each breast cancer cell line (MDA-MB-231 and MCF7) were lysed as previously explained. After the lysis, two aliquots of each sample were analyzed independently with the two assays. The results of both sets analyses are shown in Table 1. As observed, the results are in good agreement in both cell lines, with relative differences around 7-8.3% for the modified ELISA with respect to the commercial assay. These values suggest the usefulness of the Nd-labelled antibody and its stoichiometric characterization for further analysis.

Regarding the comparative values within the two cell lines analyzed, the MDA-MB-231 (most malignant and proliferating phenotype) shows a much higher number of receptors than the MCF-7, in agreement with previously published work[27]. However, the bulk analysis reflects the concentration of the receptors that are present at the cell surface but also inside the cell cytosol from previously endocyted Tf/TfR1 assemblies. The aim of the present work is the possibility to distinguish among both, to the best of our knowledge never reported before, and with different biological implications than the total TfR1 concentration.

Determination of the sample introduction efficiency for single cell (SC)-ICP-MS.- For single cell analysis, a sample introduction set-up consisting on a high-performance concentric nebulizer (HPCN) and a small-volume on-axis spray chamber utilizing a sheath gas flow were used as described previously[23]. In order to characterize the system and to obtain the sample introduction efficiency, two different models were evaluated. Europium-doped polystyrene beads of about 3 µm of diameter (lower diameter than eukaryotic cells but higher density) and the same MDA-MB-231 human cells used for the

experiments. Whereas the calibration beads are a good and stable option for a daily performance test, the use of the actual cells is preferred for the calculation of the transport efficiency because the differences in size, density and general behavior during the nebulization are avoided. The calibration beads were diluted to 33000 beads·mL^{-1}. In the case of the cells, they were precisely counted by flow cytometry after labelling, and the suspensions were adjusted to around 2.5·10^{5} cells·mL^{-1}. The suspensions were sequentially measured by SC-ICP-MS monitoring ^{142}Nd and ^{31}P for the cells and ^{153}Eu for the beads. To obtain the transport efficiency, the expected events were compared with the obtained events, according to a previous publication and using the statistical restrictions previously detailed.[18]

Figure S2a shows histograms of the Eu-doped beads measured by ICP-TQ-MS and applying 5 ms as dwell time. As can be seen, since these are manufactured beads, the dispersion in the intensity of the events is very low, and the detection of multiple particles in a single event can be considered negligible. By comparing the number events detected and the expected ones it is possible to calculate a bead transport efficiency of 52%. Similarly, in the case of the cells, P was chosen as constitutive element to be measured off-mass using the oxide formation ($^{31}P^{16}O^{+}$ at m/z 47). Since these cells were already labelled with the anti-TfR1 antibody, the Nd^{+} was also used for the same purpose. The results observed for these cells can be seen in Figure S2b and S2c, respectively. In this case, the results are due to the biological variety more dispersed than in the case of the beads. More importantly, the transport efficiency calculated for cells turned out to be very similar, 55% for P and 50% for the Nd label. These results revealed that the antibody labelling on the cell surface was successful.

Analysis of TfR1 present at the cell surface by single cell ICP-MS in MDA-MB-231 and MCF7 cells.- Tagging of breast cancer cells with the labelled antibody was conducted as described before, using formaldehyde for fixation. In order to ensure that enough labelled antibody was added to tag every receptor at the cell surface, three different antibody concentrations were added to three independent cell suspensions each containing 10^6 cells. It is expected that different antibody concentrations could affect the signal in two different ways: 1) increasing the height of the detected events if more TfR1 are tagged upon increasing the concentration of the antibody and 2) increasing the number of events since cells with lower number of TfR1 could be tagged and, therefore, visualized at higher antibody concentrations. The results are summarized in the boxplot of Figure 3. As can be seen, the height of the detected events increases when the cells are treated with 1 $\mu g \cdot mL^{-1}$ antibody, but there is neither significant variation in the height of the events nor the number of detected events above this concentration. Therefore, the antibody concentration of 1 $\mu g \cdot mL^{-1}$ seems to represent sufficient excess to tag all the TfR1 present in the cell surface of MDA-MB-231.

Then, this concentration was used to tag the two cell lines of breast cancer: MCF7 (less malignant) and MDA-MB-231 (more aggressive and proliferative phenotype). The type of detected events can be seen in Figure 5 where the signal for $^{142}Nd^+$ are plotted for the two cell lines. In this case, the phosphorous signal has been monitored as internal control to verify that the cell introduction system is working correctly and that the number of cell events is approximately constant among different days.

The first finding was that the lower height of the events detected for phosphorous (data not shown here) in the MCF7 agrees with the fact that this cell line

shows a different morphology and probably size than MDA-MB-231[28]. Lower signal intensities of $^{142}Nd^{+}$ in the events were observed for the MCF7 cell line in comparison to MDA-MB-231. Although the analyzed suspensions contained the same number of cells, this observation reflects a descent in the number of TfR1 as expected from the bulk results obtained in the first part of the work (Table 1). To convert the height of the Nd events into Nd mass per cell, a calibration curve was constructed using Nd standards. In this case, the transport efficiency used for the calculations was 70% based on the analysis of a 30 nm gold nanoparticles standard with the same system[29]. The Nd mass in each cell was then easily converted to the mass of antibody using the obtained stoichiometry (20 Nd ions per antibody) and further, to the number of antigen molecules (TfR1) per cell.

The boxplot of the results corresponding to three independent cell cultures can be seen in Figure 5. Mean values of about $2.3 \cdot 10^4$ receptors per individual cell surface in the MDA-MB-231 while only $6.4 \cdot 10^3$ receptors per individual cell surface in the MCF7 were obtained. These results showed, consistently, that the number of TfR1 per cell is different between cell lines being about 4-fold higher in the case of the most malignant phenotype (MDA-MB-231).

Such finding confirms that fast proliferating cells, like MDA-MB-231 express TfR1 at very high levels, and this expression can even increase in response to iron deficiency, which is also sustained by previous measurements of intracellular Fe concentration in these two models.[30] This shows the importance of the developed methodology that could be further implemented for the absolute measurement of other malignancy biomarkers at

the single cell level with high sensitivity and minimum sample handling (no need for cell lysis).

Membrane and intracellular TfR1.- The use of the Nd-labelled antibody permitted to address the total TfR1 concentration present in the cell lysate, as shown in a previous section, with comparably good results to the commercial ELISA (Table 1). Such results can be converted in the number of TfR1 per cell by taking into account the precise number of cells in each sample obtained by flow cytometry. In addition, the single cell experiments allow the determination of the TfR1 per cell present at the cell membrane. Thus, the difference between these two sets of values permits to illustrate the intracellular level of TfR1.

These results are shown in Table 2 for three independent cell cultures of each of the cell lines. As can be seen, the bulk data results reveal mean values of $2.3 \cdot 10^5$ receptors per cell for MCF7 and $1.3 \cdot 10^6$ in the case of MDA-MB-231. Thus, the MDA-MB-231 shows, in both cases, substantially higher TfR1 levels. These values are in the order of other reported by using indirect methods like those based on the radioactive tagging of transferrin and measuring the induced radioactivity of cells after formation of the Tf/TfR1 assembly that is rapidly internalized (min)[31]. However, regarding the number of receptors present at the cellular membrane, no information from the literature could be obtained. Since an important research line focuses on the use of TfR1 as potential entrance mechanism for viruses into cells[32], this is an extremely valuable information. Moreover, nanomedicine and antibodies targeting TfR1 (at the cell membrane) have been developed for tumor-specific targeted therapy, in the field of precision oncology.[33] Thus, the obtained information reveals the extraordinary potential of the single cell

strategy to address, quantitatively, the expression of cell biomarkers present only on the cell surface at extremely low concentration levels thanks to the excellent capabilities of ICP-MS detection.

Conclusions

The use of labelled antibodies in combination with cell tagging and single cell analysis via ICP-MS seems to be a promising approach for the determination of clinical biomarkers, particularly present at the cell surface. For this aim, the MAXPAR® labelling kit has proved to be successful for labelling the anti-TfR1 monoclonal antibody obtaining a stoichiometry of approximately 20 Nd ions per antibody molecule that might correlate with the presence of one chelating polymer attached to an antibody molecule. The tagging of cells after fixation with this antibody showed throughout this work to occur efficiently and in a reproducible way. Furthermore, the tagged cells are transported into the ICP-MS through a high-efficiency nebulizer and spray chamber and can be detected by P and Nd monitoring. The quantitative analysis of Nd in the obtained cell events can be used for further evaluation of the number of TfR1 on the surface of different cell cultures of breast cancer. The obtained results showed that the developed strategy exhibits a great potential for the quantitative analysis of TfR1 at the single cell level without sorting to cell disruption and minimum handling.

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Tables and Figures

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Table 1. Comparison of the results obtained by the commercial ELISA and the modified ELISA using the Nd-labelled antibody and ICP-MS detection. Each sample corresponds to three independent replicates.

	Nd-labelled ELISA	**Commercial ELISA**	**Relative differences**
Sample	**TfR1 (nmol L^{-1})**	**TfR1 (nmol L^{-1})**	**(%)**
MDA-MB-231	24 ± 3	22 ± 2	8.3
MCF7	6.1 ± 0.2	5.67 ± 0.02	7.0

Table 2. Average number of transferrin receptors per cell (TfR1/cell) obtained by the commercial ELISA and the developed SC-ICP-MS method.

Samples	**TfR1/cell** Total (bulk)	**TfR1/cell** Cell membrane (SC-ICP-MS)	**TfR1/cell** Intracellular
MDA-MB-231	$(1.27 \pm 0.01)\cdot10^6$	$(2.3 \pm 0.4)\cdot10^4$	$(1.25 \pm 0.4)\cdot10^6$
MCF7	$(2.3 \pm 0.1)\cdot10^5$	$(6.4 \pm 0.9)\cdot10^3$	$(2.2 \pm 0.8)\cdot10^5$

Figure 1. Chromatograms obtained by SEC-UV at 280 nm (A) and SEC-ICP-MS measuring $^{142}Nd^{+}$ (B). A) The blue line corresponds to the original anti-TfR1 antibody (offset: 2 A.U.), the red line correspond to the reduced antibody (offset: 1 A.U.) and the green line corresponds to the labelled antibody containing the MAXPAR® chelator and the Nd atoms incorporated. (B) ICP-MS trace of $^{142}Nd^{+}$ showing the labelled antibody by SEC-ICP-MS.

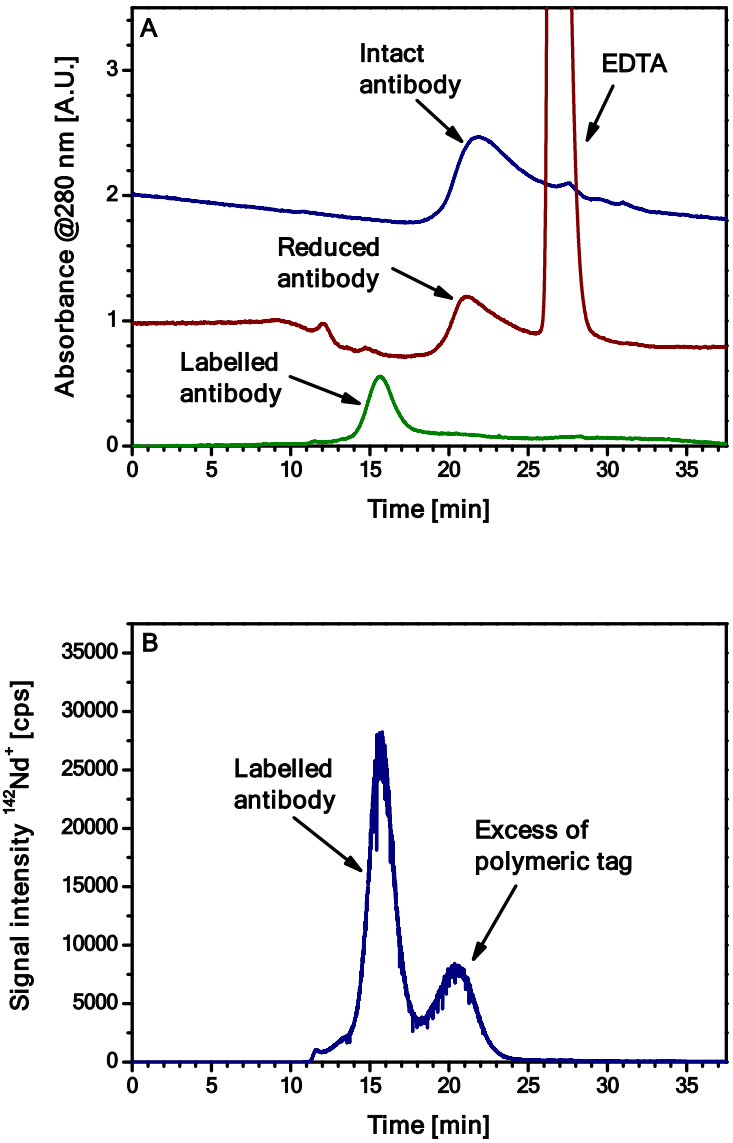
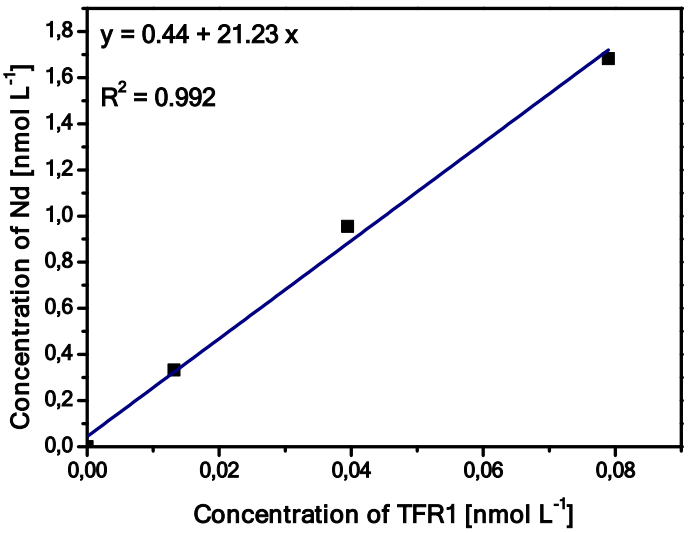


Figure 2. Evaluation of the stoichiometry of the labelled antibody by plotting the antigen concentration versus the Nd concentration obtained by ICP-MS.



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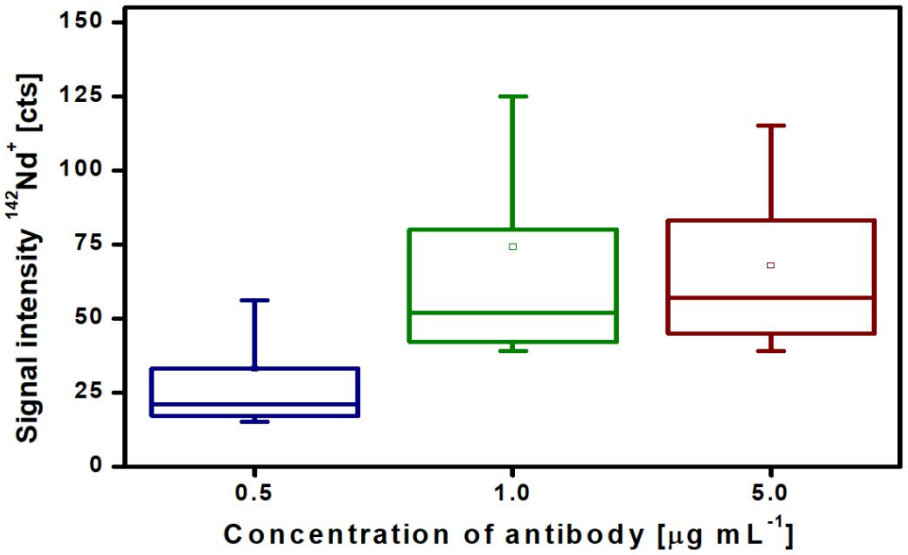
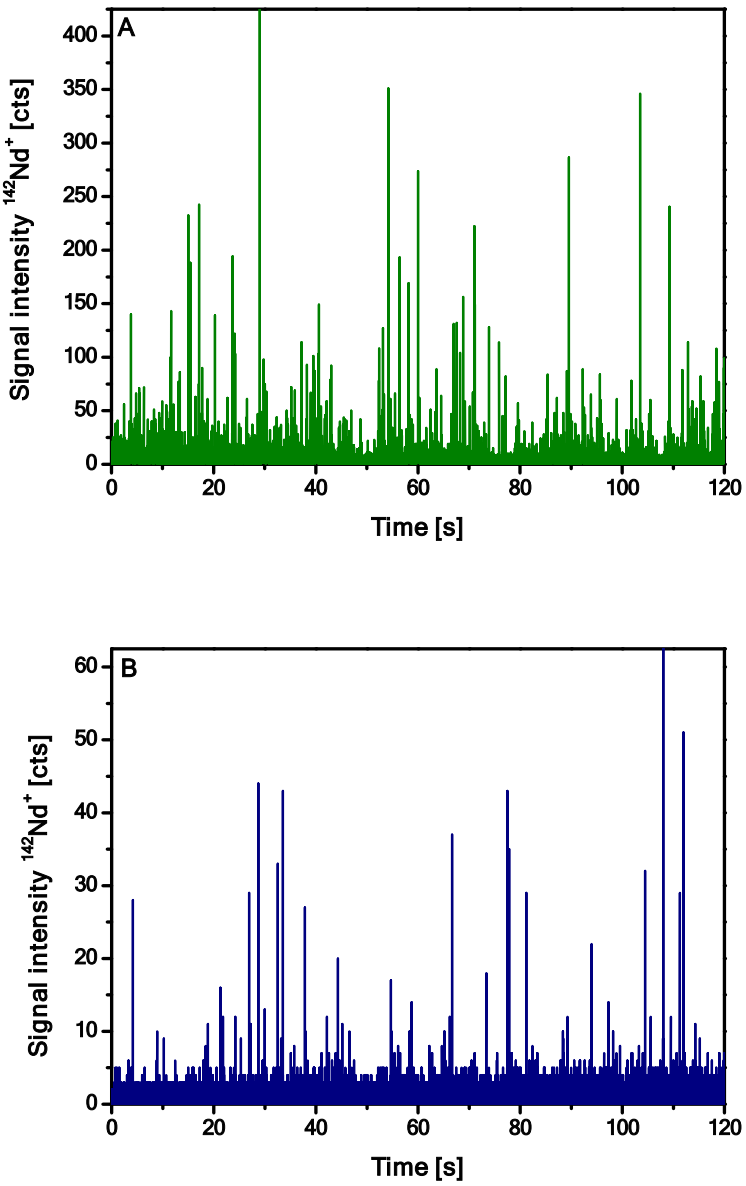


Figure 4. Example of cell events obtained detecting Nd by SC-ICP-MS in the (A) MDA-MB-231 and (B) MCF7 cells.



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