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Gold nanoclusters as elemental label for the sequential quantification of apolipoprotein E and metallothionein 2A in individual human cells of the retinal pigment epithelium using single cell-ICP-MS

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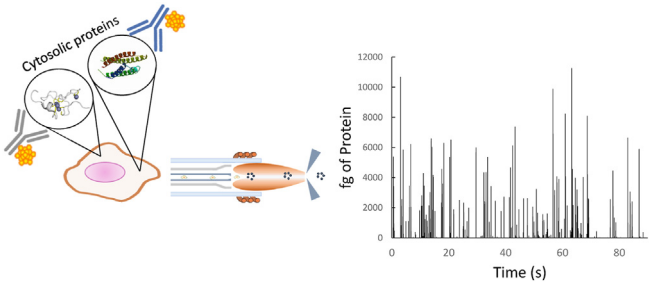
HIGHLIGHTS

- AuNCs were used as label for determination of cytosolic proteins in sc-ICP-MS.
- APOE and MT2A were compared in cells under pro-inflammatory and control conditions.
- Fe was measured to check the integrity of the cells after the immunoassay.
- APOE and MT2A amounts were corroborated with commercial ELISA kits.

GRAPHICAL ABSTRACT



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ABSTRACT

Gold nanoclusters (AuNCs) with a diameter of 1.99 nm on average were synthesized and applied as labels in immunoprobes for the determination of cytosolic proteins in individual human retinal pigment epithelium (HRPEsv) cells by single cell – inductively coupled plasma – mass spectrometry (sc-ICP-MS). For quantitative purposes, the number of gold atoms per immunoprobe (i.e., the amplification factor) was determined; 466 gold atoms on average were obtained. Human metallothioneins (MT), including the 2A isoform (MT2A), and apolipoprotein E (APOE) play an important role under inflammation and oxidation processes in the RPE. The new single biomarker strategy introduced was applied to the sequential determination of MT2A and APOE in HRPEsv cells under pro-inflammatory and control conditions through the development of immunoassays with the corresponding AuNCs immunoprobes and the measurement of the $^{197}Au^{+}$ signal by sc-ICP-MS. In addition, $^{56}Fe^{+}$ signal was measured as constituent element of HRPEsv cells in order to check the integrity of the cells after the immunoassay and to confirm the number of cell events detected when monitoring the protein label ($^{197}Au^{+}$). Optimisation of parameters related with the sample preparation for the analysis of cytosolic proteins in intact HRPEsv cells

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was carried out. The method was successfully applied to the determination of both proteins in control cells and cells treated with the recombinant human interleukin-1α. Quantitative results obtained per cell for the average protein amounts of APOE and MT2A using the sc-ICP-MS procedure were corroborated with commercial ELISA kits.

1. Introduction

Age-related macular degeneration (AMD), the leading cause of irreversible blindness and visual disability in developed countries, is characterised by the accumulation of extracellular deposits (known as drusen), along with progressive damage of retina tissues inducing photoreceptor atrophy [1]. The retinal pigment epithelium (RPE) constitutes the outermost layer of the retina and has many important functions in the homeostasis of the eye to maintain visual function. However, this monolayer of cells is constantly subjected to high levels of oxidative stress, which may trigger a vicious cycle of chronic inflammatory response and consequent development of AMD disease [1]. In this context, human metallothioneins (MT), including the 2A isoform (MT2A), play an important role in Zn buffering and muffling, as a reactive oxygen species chelator or as an inhibitor of pro-inflammatory cytokine activation, which are released under oxidative stress processes [2]. Additionally, the functions of apolipoprotein E (APOE), one of the most abundant proteins accumulated in drusen, are also highly modulated under inflammation and oxidation processes [3]. Thus, inflammatory cytokines can either up- or down-regulate the synthesis of APOE or MTs in tissues [4,5]. The study of dual but antagonistic effects of pro-inflammatory cytokines in protein synthesis in RPE cells may provide valuable information about altered molecular pathways during inflammatory stress and contribute to the knowledge of AMD disease.

Heterogeneity of cell populations is well-known in all biological systems. In fact, cells from the same cell type, even under equal physiological conditions or external stimuli, may differ in the level of biomolecule expression. However, it can be difficult to assess and correctly interpret possible differences between cell populations, unless biological systems are investigated on a cell-by-cell basis [6]. Therefore, there is a need for innovative analytical techniques that allow for the analysis of individual cells, as well as quantitation of their molecules and elemental content.

Single cell inductively coupled plasma - mass spectrometry (sc-ICP-MS) has demonstrated a huge potential for the determination of elemental compositions in individual cells [7,8]. Nowadays, sc-ICP-MS has proved to be a versatile tool for studies on the determination of naturally occurring metals within the cells [9,10], the cellular uptake of metal and metalloid components [11–14], as well as the cellular uptake of metal-containing nanoparticles (NPs) [15,16].

The quantification of endogenous cellular proteins by sc-ICP-MS still remains a challenge. In this case it is necessary to perform an immunoassay in suspended cells by using an immunoprobe conjugated with an elemental label for detection. However, this step can compromise cells integrity. So far, a limited number of studies have reported on the combination of an immunoprobe and sc-ICP-MS for protein analysis [17–21]. Furthermore, the applications related to quantitative protein analysis are very scarce, and in all cases the target biomolecule is a membrane biomarker [17,20,21]. For protein determination, the amplification factor (i.e., the number of elemental labels per immunoprobe) must be known, thus requiring the use of a well characterised immunoprobe. For example, the quantification of a breast cancer cell membrane biomarker in two human cell lines was reported using a Nd-labelled antibody (MAXPAR® label with 21 Nd atoms per immunoprobe) [17]. In a similar fashion using Lu-labelled MAXPAR®, the targeting of HER2 (breast biomarker) in mixtures of human cell lines was performed [20]. In addition, Liu et al. [21] reported the use of an aminofluorescein-DOTA-Nd bimodal detection label (by MS and near infrared, NIR) for the identification and determination of prostate-specific membrane antigen in circulating tumour cells. At this point, it should be also noted that the technology called as mass cytometry allows detection of several biomolecules (up to 40 cellular parameters) at single-cell resolution [22,23], using a time of flight mass spectrometer (TOFMS).

Increasing the amplification provided by the elemental labels is a critical need especially for the quantification of low abundance proteins by sc-ICP-MS. Therefore, the use of nanostructures providing a larger number of elemental labels per immunoprobe has emerged. For example, virus-like NPs have been shown to provide significant signal amplification [24]. However, relatively big NPs can block the recognition sites of the immunoprobe. As an alternative, metal nanoclusters (NCs) have been recently proposed as elemental labels in ICP-MS detection due to their small metal core size (below 3 nm), along with the resulting high amplification factor they provide (NCs are composed of hundreds of metal atoms) [25]. Several types of metal NCs (e.g., AgNCs [26] and PtNCs [27]) have been successfully employed for the determination of proteins in biological fluids by ICP-MS, as well as in tissue sections by laser ablation ICP-MS [28,29].

In the current work, a single biomarker strategy using AuNCs as elemental labels for the sequential determination of cytosolic proteins in individual human RPE cells (HRPEsv) by sc-ICP-MS is investigated for the first time. The proposed methodology is based on immunoassay performed in a cell suspension by using AuNCs conjugated to protein-specific antibodies for the sequential determination of the cytosolic proteins APOE and MT2A in HRPEsv cells under pro-inflammatory and control conditions. The Au to antibody stoichiometry in the Au-labelled immunoprobes was determined in order to provide quantitative determination of the aforementioned proteins in HRPEsv cells. Furthermore, $^{56}Fe^{+}$ intensity signal was measured as constituent element of HRPEsv cells, assisting to check the integrity of the cells after the immunoassay in suspension and also to confirm the number of cell events detected when monitoring the protein label ($^{197}Au^{+}$). Importantly, our strategy makes it possible to quantify the expression levels of cytosolic proteins on a cell-by-cell basis under the same stimulus, providing insight into biological heterogeneity. Results obtained per cell for the average protein amounts of APOE and MT2A using the developed procedure were corroborated with commercial ELISA kits.

2. Materials and methods

2.1. Chemicals and materials

2.1.1. AuNCs synthesis

Water dispersible AuNCs were synthesized using $AuCl_4Na \cdot H_2O$

(99% powder; Sigma-Aldrich), lipoic acid (>98% powder; Across Organics) and $NaBH_4$ (98% powder, Sigma Aldrich) as the metal salt precursor, stabilising ligand and the reductant, respectively. Other reagents and solvents were also employed during the sample preparation protocols: NaOH (Sigma-Aldrich) to adjust the water solution's pH in order to help with ligand solubilisation and propan-2-ol (Fisher Scientific) to prepare the reductant solution.

2.1.2. Synthesis and purification of the AuNCs immunoprobes

To bind the AuNCs with specific primary antibodies, the carbodiimide strategy was selected. 1-Ethyl-3-(3-Dimethylaminopropyl) Carbodiimide (EDC) (98% powder; Across Organics) and *N*-hydroxysuccinimide (NHS) (>98% powder; Sigma-Aldrich) were the reagents employed for such a purpose. Goat polyclonal anti-human apolipoprotein E (anti-h-APOE) (Fisher Scientific) and rabbit polyclonal anti-human metallothionein 2A (anti-h-MT2A) (Cloud Clone Corporation) were the primary antibodies employed for the immunoprobes. The synthesis and characterisation of the Au-labelled immunoprobes (AuNCs:anti-h-APOE and AuNCs:anti-h-MT2A) was performed following a protocol similar to that previously reported [27,28]. For the purification of the AuNCs and the AuNCs labelled immunoprobes, 3 and 100 kDa pore size Amicon ultra centrifugal filter units (Merck Millipore) were used respectively.

Deionised ultrapure water, resistivity 18.2 MΩ cm, (Purelab Flex 3&4; ELGA-Veolia) was used throughout, as well as HNO_3 (67–69%, trace analysis; VWR chemicals) to prepare solutions before conventional ICP-MS measurements.

2.1.3. Cell line and culture conditions

The cell line used was the human immortalised RPEsv40 (HRPEsv) [30]. For the growing and preparation of the cell suspension, two culture media and several supplementations were used. At first, during the growing and expansion of the cell line, Dulbecco's modified eagle medium/nutrient mixture F-12 (DMEMF12) (Sigma Aldrich), supplemented with 1% (v/v) penicillin/streptomycin (P/S) and 10% (v/v) fetal bovine serum, qualified, heat inactivated (FBSi) (both purchased in Gibco™ Thermo Scientific) was used. Once cells were confluent, the medium was changed to hybridoma serum free (Gibco™, Thermo Fisher Scientific), supplemented with L-glutamine (Thermo Fisher Scientific) and P/S. For HRPEsv cells treated with the pro-inflammatory stressor, the recombinant human interleukin-1α (IL1α, Gold Bio) was employed. Afterwards, 0.25% trypsin-EDTA (Gibco™, Thermo Fisher) and 4% paraformaldehyde (PFA) from VWR chemicals were used to remove adherent cells and collect them from the seeding flasks, as well as to perform the cell fixation, respectively. Cells pellets were stored into the fridge at 4 °C in phosphate-buffered saline (PBS) 1x (pH 7.4) until further use. All the material and reagents used were autoclaved.

2.1.4. Immunoassay in cell suspension

To perform the immunoassay in cell suspension, apart from the Au-labelled immunoprobes, several reagents were employed: Triton X-100 (Sigma-Aldrich) for cell membrane permeabilisation, bovine serum albumin (BSA) (99% powder, Merck) and donkey serum (Sigma-Aldrich) to block non-specific interactions. Washing steps were carried out with 10 mM PBS, pH 7.4 (Sigma Aldrich) and surfactant tween 20 (Sigma-Aldrich). Additionally, 4% paraformaldehyde (PFA) solution (Sigma Aldrich) was used for the cell fixation.

2.1.5. Solution and suspension preparation for single-cell ICP-MS

For single-cell analysis, cells were suspended in a buffer composed of 50 mM Trizma base (primary standard and buffer >99.9% crystalline; Sigma Aldrich) and 17 mM NaCl (Sigma Aldrich), while pH was adjusted to 7.4 with diluted HCl (VWR chemicals). The sample transport efficiency of the ICP-MS introduction system was calculated using 60 nm BPEI-coated AgNPs (NanoComposix). The calibration curve for Au quantification was established using dissolved Au standards (7% HCl, Supelco, 1000 μg mL^{-1} ICP standard, Merck-Millipore).

2.1.6. ELISA kits for MT2A and APOE quantitative determination

Two commercial enzyme-linked immunosorbent assay kits from Cloud-Clone Corporation were employed for the determination of APOE (SEA704Hu 96 Tests) and MT2A (SEB868Hu 96 Tests) protein concentrations in the HRPEsv cells (both in control cells and cells treated with IL1α). For the extraction of the cytosolic content, cells were centrifuged, re-suspended in a buffer solution of Tris-HCl at pH 7.4 (Sigma Aldrich) and lysed using the ultrasound probe (Bandelin sonoplus HD2070). The cell lysate was then centrifuged (15,700 g, 30 min at 4 °C) and the supernatant (i.e., the cytosolic fraction) was collected for protein quantification with ELISA kits.

2.2. Instrumentation

NexION 350 X ICP−MS (PerkinElmer) was used to detect $^{197}Au^{+}$ and $^{56}Fe^{+}$ intensity signals in individual HRPEsv cells. The sample introduction system consists of a high-efficiency nebuliser (HEN) from Meinhard, which was fitted into the Asperon^{R} spray chamber (PerkinElmer). Sample introduction was carried out in self-aspiration mode at the determined sample introduction rate of 40 μL min^{-1}. Data processing following sc-ICP-MS analysis was performed manually based on an iterative algorithm using Microsoft Excel software, enabling the discrimination of cell events from the background signal, which was attributed to the dissolved metal species. Data presentation (i.e., charts, histograms and cell event profiles) was also carried out using Microsoft Excel.

For cell counting, a Neubauer haemocytometer (Sigma Aldrich) plate was used under an optical microscope (Inverted Microscope Leica DM IL LED, Leica Microsystems, Wetzlar, Germany) at 10-fold magnification. ELISA measurements were carried out with the spectrophotometer PerkinElmer 2030 Multilabel reader VICTOR™ X5.

2.3. Experimental methods

2.3.1. Synthesis and characterisation of monodisperse AuNCs and AuNCs immunoprobes

The protocol for the synthesis of AuNCs has been previously reported [31]. Briefly, the protocol is described as follows: 200 μL of 50 mM $NaAuCl_4$ were added into a cylindrical flask, along with an aqueous solution of lipoic acid (0.035 g in 20 mL of water) adjusted at pH 8 (adjusted by adding 50 μL of 2 M NaOH). After mixing the metal precursor and the capping ligand, $NaBH_4$ (400 μL of 50 mM in propanol solvent) was added dropwise into the solution, and the mixture was left for 4 h at room temperature under continuous stirring. The AuNCs were then subjected to an aging post-treatment (50 °C for 2 h) to improve NCs monodispersity [32]. The average diameter of AuNCs determined by high resolution transmission electron microscopy was 1.99 ± 0.04 nm.

Once the AuNCs were purified by ultracentrifugation, they were bioconjugated to the protein-specific antibodies (anti-h-APOE and anti-h-MT2A). For this purpose, the carbodiimide strategy was used and a protocol similar to that described in the literature was followed [27,29]. Isolation of labelled immunoprobe from the nude AuNCs was also carried out by proper ultracentrifugation. Procedures are briefly described in the Supplementary Material.

To determine the Au-to-antibody stoichiometry in the

immunoprobes, the quantification of the available antibody and the Au concentration in the Au-labelled immunoprobes was required. Thus, a similar procedure to the previously described by Cruz-Alonso et al. [29] was performed for the AuNCs:anti-h-APOE and AuNCs:anti-h-MT2A immunoprobes. A quantitative direct ELISA with an IgG standard was carried out to determine the available concentration of antibody in the Au-labelled immunoprobe solution followed by conventional ICP-MS analysis to determine the Au concentration. The ratio of these two quantities defined the stoichiometry of Au-to-antibody in the immunoprobes.

2.3.2. HRPEsv cell treatment

The HRPEsv immortalised cells were cultured at 37 °C in a 5% CO_2 incubator using DMEMF12 complete medium supplemented with 10% FBSi and 1% of P/S. Once cells achieved 80% of confluence, they were washed with PBS 1x and their medium was changed to hybridoma free serum supplemented with 5% L-glutamine and 1% P/S. Twenty-four hours later, cultured HRPEsv cells were either non-treated (i.e., control cells only with hybridoma medium) or treated with the pro-inflammatory cytokine IL1α (100 ng mL^{-1}) for 48 h, according to previous publications [4]. Subsequently, cultured cells were trypsinized by incubation (37 °C in 5% CO_2) with 3 mL per flask of 0.25% trypsin-EDTA for 3 min. Later, 10 mL per flask of DMENF12 were added to stop the trypsin activity and the cells were collected into different falcon tubes to obtain the cellular pellet from the supernatant by mild centrifugation (3 min at 100 g). Finally, to remove possible dead cells that remained in the medium, cells were washed once with PBS and collected to obtain the cell suspension. This sample preparation procedure was followed both for cell samples subjected to ELISA assays and for those prepared for analysis with sc-ICP-MS. Fig. S1 (Supplementary Material) shows a schematic diagram of the experimental implemented for the preparation of HRPEsv cells for analysis with sc-ICP-MS.

Afterwards, the number of cells in the suspension was determined under the microscope using a Neubauer haemocytometer chamber. Cells at a concentration of $1.2–1.5 \cdot 10^6$ (final concentration after fixation ~$1 \cdot 10^6$ cells mL^{-1}) were carefully separated in different Eppendorf tubes where fixation procedure took place, both for control and IL1α treated cells. Two protocols were evaluated for the fixation of cell cultures before the immunoassay with the AuNCs immunoprobes, denoted as traditional [17] and "mild fixation" conditions. The steps included in both procedures are collected in Table 1. Cells were stored at 4° in the fridge in PBS 1x until further use. The cells morphology was examined with a bright field microscope after every single experimental step to visually ensure the integrity of the cells.

Each of the two target proteins were analysed in three biological replicates for control (CT) and IL1α−treated cells. In addition, two negative controls were prepared, one for the control cells and another for the IL1α−treated cells. Such negative controls were subjected to the immunoassay protocol, but the AuNCs immunoprobes were not added. $^{56}Fe^+$ and $^{197}Au^+$ intensity signals were detected by sc-ICP-MS for CT and IL1α HRPEsv cells, whereas only $^{56}Fe^+$ was monitored for the negative controls (neg-CT and neg-IL1α).

2.3.3. Immunoassay with cells suspension

The immunoassay with the two Au-labelled immunoprobes was independently performed with the CT and IL1α−treated hRPEsv cells in suspension at a concentration of $1 \cdot 10^6$ fixed cells. All steps were carried out at room temperature and they are described as follows: (i) Permeabilisation of the membranes with a surfactant: 500 μL of PBS 1x with 0.1% Triton X-100 for 15 min; (ii) Washing: 500 μL of PBS 1x-0.05% tween-20 and spin at 100 g; (iii) Blocking: 500 μL PBS 1x with 10% donkey serum and 1% BSA for 30 min of incubation; (iv) Incubation with the AuNCs immunoprobes: 200 μL of AuNCs:anti-h-MT2A or AuNCs:anti-h-APOE) in PBS 1x with 5% donkey serum and 1% BSA of AuNCs:anti-h-MT2A or AuNCs:anti-h-APOE were respectively added to the cells in suspension; and (v) Washing with 500 μL PBS 1x. This last stage was carefully evaluated since an incorrect washing could generate either undesirable signal events or an increase of the background during sc-ICP-MS analysis.

2.3.4. sc-ICP-MS analysis in individual cells and data processing

Upon 1 h incubation with the Au-labelled immunoprobes, the cell pellets were re-suspended in 50 mM Trizma and 17 mM NaCl (pH 7.4) and diluted to a final cell concentration of $1 \cdot 10^5$ cells mL^{-1}. The cell suspension samples were introduced into the ICP-MS through the HEN nebuliser which was operated in self-aspiration mode at 40 μL min^{-1} sample introduction rate. During the analytical run, the cell suspension was subjected to a soft and continuous shaking on a vortex apparatus.

ICP-MS was operated in time-resolved analysis mode. Experimental parameters set are presented in Table 2. For the data treatment after sc-ICP-MS analysis, an established iterative procedure was performed to discriminate the cell events from the background [33]. Such strategy is based on averaging the entire data set and collecting all data points that are three or five standard deviations (3σ or 5σ) 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 threshold. Two different criteria were applied for a correct data treatment: 5σ threshold for $^{197}Au^+$ and 3σ for $^{56}Fe^+$, as discussed in the Results and Discussion section.

Additionally, to determine APOE and MT2A amount per individual cell from sc-ICP-MS data, different steps must be followed, which involve: (i) Filtering the cell events from the ionic background (ionic $^{197}Au^+$ from AuNCs immunoprobes); (ii) Converting

Table 1
Comparison between the conditions employed to perform the fixation process of the HRPEsv cells with the traditional and mild procedures.

	Traditional fixation	Mild fixation
Steps of the fixation protocol	1. Spin off all the PBS 1x (dry cellular pellet)	1. Add 250 μL of 4% PFA into the cell suspension (collected in 250 μL PBS 1x, not dry cellular pellet)
	2. Add 500 μL of 4% PFA directly on the cells pellet	2. Incubation at room temperature (10 min)
	3. Incubation at room temperature (10 min)	3. Spin off the liquid (smooth centrifugation)
	4. Spin off the 4% PFA (smooth centrifugation)	4. Add other 250 μL of 4% PFA on the cell pellet
	5. Washing with PBS 1x (once)	5. Incubation at RT (10 min)
	6. Spin off and remove the PBS 1x with the damage cells	6. Spin off and remove PFA
	7. Add 1 mL of PBS 1x for storage	7. Washing with PBS 1x (once)
		8. Spin off and remove the PBS and damage cells
		9. Add 1 mL of PBS 1x for storage

Final step: Cell counting to check the cells number and storage of cell suspensions (cells in PBS 1x) in the fridge (4 °C) until further use.

Table 2
Operating conditions for sc-ICP-MS measurements of CT and IL1α treated HRPEsv cells.

Parameter	Values
ICP RF power (W)	1400
Plasma gas flow (mL·min^{-1})	18
Make up gas flow (mL·min^{-1})	0.6
Nebulizer gas flow (mL·min^{-1})	0.3
Isotopes (m/z)	$^{197}Au^+$, $^{56}Fe^+$ (collision cell, KED)
He gas flow (KED) (mL·min^{-1})	4
Dwell time (ms)	10

the $^{197}Au^+$ intensity cell detection signals to mass of Au per cell; and (iii) Calculating the absolute amount of protein per cell from the pre-determined Au-to-antibody stoichiometry. More specifically, for the determination of the absolute mass of Au per cell event, the intensity of $^{197}Au^+$ acquired per cell event must be transformed to absolute mass of Au. For such purpose, an external calibration using Au liquid standards for ICP-MS (0–50 μg mL^{-1}) was employed and the following relationship was established [7,8,34]:where m_c is the mass of Au, F is the sample flow rate, t is the dwell time, Int is the net event intensity, b is the slope of the calibration curve and η is the transport efficiency. To determine the transport efficiency, a suspension of a known concentration of commercial 60 nm AgNPs nanoparticles was measured (50 ng L^{-1}) [35].

Finally, to transform the amount of Au into the corresponding protein mass (APOE and MT2A) it was followed a similar methodology to that previously reported for tissue sections [28]. For such purpose, the number of Au atoms per immunoprobe (466 atoms on average) as well as the molecular mass of each protein (6 kDa for MT2A and 34 kDa for APOE) must be considered.

3. Results and discussion

3.1. Sample preparation for the analysis of cytosolic proteins in intact HRPEsv cells by sc-ICP-MS

3.1.1. Monitoring $^{56}Fe^+$ in individual HRPEsv cells.

Maintaining cell integrity while achieving maximum cell transport efficiency to the ICP is of critical importance in sc-ICP-MS. The use of a sample introduction system that triggers breakage of cells during nebulisation and transport would generate not only low transport efficiencies, but also high background signals (resulted from the liberation and solubilisation of the elements present inside the cells, as well as mixing of fragments from different cells). For this reason, the possibility to monitor a cell intrinsic element, along with $^{197}Au^+$ detection from the Au-labelled immunoprobes within the cells, was explored. These studies provide validation for the number of $^{197}Au^+$ signal pulses that corresponded to cell detection events. Also, the measurement of an intrinsic element is valuable to evaluate if the treatment with the IL1α, or the use of AuNCs immunoprobes, can affect the number of detected cell events compared to those detected for negative control HRPEsv cell suspensions.

Thus, Mg, Ca, Cu, P, S, Mn, Co, Zn and Fe were all evaluated as intrinsic elements of HRPEsv cells using a negative control sample (neg-CT: HRPEsv cells without the AuNCs immunoprobe and without IL1α treatment). However, selection was limited to Ca, Cu and Fe which provided detectable cell events. Although the number of detected cell events was comparable for all three isotopes ($^{44}Ca^+$, $^{65}Cu^+$ and $^{56}Fe^+$), monitoring $^{56}Fe^+$ provided higher mean intensities per cell detection event which was 169.7 counts as opposed to 13.71 and 3.16 recorded for $^{44}Ca^+$ and $^{65}Cu^+$, respectively. Therefore, $^{56}Fe^+$ was monitored as an intrinsic element throughout this study.

Experimental results also showed agreement in the number of cell events detected for different cell suspensions, such as neg-CT, neg-IL1α, CT and IL1α; telisahus, demonstrating that the IL1α-treatment or the immunoassay with the AuNCs labels did not compromise the integrity of the HRPEsv cells. Although dual isotope detection would achieve the simultaneous detection of $^{197}Au^+$ and $^{56}Fe^+$ per single cell, such possibility was not available in the ICP-MS instrument employed. Therefore, $^{197}Au^+$ and $^{56}Fe^+$ detection was conducted sequentially for the same cell suspension, with a delay of 1 min; the time required by the instrument to switch from $^{197}Au^+$ detection (standard mode) to $^{56}Fe^+$ detection (KED mode).

3.1.1. Cell fixation

Fixation of the cells is needed prior to the immunoassay. Moreover, it has been demonstrated that fixation strengthens the cellular structure [7]. Reagents such as PFA or methanol [36] are commonly used for such purpose. However, the use of non-optimized concentrations and/or conditions (e.g., high concentration or large times of incubation) might cause extensive cell breakage and thus cell loss. Thus, a novel fixation protocol, referred here as “mild fixation” was developed in this work to lessen the PFA effect in comparison with traditional fixation protocols (Table 1).

To study both fixation protocols with the HRPEsv cells, the cells (intact and with well-defined shape) were counted using the Neubauer chamber. The counting was repeated 3 times for each sample, before and after the fixation, to obtain the standard deviation associated with the measurement, which was in all cases below 20% RSD (relative standard deviation). Five cell suspensions treated separately with 4% PFA (biological replicates) were employed for cell counting after fixation. Results (Table 3) showed that the percentage of cells loss was reduced from more than 40% of cells with the traditional fixation to only 15% using the “mild fixation” protocol proposed in this work.

3.1.2. Optimisation of the immunoassay

To perform the immunoassay in cell suspensions, in addition to the cell fixation, attention must be paid to other required preparation steps, such as incubation times, to ensure HRPEsv cell integrity and avoid cell structure damage. Three aliquots from CT cells were selected to carry out the immunoassay for the determination of APOE. The samples were analysed by sc-ICP-MS monitoring the $^{197}Au^+$ signal from the elemental label (evaluation of the immunoassay and the cells integrity) and $^{56}Fe^+$ naturally present in the cells (evaluation of the cells integrity). Two times were evaluated for the blocking stage (30 min and 1 h) and the immunoprobe incubation (overnight at 4 °C and 1 h at room temperature, [17]). No significant differences were observed for the two-blocking times evaluated, whereas the use of an overnight incubation for the AuNCs immunoprobe showed a strong effect on the HRPEsv cell integrity. As an example, Fig. S2 (Supplementary Material) presents the time-resolved profiles obtained for $^{197}Au^+$ and $^{56}Fe^+$ isotopes using the overnight incubation of the AuNCs immunoprobe. A significantly lower number of cell events were counted after an overnight incubation (46 events on average; n = 3) compared to 1 h incubation (406 events on average; n = 3). Furthermore, the $^{197}Au^+$ signal coming from the ionic Au (i.e., dissolved) increased from 7 up to 74 counts (zoom at Fig. S2B), indicating that a significant number of cells had ruptured. An increase of the background signal was also observed for $^{56}Fe^+$.

Additionally, the washing step following the incubation with the immunoprobe (AuNCs:anti-h-APOE) must be optimized since it is necessary to ensure that the excess of AuNCs-labelled antibody

Table 3
Experimental results obtained for the cell counting, comparing the loss of cells versus the total cells number before and after the two fixation processes (traditional and mild fixation) investigated for HRPEsv cells.

Counting	Traditional fixation (cells mL^{-1})	Mild fixation (cells mL^{-1})
Before fixation **(instrumental replicates; n = 3)**	$1.12 \cdot 10^6 \pm 1.7 \cdot 10^5$	$1.73 \cdot 10^6 \pm 8.9 \cdot 10^4$
After fixation & washing **(biological replicates; n = 5)**	$5.93 \cdot 10^5 \pm 8.7 \cdot 10^4$	$1.50 \cdot 10^6 \pm 1.9 \cdot 10^5$
Loss percentage	41.9%	13.6%

does not remain in the cell suspension while a thorough washing procedure may affect the number of cells. A single or double washing step was explored with the aim to effectively remove the unbound immunoprobes and simultaneously minimize cellular breakage and loss. By comparing the results of these two steps, it was observed that a double washing step dropped the number of $^{56}Fe^+$ detected cell events from 123 to 50. Therefore, the double washing step was discarded, and instead the cell suspensions were washed once prior to sc-ICP-MS analysis as both the number of $^{197}Au^+$ and $^{56}Fe^+$ detected cell events agreed. Therefore, it was observed that around 70% of the HRPEsv cells were lost by performing two washing steps and, therefore, one washing step with PBS 1x, spin at 100 g in the centrifuge and resuspension of the cellular pellet with the buffer was selected as the optimal washing step.

3.1.3. Sample introduction set-up

The samples were directly introduced from the Eppendorf tubes where the HRPEsv cell aliquots were stored and prepared. To ensure the load of a homogeneous cell suspension into the ICP-MS as well as no-sedimentation and cell integrity, a soft shaking was continuously applied to the tubes using a vortex. In addition, two sample introduction configuration set-ups were compared: (i) an external peristaltic pump to deliver the HRPEsv cell suspension at 20–25 μL min^{-1}, and (ii) self-aspiration directly from the Eppendorf tube at 40 μL min^{-1}.

In the case of using the external peristaltic pump, a relatively long tubbing was needed, resulting in significant adherence of the cells onto the tube walls. For this reason, frequent and time-consuming cleaning steps were carried out between samples. In contrast, the use of self-aspiration proved to be a better choice for sc-ICP-MS analysis of individual HRPEsv cells. The flow rate was two-fold higher compared to the use of the peristaltic pump, but considering the concentration of the cells introduced into the ICP-MS and the ICP-MS dwell time, it can be assured that less than one cell per dwell time entered into the ICP-MS. The transport efficiency was evaluated by measuring a suspension of a reference material (60 nm AgNPs; 50 ng L^{-1}). Experimental results showed a transport efficiency of 23.8% for the use of the self-aspiration set-up.

3.2. Optimisation of the sample dilution for sc-ICP-MS analysis

The detection of only one cell per event is required for accurate sc-ICP-MS analysis. The selection of an adequate concentration of HRPEsv cells in suspension was performed using a serial dilution with CT and IL1α–treated cells, acquiring both $^{197}Au^+$ (label on the immunoprobes) and $^{56}Fe^+$ (naturally present in the cells) signals. Comparable numbers of cell events must be obtained for both isotopes by sc-ICP-MS, providing that the conditions selected for the analysis are optimized correctly. As an example, Fig. 1 shows the time-resolved profiles obtained for $^{197}Au^+$ by sc-ICP-MS analysis for different serial dilutions of CT HRPEsv cells (AuNCs:anti-h-APOE immunoprobe was used in this case). As can be seen, the number of cell events increased from $1 \cdot 10^4$ cell mL^{-1} (Fig. 1A) up to $1 \cdot 10^6$ cell mL^{-1} (Fig. 1E) as the concentration of the cells in the suspension increased (the same pattern was found for $^{56}Fe^+$ signal, data not shown).

After data analysis, once the threshold was applied to the raw data to subtract the background (ionic Au) and determine the number of cell events, a linear increase in detected cell numbers with the sample concentration, from $1 \cdot 10^4$ to $5 \cdot 10^5$ cell mL^{-1} (depicted in Fig. 2 as circles) were observed. Nevertheless, the $^{197}Au^+$ average intensity was constant with dilution (depicted as the bar graph in Fig. 2). In addition, the signal coming from the dissolved Au was always below 10 counts, thus demonstrating that the AuNC immunoprobe was efficiently labelled to the specific protein (APOE in this particular case) inside the cell.

The behaviour observed for HRPEsv cell concentrations ranging from $1 \cdot 10^4$ to $5 \cdot 10^5$ cell mL^{-1} was not followed for the highest concentration ($1 \cdot 10^6$ cell mL^{-1}): the number of events did not show a linear relationship when compared with the dilutions and the $^{197}Au^+$ average intensity signal increased almost two-fold. As it can be seen at the enlarged $^{197}Au^+$ time-resolved profile (Fig. 1F) for such sample, some events were defined by several acquisition times, which is not the case for the lower cells concentration. For the highest concentration cell sample, a high probability for overlapping of two cells in the same event using the established analysis conditions (10 ms dwell time and 0.04 mL min^{-1} flow rate) can take place. Thus, the concentration selected for further sc-ICP-MS analysis was $1 \cdot 10^5$ cells mL^{-1}, at which time-resolved profile signals are well defined and the possibility of multiple cell overlapping is minimal.

Concerning $^{197}Au^+$ and $^{56}Fe^+$ signals from HRPEsv cell suspensions, results showed that a different discrimination criterion (3σ or 5σ) must be applied to the selected two isotopes for the correct determination of the cell events. When applying the 5σ discrimination criterion for both $^{197}Au^+$ and $^{56}Fe^+$ intensity signals resulting from the analysis of HRPEsv cells, the number of cell events counted with the $^{56}Fe^+$ isotope was lower than with $^{197}Au^+$. This observation can be attributed to an underestimation of the cell events using the 5σ criterion for $^{56}Fe^+$. As can be seen at the $^{56}Fe^+$ time-resolved profile (Fig. 3), several cell events with intensities lower than the (5σ) threshold applied for discrimination can be clearly observed. These events (marked in the graph with arrows) should be included since they represent individual cell detection events. As demonstrated in Fig. 3, by applying the 3σ criterion, a higher number of cell events was detected; a number which compares well to the number of cell events determined for a $^{197}Au^+$ acquisition when a 5σ criterion was applied. This close agreement in the number of cell events was observed for several analyses of CT HRPEsv cell suspensions and is also reflected in the slope obtained from the relationship between the number of cells detected and the cell concentration in the suspension ($^{197}Au^+$ slope = 0.0042; and $^{56}Fe^+$ slope = 0.0041). In addition, it must be noted that the 5σ criterion was used to extract the cell detection events when $^{197}Au^+$ was acquired, allowing to discriminate the cell detection events from the free Au-labelled immunoprobes that were not bound to the cytosolic proteins and were

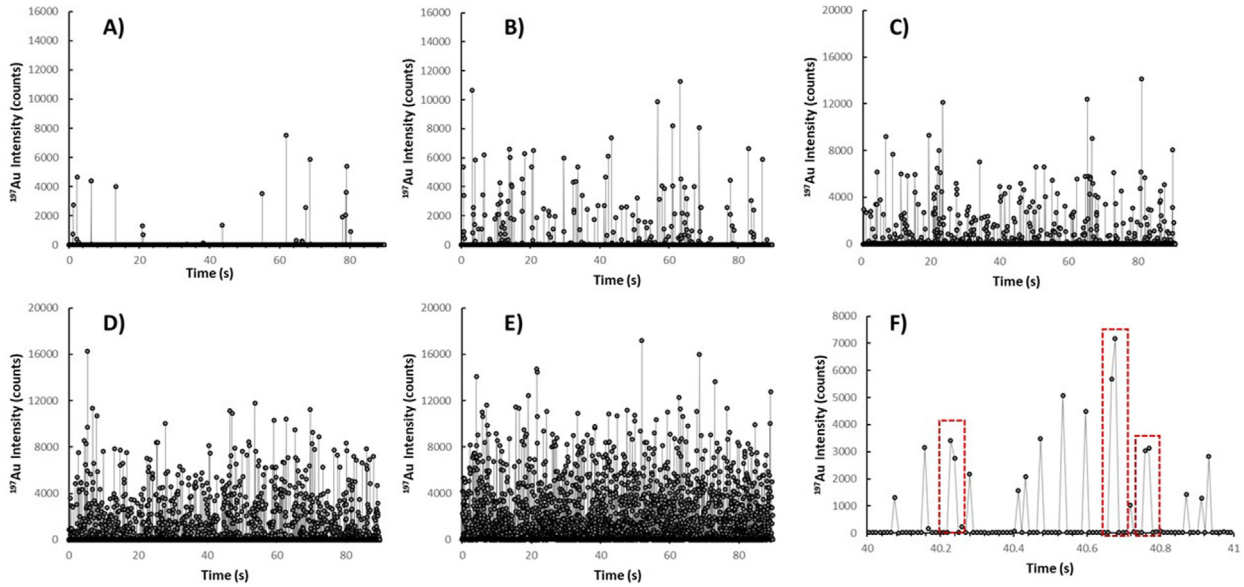

Fig. 1. $^{197}Au^{+}$ time-resolved profiles obtained for the analysis of CT HRPEsv cells (AuNCs:anti-h-APOE immunoprobe) by sc-ICP-MS using serial dilutions for the sample. A) $1{\cdot}10^{4}$ cells mL^{-1}; B) $1{\cdot}10^{5}$ cells mL^{-1}; C) $2.5{\cdot}10^{5}$ cells mL^{-1}; D) $5{\cdot}10^{5}$ cells mL^{-1}; E) $1{\cdot}10^{6}$ cells mL^{-1}; and F) Enlarged profile of the $1{\cdot}10^{6}$ cells mL^{-1} concentration for the acquisition time between 40 and 41 s.

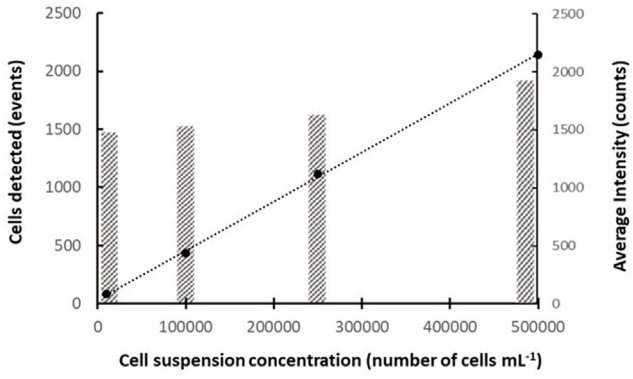

Fig. 2. Trends observed correlating the number of cell events and $^{197}Au^{+}$ intensities for the analysis of CT HRPEsv CT cells (AuNCs:anti-h-APOE immunoprobe) by sc-ICP-MS using different cell suspension concentrations. The number of cells detected in 175 s of acquisition time are shown in the graph as circles and the dotted line (left y-axis), whereas average $^{197}Au^{+}$ signal intensities are depicted as bars (secondary y-axis).

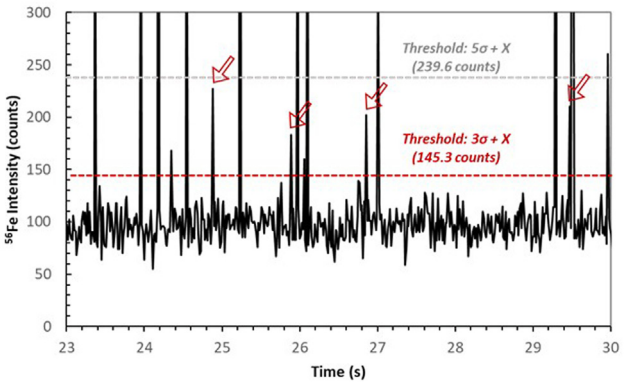

Fig. 3. sc-ICP-MS analysis of CT HRPEsv cells (AuNCs:anti-h-APOE immunoprobe). $^{56}Fe^{+}$ time-resolved profile (23–30 s acquisition time) obtained for the analysis of a cells suspension with $1{\cdot}10^{5}$ cells mL^{-1} concentration. Two thresholds are plotted applying the 3σ and 5σ criteria (as an example, the arrows mark the individual events that are not counted in the case of 5σ criterion).

possibly not removed with the washing steps.

3.3. Determination of APOE and MT2A in individual HRPEsv cells by sc-ICP-MS

The determination of APOE and MT2A was independently carried out in the cell suspensions submitted to the immunoassay with AuNCs immunoprobes. Taking into account that the same AuNCs label is used for the two selected proteins, the HRPEsv cells were divided in two groups for the individual determination of APOE and MT2A using AuNCs:anti-h-APOE and AuNCs:anti-h-MT2A immunoprobes, respectively. Each protein was independently quantified in the cultured cells previously subjected to the pro-inflammatory treatment with IL1α and the non-treated cells (CT).

The data obtained by sc-ICP-MS for $^{197}Au^{+}$ were used to evaluate the possible changes of the APOE and MT2A concentrations (fg of protein/cell) in individual HRPEsv cells after the pro-inflammatory treatment with IL1α. For this purpose, after discriminating the cell events from the background by applying the selected threshold, the $^{197}Au^{+}$ intensity was transformed into absolute mass of Au by the external calibration using ionic Au standards (see the Experimental Methods Section). Finally, the mass of Au was converted into protein mass following a protocol previously reported for tissue sections [28,29]. In the case of AuNCs:h-anti-APOE and AuNCs:h-anti-MT2A immunoprobes, the number of Au atoms per immunoprobe (i.e., the amplification factor) was determined as 466 on average. For an easier comprehension of the methodology, the steps carried out are summarized in Fig. S3.

3.3.1. MT2A concentration in individual HRPEsv cells: CT versus IL1α-treatment

To evaluate if MT2A expression can be induced by a cytokine inflammatory mediator, HRPEsv cells were treated with IL1α. As an example, Fig. 4A and B collect the $^{197}Au^{+}$ time-resolved profiles obtained by sc-ICP-MS for CT and IL1α–treated HRPEsv cells,

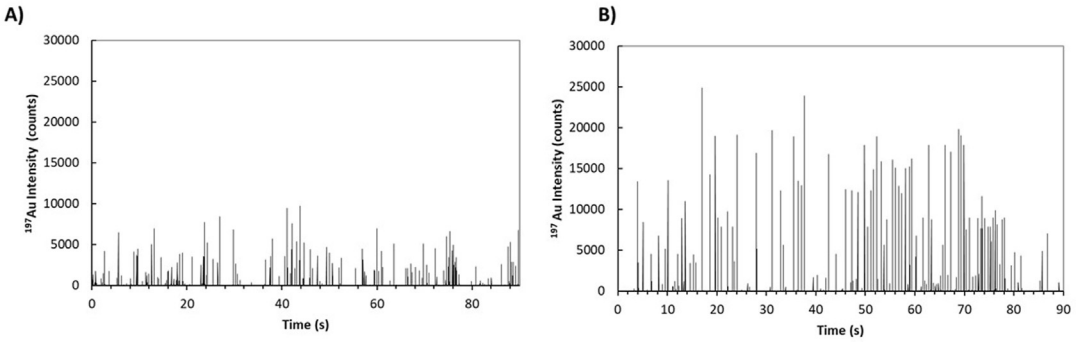

Fig. 4. $^{197}Au^{+}$ time-resolved profile obtained for the analysis of MT2A protein in HRPEsv cells (AuNCs:anti-h-MT2A immunoprobe) by sc-ICP-MS. A) CT cells; B) IL1a−treated cells (100 ng mL^{-1}; 48 h).

respectively. It can be seen that the intensity of the Au events (corresponding to the AuNCs immunoprobe and, thus, to the MT2A) increased with the IL1α treatment (Fig. 4B). This fact suggests an overexpression of MT2A after the pro-inflammatory treatment.

The trend suggested by $^{197}Au^{+}$ intensity signals from qualitative profiles were confirmed after quantitative data analysis (the $^{197}Au^{+}$ intensities were transformed into MT2A concentration, expressed as fg of the protein per cell). Table 4 contains the results obtained by sc-ICP-MS analysis for the MT2A for both CT conditions and IL1α−treated HRPEsv cells (three biological replicates per condition and each of them with three analytical replicates). The treatment of HRPEsv cells with 100 ng mL^{-1} of IL1α for 48 h produced a significant increase of MT2A intracellular levels: a 1.8-fold change was observed between both HRPEsv cells groups. The uncertainty associated to the concentration of MT2A obtained by sc-ICP-MS is high, but it can be mainly attributed to the biological variability between cells suspensions. The standard deviation associated to the MT2A concentration in CT cells was particularly large; CT2 replicate exhibited a concentration significantly lower than other replicates. Such value was included to determine the average protein concentration in the cells, but even discarding this value an overexpression of MT2A in IL1α−treated cells was observed.

To validate the MT2A concentration (fg MT2A/cell) obtained for HRPEsv cells, the average values determined by sc-ICP-MS were compared to those obtained by a quantitative commercial ELISA kit in lysed HRPEsv cells. The MT2A concentration determined by the ELISA kit was found to be 1.74 ± 0.18 fg MT2A/cell for CT HRPEsv cells and 3.36 ± 0.50 fg MT2A/cell for IL1α−treated cells (1.9-fold change), confirming an overexpression of MT2A after the pro-inflammatory treatment. Therefore, the changes observed indicate the same tendency, i.e., there is an overexpression of MT2A after IL1α treatment of HRPEsv cells. Although averaged concentrations obtained by sc-ICP-MS and ELISA analysis were in the same range, some differences can be observed. This can be attributed to the fact that a different cell culture was dedicated for the ELISA measurements as well as to specificities of the used antibodies in each method.

The study of the mass frequency histograms obtained by sc-ICP-MS can provide valuable information to understand the effect of the pro-inflammatory treatment on the HRPEsv cells population. As an example, in Fig. 5 shows histograms obtained for one CT condition and one IL1α−treated cells, representing the percentage of cells containing a certain amount of MT2A (expressed in fg). A high number of cells with very low MT2A amounts were found in both conditions (0.5−1.0 fg MT2A) In addition, it was observed that about 20% of the cells from the total population were significantly affected by the IL1α-treatment, and the mass of MT2A in such cells was in the range of 1.0−3.5 fg of MT2A. This percentage of cells increased significantly the expression levels of the protein, whereas the remaining cell population exhibited similar values for CT and IL1α−treated cells, indicating a different biochemical response of

Table 4
Experimental results obtained for the determination of MT2A in HRPEsv cells by sc-ICP-MS using AuNCs immunoprobe as the elemental label. Three biological replicates per conditions were analysed (CT and IL) and each of such replicates were analysed three independent times (analytical replicates).

	NUMBER OF EVENTS (90 s)	CELL INTENSITY $^{197}Au^{+}$	fg Au/CELL	fg MT2A/CELL	RANGE OF fg MT2A/CELL	INT. AVERAGE fg MT2A/CELL	BIOLOGICAL AVERAGE fg MT2A/CELL
CT1 R1	473	375.6	17.2	1.16	0.032−38.4	1.05	**0.66 ± 0.45**
R2	716	231.1	10.97	1.06	0.092−18.1		
R3	713	210.1	9.94	0.94	0.1−18.87		
CT2 R1	206	43.92	2.07	0.17	0.023−2.7	0.16	
R2	229	44.11	2.08	0.15	0.02−4.8		
R3	–	–	–	–	–		
CT3 R1	368	265.6	12.57	0.83	0.026−28.5	0.77	
R2	396	283.1	13.39	0.76	0.026−27.2		
R3	467	230.6	10.91	0.72	0.023−31.0		
IL1 R1	469	326.0	15.43	1.04	0.026−23.4	1.04	**1.19 ± 0.14**
R2	500	165.9	7.87	0.52	0.026−37.2		
R3	563	140.3	6.64	0.44	0.029−38.72		
IL2 R1	209	86.2	4.08	0.26	0.023−17.5	1.2	
R2	215	47.02	2.22	0.15	0.023−6.48		
R3	483	384.8	18.21	1.2	0.03−48.37		
IL3 R1	422	444.7	21.04	1.38	0.026−30.72	1.34	
R2	455	408.2	19.31	1.27	0.026−33.21		
R3	481	414.3	19.6	1.36	0.026−39.11		

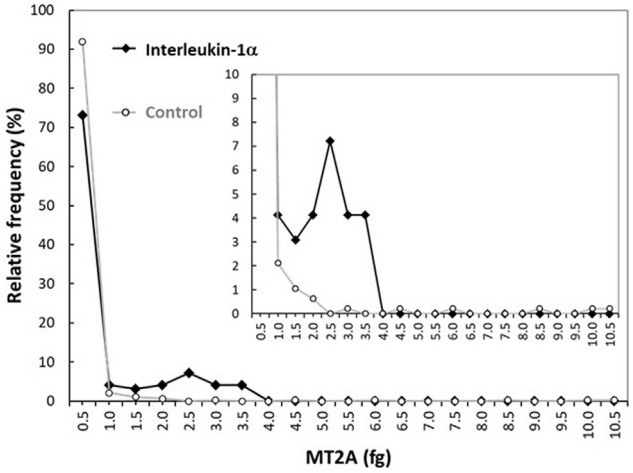

Fig. 5. Mass frequency histogram for MT2A protein in CT (CT2-R2; grey line) and IL1α−treated (IL3-R2; black line) HRPEsv cells obtained by sc-ICP-MS analysis. AuNCs:anti-h-MT2A immunoprobe was employed for the analysis of MT2A protein in HRPEsv cells.

individual cells within a population to a pro-inflammatory stimulus in *in vitro* conditions.

3.3.2. APOE concentration in individual HRPEsv cells: CT versus IL1α-treatment

The content of APOE in HRPEsv cells was determined by sc-ICP-MS using AuNCs:anti-h-APOE immunoprobe to evaluate the influence of the pro-inflammatory treatment with IL1α in individual cells. Fig. S4 (Supplementary Material) shows an example of the time profiles obtained for CT and IL1α−treated HRPEsv cells. Table 5 contains the results obtained by sc-ICP-MS analysis for the APOE in CT and IL1α−treated HRPEsv cells. It can be observed that the ranges and the average values of concentration obtained for APOE in each condition showed a significant decrease in protein expression, suggesting that APOE levels diminished after the pro-inflammatory treatment. These results were validated by the quantification of the APOE in the lysed HRPEsv cells using the commercial ELISA kit. The averaged APOE concentration determined by the ELISA was 17.9 ± 1.3 fg APOE/cell for CT cells and 10.2 ± 0.53 fg APOE/cell for IL1α−treated cells, which were in good agreement with the average protein content determined by sc-ICP-MS. In contrast to the overexpression of MT2A observed after the pro-inflammatory treatment, 0.7 and 0.6-fold changes were observed respectively for APOE by sc-ICP-MS and ELISA between CT and IL1α−treated HRPEsv cells. Finally, Fig. S5 (Supplementary Material) depicts the mass frequency histogram per cell obtained for the APOE by sc-ICP-MS for one CT condition and one IL1α−-treated cells. As can be observed, only around 11% of the cells showed a decrease in protein expression after IL1α treatment. Moreover, the percentage of cells which had a lower concentration of APOE after IL1α treatment was in the range of 40−140 fg APOE.

4. Conclusions

In this work, immunoprobes labelled with monodisperse AuNCs were synthesized and the Au:antibody stoichiometry characterized for the sequential determination by sc-ICP-MS of two cytosolic proteins (MT2A and APOE) in individual HRPEsv cells, comparing their concentrations in cells under pro-inflammatory and control conditions. The quantitative results were successfully corroborated with commercial ELISA kits.

Several aspects of the research presented must be highlighted, including the convenience to set different thresholds to differentiate events from metal atoms intrinsically constituent of the cells from the gold atoms of the immunoprobe (466 atoms of Au on average per probe), as well as the steps required to develop the immunoassay of the cytosolic proteins with a minimum destruction of the cell membrane.

Therefore, this work paves the way for the further analysis of cytosolic proteins, as well as for the use of this characterized AuNCs-labelled immunoprobes for the determination of other type of proteins or other cell cultures by sc-ICP-MS. In addition, the application of labels constituted of other metal NCs (e.g., Pt, Au, Ir) would allow the simultaneous sensitive determination (NCs contain a high number of the detected isotope per label size) of different proteins in single cells when using TOFMS for detection.

CRediT authorship contribution statement

Ana Lores-Padín: Investigation, Methodology, Writing − original draft. **Emmanouil Mavrakis:** Methodology, Validation, Writing

Table 5
Experimental results obtained for the determination of APOE in HRPEsv cells by sc-ICP-MS using AuNCs immunoprobe as the elemental label. Three biological replicates per conditions were analysed (CT and IL) and each of such replicates were analysed three independent times (analytical replicates).

	NUMBER OF EVENTS (90 s)	CELL INTENSITY $^{197}Au^{+}$	fg Au/CELL	fg APOE/CELL	RANGE OF fg APOE/CELL	INT. AVERAGE fg APOE/CELL	BIOLOGICAL AVERAGE fg APOE/CELL
CT1 R1	237	1470.7	69.58	24.99	0.28−230	20.7	**18.1 ± 2.1**
R2	250	1354.88	64.1	23.02	0.25−215		
R3	292	829.57	39.248	14.09	0.23−199		
CT2 R1	−	−	−	−	−	16.6	
R2	222	1091	51.65	18.55	0.24−498		
R3	286	857	40.54	14.56	0.26−284		
CT3 R1	341	901.81	42.67	15.31	0.31−238	17.0	
R2	373	1249	59.13	21.09	0.30−211		
R3	424	864.34	40.89	14.68	0.30−186		
IL1 R1	307	637	34.74	13.10	0.10−111	12.8	**13.3 ± 0.4**
R2	345	752	35.60	12.78	0.09−124		
R3	320	689	35.07	12.59	0.52−169		
IL2 R1	213	786	37.17	13.35	0.23−151	13.5	
R2	180	879	41.58	14.93	0.23−164		
R3	184	728	33.96	12.2	0.26−230		
IL3 R1	313	1011	47.84	17.18	0.29−206	13.7	
R2	349	739	34.53	12.4	0.28−173		
R3	345	678	32.06	11.39	0.27−191		

— review & editing. **Beatriz Fernández:** Supervision, ConceptualizationConceptulization, Writing — review & editing. **Montserrat García:** Resources, Visualization. **Héctor González-Iglesias:** Visualization, Conceptualization. **Rosario Pereiro:** Supervision, Funding acquisition. **Spiros A. Pergantis:** Supervision, Writing — review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2022.339701.

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