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Iridium nanoclusters as high sensitive-tunable elemental labels for immunoassays: Determination of IgE and APOE in aqueous humor by inductively coupled plasma-mass spectrometry

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ABSTRACT

Iridium nanoclusters (IrNCs) stabilized with citrate were synthesized and then the ligand was exchanged by lipoic acid (LA). The IrNCs@LA were bioconjugated through carbodiimide chemistry with specific antibodies to prepare IrNCs-labelled immunoprobes. The IrNCs-immunoprobes were employed in competitive immunoassays for immunoglobulin E (IgE) and apolipoprotein E (APOE) determination by detecting the iridium through inductively coupled plasma – mass spectrometry (ICP-MS). The IrNCs@LA have a 1.89 nm diameter at average and each NC contains 250 Ir atoms. Labelling of specific antibodies with IrNCs was optimized in terms of recognition capabilities and signal amplification by ICP-MS. Amplification and detection limits can be tuned by selecting the IrNCs:Ab molar ratio. An immunoprobe prepared by mixing a 10:1 IrNC:Ab molar ratio was selected for the determination of IgE and APOE in aqueous humor, achieving a signal amplification of 1760 iridium atoms per molecule of the sought protein and limits of detection in the tens of pg mL^{-1} of protein. The IrNCs-immunoprobes were evaluated for IgE determination in serum samples as well as for IgE and APOE in aqueous humor (from controls subjects and patients affected by primary open angle glaucoma) by ICP-MS, being required just sample dilution as pre-treatment. Results were corroborated with commercial ELISA kits.

1. Introduction

Glaucoma is a complex group of neurodegenerative eye disorders of multifactorial origin characterized by optic nerve damage, retinal ganglion cell death and irreversible loss of visual field [1]. Among all the subtypes of glaucoma, primary open angle glaucoma (POAG) is the most prevalent in developed countries [2], which causes excessive production of aqueous humor or outflow system obstruction. Dysfunction in the aqueous humor outflow results in the elevation of the intraocular pressure, which is considered the main risk factor for glaucoma disease. The aqueous humor fluid, secreted by the ciliary epithelium, contains proteins, metabolites, and essential elements, which content may be altered during glaucoma development [3]. Many candidate glaucoma biomarkers identified in eye tissues and biofluids have been published up to date [4]. Among the most frequently biomarkers identified in aqueous humor, higher levels of apolipoprotein E (APOE) were observed in POAG patients [5,6]. This major apolipoprotein of the central nervous system controls the homeostasis of cholesterol and other lipids, whose up-regulation may be related with the microglial reactivity during glaucoma [7,8]. Similarly, altered immune response in the aqueous humor has been related to glaucoma neurodegeneration (differences in antibody patterns increased evidence for an autoimmune involvement in the pathogenesis of the disease [9,10]). In this line, a recent investigation suggested a possible association between glaucoma and immunoglobulin E (IgE) response to allergens [11].

However, current methods for the quantification of APOE and IgE provide a high heterogeneity of results depending on the methodology used. Concentration of APOE in aqueous humor has been reported to be

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below 500 ng mL^{-1} [12], while IgE is present in the aqueous humor proteome at a very low concentration (below 1.8 ng mL^{-1}) [13]. In addition, it must be kept in mind that just a few microliters of aqueous humor can be obtained from the anterior chamber in *in vivo* sampling. Thus, highly sensitive analytical techniques requiring a minute amount of sample are needed.

In the last years, inductively coupled plasma – mass spectrometry (ICP-MS) has shown its excellent capabilities for the sensitive and multiplexed analysis of biomolecules after specific reactions, such as immunoassays with metal-labelled immunoprobes. A wide variety of metal-labels has been investigated for the development of specific probes for ICP-MS detection. The first work was published in 2001 and used Eu^{3+}-labelled streptavidin [14]. Labelling with lanthanide complexes has been widely reported along the years [15]. In order to get a higher signal amplification (i.e., a higher number of the detectable element per immunoprobe), polymers containing several atoms of a given lanthanide have been also developed. Such approach has given rise to the commercial Maxpar® labelling reagents, widely employed in combination with ICP-MS for the analysis of biomolecules in biological fluids and cells [16,17].

The application of metal nanoparticles (NPs), such as AuNPs [18,19], AgNPs and PtNPs [19] as labels for detection of biomolecules by ICP-MS has been also investigated. The approach has been extended to different NPs, including quantum dots (QDs) [20], or $NaEuF_4$, $NaTbF_4$ and $NaHoF_4$ NPs [21], among many other types of NPs [22,23]. However, an inconvenience of QDs and other NPs constituted by a small percentage of the measured element is that a considerable part of such NP-types does not contribute to the amplification of the analytical signal. Therefore, an advantage of metal NPs, compared to those NPs or to chelates and polymers that contain a considerable non-metallic part, is the benefit expected due to the higher ratio of the detected metal atoms per label-size.

The ICP-MS sensitivity will be affected by the detectability characteristics of the isotope acting as reporter (e.g., relative abundance, ionization efficiency) and by the number of reporter metal atoms per immunoprobe. However, the labeling of a biological molecule with a rather "massive" NP can decrease its efficacy as recognition biomolecule. Spherical or quasi-spherical metal nanoclusters (NCs) with diameters below 3 nm constitute a specific type of metal NP [24], whose small size as compared to conventional NPs make them advantageous as labels [25–27].

Previously reported work has shown that ICP-MS limits of detection (LODs) for ^{193}Ir are similar or lower than for ^{197}Au or ^{195}Pt [28–30]. Thus, aiming to increase the availability of different metal NCs as labels to further achieve multiplexed determinations of low levels of proteins by ICP-MS, IrNCs were investigated in this work as innovative immunoprobe labels for the determination of two specific proteins related with glaucoma in human aqueous humor microsamples. The IrNCs have been synthesized, bioconjugated and applied to the challenging determination of APOE and IgE proteins by ICP-MS.

2. Experimental

2.1. Reagents and materials

IrNCs were synthesized employing $IrCl_3{\cdot}xH_2O$ (99.9% crystals, Sigma-Aldrich, USA) as metal precursor, tri-sodium citrate dihydrate (99% crystalline powder, Merck, Germany) and lipoic acid (98% powder, Across Organics, Belgium) as surface ligands and $NaBH_4$ (98% powder, Sigma-Aldrich) as reducing agent. Bioconjugation of the IrNCs to the selected antibodies was performed with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (98% powder, Across Organics) and N-hydroxysuccinimide (NHS) (>98% powder, Sigma-Aldrich). Purification steps were carried out with Amicon ultra centrifugal filter units (3 and 100 kDa pore size, Merck).

The competitive immunoassay for IgE determination was accomplished employing native human (h) IgE protein (Abcam, UK) as standard. The immunoprobe was prepared in our laboratory, labelling a polyclonal anti-h IgE antibody (Ab) (ε-chain specific, produced in goat, Sigma Aldrich) with IrNCs. Similarly, APOE determination was performed employing recombinant human APOE (Active) (Lyophilized, Abcam) and a polyclonal anti-h APOE Ab (produced in goat, Thermo Fischer Scientific, Germany). The assays were carried out on ELISA microtitration plates (96 well, Thermo Fisher Scientific). Bovine serum albumin (BSA) (99% powder, Merck) was used as blocking agent and a washing solution prepared with 10 mM phosphate buffered saline (PBS) solution (pH 7.4) with 0.05% of Tween 20 (Sigma-Aldrich) was used in the washing steps of the immunoassay protocol.

For evaluation of immunoprobe functionality, a polyclonal anti-goat IgG Ab labelled with horseradish peroxidase (whole molecule, produced in rabbit, Sigma-Aldrich) and a TMB substrate kit (Thermo Fisher Scientific) were employed. This study was performed on ELISA microtitration plates (96 well, Thermo Fisher Scientific).

Method validation was carried out with commercial IgE (human) ELISA Kit (Abnova, Taiwan) and APOE (human) ELISA Kit (Cloud-Clone, USA). Other reagents were nitric acid (65%, Merck), sulfuric acid (Thermo Fisher Scientific), sodium hydroxide (97%, Merck). Deionized ultrapure water (resistivity 18.2 MΩ cm^{-1}) (Purelab Flex 3&4, UK) was used.

Aqueous humor samples from glaucoma patients (POAG) and control subjects (CT) were collected from human eyes obtained 24 h postmortem through the National Disease Research Interchange (Philadelphia, PA). The procedures adhered to the tenets of the Declaration of Helsinki on biomedical research involving human subjects. Clinical history of all individuals includes ophthalmic examination for eye disease classification. All of them suffered from cataracts and did not present relevant ocular pathologies such as retinopathies or maculopathies. Once collected, samples were stored at −80 °C until analysis. Full ethical approval was obtained for the Clinical Research Ethics Committee at the Principality of Asturias (Oviedo, Spain).

Table 1
Operating conditions for ICP-MS measurements.

Parameter	Values
ICP RF power (W)	1500
Plasma gas flow ($L{\cdot}min^{-1}$)	15.0
Make up gas flow ($L{\cdot}min^{-1}$)	0.10
Nebulizer gas flow ($L{\cdot}min^{-1}$)	1.07
Isotopes (m/z)	^{193}Ir, ^{195}Pt
Dwell time (ms)	500

2.2. Instrumentation

IrNCs morphology and elemental composition were determined by high-resolution transmission electron microscopy (HR-TEM) (JEOL JEM-F200, Jeol, Tokyo). The instrument is equipped with an energy dispersive X-ray (EDX) detector. An ICP-MS (7900 series, Agilent, USA) was used to quantify IrNCs concentration in the synthesis solution and for the determination of IgE and APOE proteins concentration in the biological samples after the immunoassays with the IrNCs-labelled antibodies. Experimental parameters applied for ICP-MS analysis are collected in Table 1.

Other equipment employed includes an absorbance microplate reader (Victor X5, Perkin Elmer, USA), an ultrasound bath (JP Selecta, Spain), a centrifuge (Gyrozen and Co., South Korea), a hotplate stirrer (Fisher Scientific), a vortex mixer (Labbox Labware, Spain) and a stove (Memmert, Spain).

2.3. Synthesis and bioconjugation of IrNCs

IrNCs stabilized with citrate (IrNCs@citrate) were synthesized

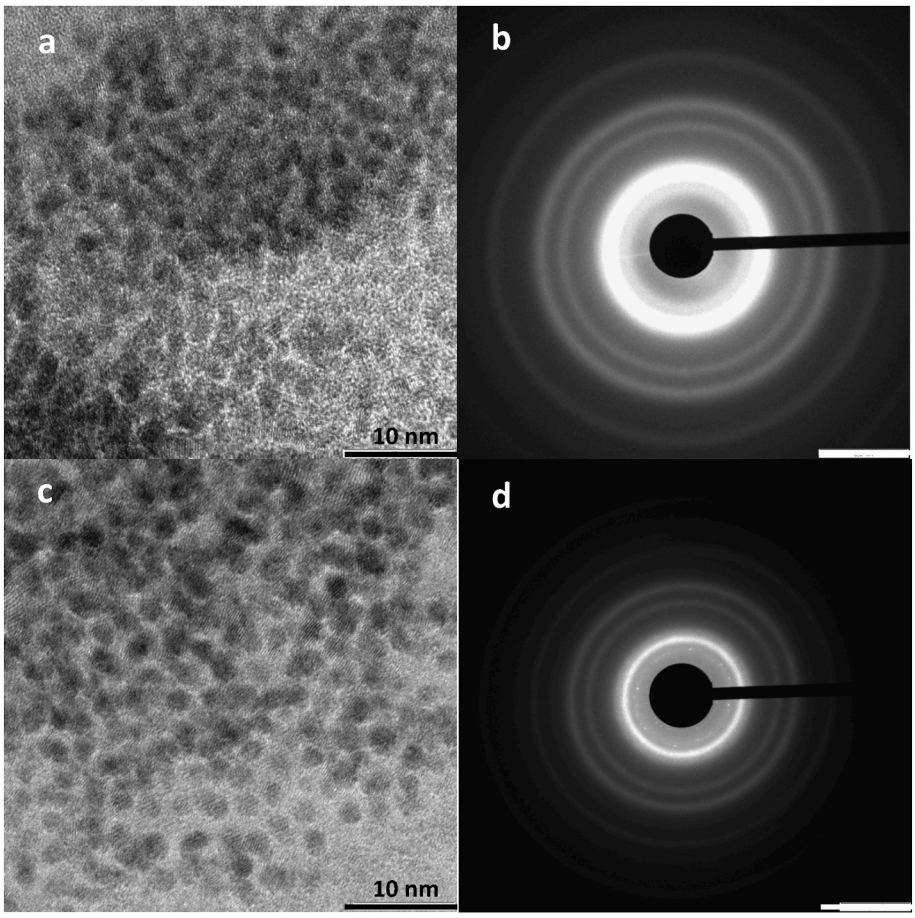

Fig. 1. Morphological characterization of the IrNCs. **(a)** HR-TEM image of IrNCs@citrate; **(b)** SAED pattern for IrNCs@citrate; **(c)** HR-TEM image of IrNCs@LA; **(d)** SAED pattern for IrNCs@LA.

following a slightly modified protocol previously described [31]. In brief, 3 mL 1% m/v trisodium citrate dihydrate solution were mixed with 20 mL 0.03% m/v $IrCl_3{\cdot}xH_2O$. The pH of the resulting solution was adjusted to 8 with 1 M NaOH and heated under reflux for 20 min. Afterwards, 1 mL of a 0.1 M solution of $NaBH_4$ was added dropwise while turning the plate off, allowing the reaction mixture to cool down and stirring was continued for 30 additional min. The resulting IrNCs suspension was purified by ultrafiltration with a 3 kDa Amicon filter to eliminate excess reagents. The purified solution of IrNCs@citrate was stored at room temperature protected from light.

In a second step, the surface of the synthesized IrNCs was modified, exchanging the citrate by a thioalkanoic ligand. Thus, the IrNCs were functionalized with carboxylic groups allowing facile bioconjugation with an Ab by EDC chemistry in a further step. Ligand-exchange approaches were employed in the literature for bioconjugation of initially citrate-stabilized AuNPs [32,33]. For ligand exchange, 10 mL of IrNCs@citrate were placed in a glass vial with 28 μmol of lipoic acid and 4 μL of 2 M NaOH and stirring was maintained for 15 h. The resulting IrNCs protected with lipoic acid (IrNCs@LA) were purified with a 3 kDa Amicon.

The immunoprobes for the immunoassays (IgE and APOE target analysis) were independently prepared by bioconjugation of the specific antibodies to the IrNCs@LA. To this end, 100 μL (100 μg mL^{-1}) of the corresponding Ab were added to an Eppendorf tube with the required volume of IrNCs@LA to obtain the desired NC:Ab molar ratio. Then, 12 μL of a solution containing EDC and NHS (Ab:EDC:NHS 1:1500:1500) in 10 mM PBS (pH 7.4) were added and the resulting mixture was stirred with a vortex for 2 h. Afterwards, the immunoprobes (i.e., IrNCs@LA labelled antibodies) were purified by ultrafiltration with a 100 kDa Amicon filter. The purified immunoprobes were diluted with PBS and stored at 4 °C for 24 h maximum.

2.4. Optimization of the IrNC:Ab molar ratio of the immunoprobe

The recognition capabilities of the immunoprobe were studied as a function of the IrNC:Ab molar ratio added to the bioconjugation reaction. The functionality of the Ab labelled with IrNCs@LA was determined by a spectrophotometric immunoassay employing a secondary HRP-labelled Ab. An IgE standard (10 μg mL^{-1}, 100 μL/well) was immobilized in an ELISA plate and incubated at 37 °C for 2 h. Excess protein solution was removed and 200 μL/well of a 1% BSA solution in 10 mM PBS (pH 7.4) were added to block the remaining sites. The plate was incubated during 15 h at 4 °C. After removing excess blocking solution, 100 μL/well of the IrNCs@LA immunoprobe (10 μg mL^{-1} referred to Ab) were added to the wells and incubated 2 h at 37 °C. In this last step, immunoprobes differing in the IrNC:Ab molar ratio, were employed for optimization purposes. Afterwards, the plate was thoroughly washed with washing solution, 100 μL/well of anti-goat-IgG-HRP (dilution 1:10000 in PBS) were added and the plate was incubated 2 h at 37 °C. Then the plate was washed several times with washing solution and 100 μL/well of the TMB substrate kit were added. The enzymatic reaction was stopped with 2 M H_2SO_4 and the absorbance was read at 450 nm with a microplate spectrometer.

2.5. Competitive immunoassay based on IrNCs

A competitive immunoassay was designed for determination of IgE and APOE. For such purpose, an ELISA microtitration plate was

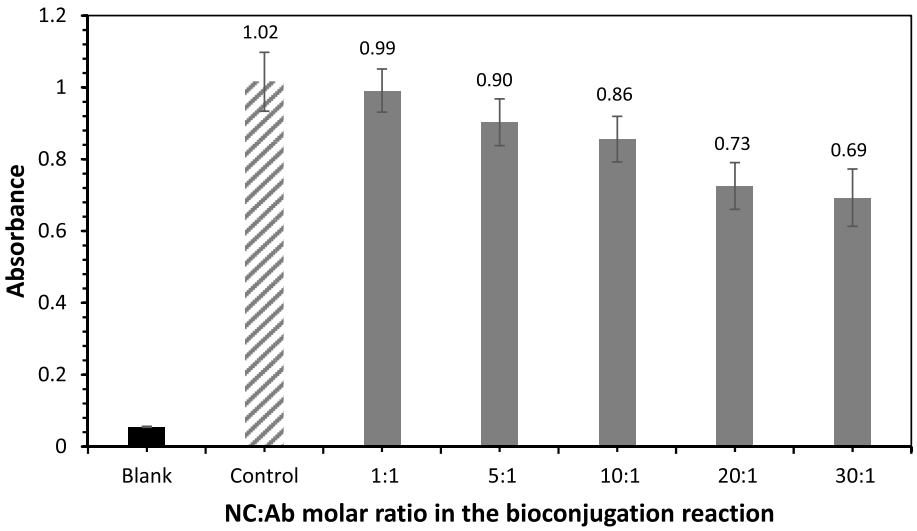

Fig. 2. Results of the spectrophotometric immunoassay for the determination of the recognition capabilities of the IrNCs@LA:Anti-h-IgE immunoprobe as a function of the NC:Ab molar ratio in the bioconjugation reaction. Absorbance of the immunoassay blank (no IgE protein) in black, immunoassay control (non-bioconjugated Anti-h-IgE Ab) in striped pattern, and the different immunoprobes with IrNCs@LA in gray. Uncertainties represent the standard deviations of the mean of three independent measurements.

employed. 100 μL/well of 10 μg mL^{-1} protein standard were incubated for 2 h at 37 °C. After removing excess protein solution, free binding sites on the wells were blocked incubating with 1% BSA solution in PBS for 15 h at 4 °C.

Simultaneously, 100 μL of the specific IrNCs@LA immunoprobe (10 μg mL^{-1} referred to Ab) were incubated in an Eppendorf tube with 100 μL of the protein standard (or sample) and stirred for 15 min with a vortex. Afterwards, this mixture was added to the previously treated wells and incubated 2 h at 37 °C. Then, the wells were exhaustively washed and 50 μL/well of a 2 M HNO_3 solution were added to extract the IrNCs. The Ir concentration was measured by conventional nebulization ICP-MS in each well.

2.6. Determination of IgE and APOE in samples of human serum and aqueous humor

The applicability of the IrNCs@LA-based immunoassay was validated by measuring the IgE concentration in three human sera from healthy subjects without significant pathologies (named S1 to S3). For such purpose, the samples were analyzed both with the developed immunoassay and with a commercial ELISA kit. Dilution of the samples was required to fit the calibration curve of the IrNCs@LA-based immunoassay (S1 was diluted 1:500, whereas S2 and S3 were diluted 1:1000).

In addition, the concentration of IgE and APOE was determined in aqueous humor samples employing the immunoassays based on the IrNCs@LA label. Two groups of samples were studied: control subjects (named CT1 to CT7) and POAG patients (designated as P1 to P5). Aqueous humor samples were properly diluted in PBS to fit the calibration curves; i.e., 1:2 dilution for IgE determination and 1:6000 dilution for quantifying APOE concentration. The values obtained for APOE concentration in the aqueous humor samples were validated with a commercial ELISA kit for APOE human protein (diluting the samples 1:3 in PBS).

3. Results and discussion

3.1. Synthesis and characterization of IrNCs

Citrate stabilized IrNCs (IrNCs@citrate) were synthesized and ligand exchange was carried out to modify the NCs surface with lipoic acid following the steps described in Section 2.3. The formation of the IrNCs was confirmed by HR-TEM. Fig. 1a and 1c shows photographs obtained for IrNCs@citrate and IrNCs@LA, respectively. The size of the IrNCs was determined measuring the diameter of randomly observed IrNCs in the HR-TEM photographs. IrNCs@citrate had an average diameter of 1.95 ± 0.07 nm (determined for n = 300, confidence interval 99%, standard normal distribution), whereas IrNCs@LA were slightly smaller and showed a lower polydispersity, with an average diameter of 1.89 ± 0.03 nm (n = 300, confidence interval 99%, standard normal distribution). The small difference observed for the IrNCs diameter and polydispersity comparing IrNCs@citrate and IrNCs@LA could be attributed to the fact that at the conditions of the ligand exchange reaction it is possible that the lipoic acid is acting as an etching agent and, thus, the size and polydispersity of the IrNCs can be slightly reduced. Both types of IrNCs show a face centered cubic crystal lattice, proved measuring the $d_{spacing}$ values from the selected area electron diffraction (SAED) pattern shown in Fig. 1b and 1d (IrNCs@citrate and IrNCs@LA, respectively). The success of the ligand exchange was confirmed with EDX analysis. In Figure S1 (Electronic Supplementary Material, ESM) it can be observed that IrNCs@LA contain sulfur in their surface composition, whereas no sulfur was detected in the IrNCs@citrate. Sulfur is an element present in the lipoic acid molecule, whereas citrate does not contain any sulfur atom in its structure, thus confirming the presence of a lipoic acid coating after the ligand exchange reaction.

In order to determine the IrNCs concentration in solution, the concentration of elemental Ir in three replicates of the purified IrNCs solutions was measured by ICP-MS after an acid digestion of the samples. Experimental results showed an Ir concentration of 0.47 ± 0.02 mM and 0.38 ± 0.04 mM for IrNCs@citrate and IrNCs@LA, respectively. Calculations to obtain the number of Ir atoms per NC are collected in the ESM. The quotient between the elemental concentration of Ir in solution and the number of Ir atoms per NC (274 atoms of Ir per IrNC@citrate, and 250 atoms of Ir per IrNC@LA) gives the concentration of IrNCs in the synthesis solution: $1.71 \cdot 10^{-6}$ mol NC L^{-1} for IrNCs@citrate and $1.51 \cdot 10^{-6}$ mol NC L^{-1} for IrNCs@LA. Such information is necessary to achieve reproducible bioconjugation processes for IrNCs prepared in different batch synthesis or in different laboratories.

3.2. Selection of the optimal NC:Ab ratio in the IrNCs immunoprobe

The NC:Ab molar ratio in the bioconjugation reaction must be optimized in order to maintain the recognition capabilities of the Ab, while having the maximum number of Ir atoms per Ab to achieve a greater amplification. For optimization purposes IrNCs@LA were bioconjugated with an Ab against human IgE (IrNCs@LA:Anti-h-IgE immunoprobe), as this particular Ab had been successfully labelled with other metallic NCs, such as AuNCs [25] and PtNCs [27]. The immunoprobes were

Table 2
Study of the influence of the added IrNC:Ab molar ratio to the bioconjugation reaction (IrNCs@LA:Anti-h-IgE immunoprobe) on the performance of the immunoassay for IgE determination. Amplification of the immunoprobe (in molarity), limit of detection and sensitivity are included for comparison between different mixed IrNC:Ab molar ratios.

IrNC:Ab Molar Ratio Added	Amplification of the immunoprobe Ir (M)/IgE (M)	LOD (ng mL^{-1}; IC10)	Sensitivity (ng Ir/ng IgE)
5:1	956	0.02	0.55
10:1	1760	0.03	1.32
20:1	1228	0.15	0.81

prepared *via* EDC crosslinking chemistry by the formation of a covalent bond between the carboxylic group of the lipoic acid coating of the IrNCs and an amino group of the Ab (see procedure in Section 2.3). Different mixed NC:Ab molar ratios in the bioconjugation reaction solution were tested (1:1, 5:1, 10:1, 20:1 and 30:1).

The recognition capability of the IrNCs@LA:Anti-h-IgE immunoprobes as a function of the added NC:Ab molar ratio was studied with a spectrophotometric immunoassay, following the steps indicated in Section 2.4 (the absorbance of the wells is proportional to the functionality of the Ab). It was observed that the Ab functionality decreased as the NC:Ab molar ratio in the immunoprobe increased, being particularly low for NC:Ab ratios higher than 10:1 (see Fig. 2). The loss of functionality when increasing IrNC:Ab molar ratio can be attributed to the fact that the iridium nanoclusters block the recognition sites of the Ab molecules.

3.3. Competitive immunoassay based on IrNCs@LA label for protein determination in serum and aqueous humor

A competitive immunoassay was developed for protein determination employing IrNCs@LA immunoprobes. For optimization purposes, the IrNCs@LA bioconjugated to Anti-h-IgE Ab was investigated. Three different immunoprobes were assayed for the determination of IgE. The immunoprobes differed in the NC:Ab molar ratios (5:1, 10:1 and 20:1) added to form the immunoprobe. The 1:1 NC:Ab molar ratio was discarded as the yield of the bioconjugation reaction is expected to be lower than 100%, implying that some Ab molecules are not labelled. On the other hand, NC:Ab molar ratios higher than 20:1 were not considered for the immunoassay due to the loss of the recognition capabilities of the Ab (Fig. 2).

The immunoassays were performed following the procedure detailed in Section 2.5. The Ir concentration in the wells was determined by ICP-MS and the calibration graph was fitted to a 4-parameter logistic (4 PL) regression with MyCurveFit. The plot of IgE molarity against detected metal molarity fitted a linear trend for the interval IC20-IC80 of the 4 PL curves. The slope of this linear interval corresponds to the metal atoms detected per IgE molecule (i.e., the amplification achieved with the immunoprobe) [27]. It can be observed in Table 2 that the amplification increased when raising the NC:Ab ratio from 5:1 to 10:1. However, a lower amplification was obtained for the 20:1 NC:Ab molar ratio, probably due to the important loss of functionality of the Ab at such ratio (Fig. 2).

The analytical performance of the immunoassay in terms of sensitivity and LOD was studied as a function of the IrNC:Ab molar ratio in the bioconjugation reaction. Experimental results showed that the sensitivity of the assay was proportional to the amplification achieved by the IrNCs labelled immunoprobes using 5:1 and 10:1 M ratios. As can be observed in Table 2, the sensitivity increased from 0.55 ng Ir/ng IgE to 1.32 ng Ir/ng IgE when increasing the IrNC:Ab molar ratio from 5:1 to 10:1. However, when IrNC:Ab molar ratio was increased up to 20:1, the sensitivity dropped to 0.81 ng Ir/ng IgE. Therefore, it can be stated that the sensitivity of the assay was determined by the number of iridium atoms detected per each protein molecule. However, at the same time there is a compromise between the number of IrNCs per Ab and the functionality of the labelled Ab (that was lower when increasing the number of IrNCs per Ab; see Fig. 2). Rising the IrNC:Ab molar ratio from 5:1 to 10:1 supposes a greater number of iridium atoms labelling the Ab molecules with a slight decrease of the Ab functionality. Nevertheless, despite having a larger number of iridium atoms per Ab at the IrNC:Ab molar ratio 20:1, the loss of functionality (around 30% compared to the non-labelled Ab) is dominating the compromise and the sensitivity achieved was lower compared to the 10:1 immunoprobe (0.81 versus 1.32 ng Ir/ng IgE). On the other hand, the LOD of the assay seems to mainly depend on the functionality of the immunoprobe and a lower limit of detection was found at higher Ab functionality (i.e., at lower IrNC:Ab molar ratios).

In this way, the analytical performance of the immunoassay can be tuned by changing the IrNC:Ab molar ratio of the immunoprobe in order to best fit the particular sample and analyte. For example, an IrNC:Ab molar ratio of 5:1 offers the best LOD sacrificing the sensitivity, but this could be of interest when analyzing proteins present in very low concentration (e.g., determination of proteins in cell media or cell lysates). Nevertheless, for the analysis of samples with larger protein concentration, an IrNC:Ab ratio of 10:1 should be employed as it provides the maximum sensitivity.

As it can be seen in Table 4, the LOD obtained with the IrNC:Ab molar ratio of 5:1 was lower than for all the representative articles collected in the Table. Regarding the amplification of the assay, the small size of IrNCs allows having a large number of detectable atoms in the Ab without hindering the recognition capabilities. Employing a 10:1 IrNC:Ab molar ratio the proposed methodology exhibited an amplification of 1760 detectable Ir atoms per Ab, that is a much higher amplification compared to methodologies which employ chelates [2],

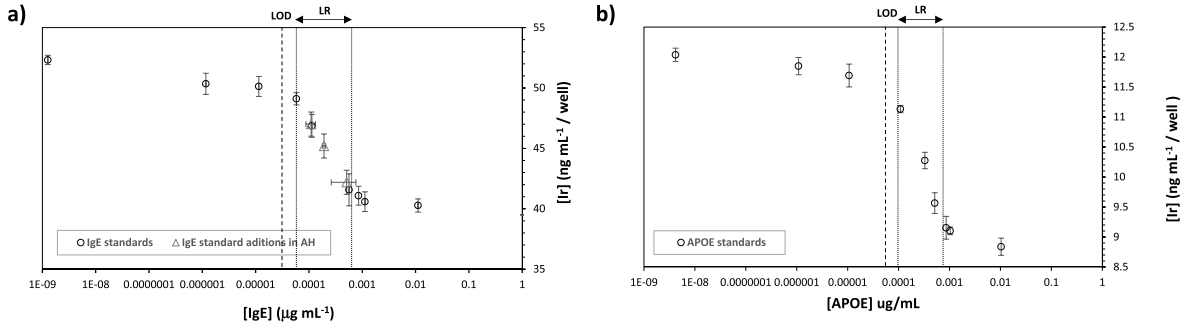

Fig. 3. Calibration curves obtained for the IrNCs@LA-based immunoassays: **(a)** Calibration curve for IgE protein. IgE standards were diluted in PBS (circles). The graph also includes triangles related to an aqueous humor sample (CT7) spiked with different concentrations of IgE standard (1:2 aqueous humor dilution). **(b)** Calibration curve for APOE protein in PBS. Uncertainties in both calibration curves represent the standard deviations of the mean of three independent measurements. LOD: Limit of detection, LR: Linear range.

Table 3
Experimental results obtained for the determination of IgE and APOE in aqueous humor samples using IrNCs@LA immunoprobes (IrNCs@LA-Anti-h-IgE and IrNCs@LA-Anti-h-APOE, respectively) by ICP-MS. Age (y: years old) and gender are collected between parentheses. The Table also collects the values obtained for APOE concentration determined by a commercial ELISA kit. Uncertainties represent the standard deviations of the mean of three independent measurements.

Aqueous Humor Sample	[IgE] (ng mL^{-1}) IrNCs@LA immunoassay	[APOE] (ng mL^{-1}) IrNCs@LA immunoassay	[APOE] (ng mL^{-1}) ELISA Kit
CT1 (80 y, male)	0.52 ± 0.03	437 ± 15	481 ± 73
CT2 (83 y, female)	5.4 ± 0.7	665 ± 5	775 ± 66
CT3 (93 y, male)	–	1388 ± 59	1653 ± 190
CT4 (83 y, female)	0.08 ± 0.01	741 ± 18	736 ± 19
CT5 (69 y, male)	0.65 ± 0.04	–	–
CT6 (76 y, male)	0.54 ± 0.08	–	–
CT7 (70 y, female)	0.24 ± 0.03	641 ± 7	674 ± 19
P1 (81 y, male)	0.27 ± 0.03	1031 ± 21	–
P2 (79 y, male)	1.31 ± 0.07	555 ± 49	–
P3 (70 y, female)	–	915 ± 25	961 ± 33
P4 (53 y, female)	1.11 ± 0.04	755 ± 30	713 ± 28
P5 (79 y, female)	0.43 ± 0.03	777 ± 9	–

QDs [3] or iodinated antibodies [6].

3.4. IgE and APOE determination in human serum and aqueous humor

The applicability of IrNCs@LA immunoprobes as elemental labels for ICP-MS detection was initially evaluated by quantifying IgE in serum samples, as the concentration levels of this protein in human serum have been thoroughly studied as an allergy biomarker [34]. In addition, IgE was determined in aqueous humor samples from CT and POAG patients. Finally, the determination of APOE in aqueous humor samples from CT and POAG patients was also addressed. For each protein, IgE and APOE, the corresponding IrNCs@LA immunoprobes (IrNCs@LA:Anti-h-IgE and IrNCs@LA:Anti-h-APOE) were synthesized by mixing a 10:1 NC:Ab molar ratio in the bioconjugation reaction because this provides the maximum sensitivity while the LODs are low enough to analyze both proteins.

3.4.1. IgE determination in serum and aqueous humor

The procedure for the analysis of IgE in sera using IrNCs@LA:Anti-h-IgE immunoprobe was detailed at Section 2.5. A calibration plot was constructed by representing the concentration of Ir in the wells as a function of the concentration of the IgE standards (see Fig. 3a). The human serum samples (previously diluted in PBS to fit the calibration curve) were analyzed with the immunoassay without any sample pretreatment. The results obtained with the IrNCs@LA-based immunoassay by ICP-MS were corroborated with experimental results obtained for the same samples by a commercial ELISA kit. As it can be seen in Table S1 (ESM), the results obtained with both methods are comparable, thus proving the applicability of the assay using IrNCs@LA labels. No significant differences were found applying a paired sample T-test at 95% confidence to the experimental results obtained with both methodologies, ELISA kit and IrNCs based immunoassay. Therefore, it can be stated that no matrix effects for IgE determination were present using the proposed method when analyzing serum samples.

For the analysis of IgE in aqueous humor samples, the presence of possible matrix effects was evaluated prior to the determination of IgE concentration in the samples. To the best of our knowledge there are no commercial kits with a low enough LOD for determining IgE protein in aqueous humor. Therefore, matrix effects could not be evaluated by comparing the data obtained with our methodology and with a validated method (as done for serum). To evaluate such matrix effects, known concentrations of IgE were added to a 1:2 PBS diluted aqueous humor sample. The data obtained for the IgE spiked aqueous humor aliquots (represented as triangles) are depicted in Fig. 3a. The points fitted into the calibration curve for IgE in PBS and, therefore, it can be concluded that no matrix effects were present. Thus, it is possible to quantify the IgE concentration in aqueous humor samples by referring it to a calibration graph made in PBS (Fig. 3a). The IgE concentration was studied in aqueous humor samples from six CT and four POAG patients (samples CT3 and P3 were not measured as there was limited volume available and they were exclusively employed for APOE determination) with the IrNCs@LA-based immunoassay. According to Shahidullah et al. [13], IgE levels in aqueous humor from healthy patients are lower than 0.75 iU mL^{-1} (1.8 ng mL^{-1}). Except for sample CT2, the experimental values obtained for IgE in the analyzed aqueous humor samples were below the previously reported levels (see Table 3). A T-test at 95% confidence assuming unequal variances was applied to the values obtained for control and glaucoma patients. No significant differences were found among the two groups. However, the number of analyzed samples was quite limited and a larger cohort needs to be studied in order to accurately assess whether there are differences in IgE protein concentration in aqueous humor is altered by POAG pathology.

3.4.2. APOE determination in aqueous humor

For the determination of APOE in the aqueous humor samples, the IrNCs@LA:Anti-h-APOE immunoprobe was employed. The calibration graph for APOE in PBS is depicted in Fig. 3b.

The concentrations of APOE in aqueous humor samples determined with the IrNCs@LA-based immunoassay and with a commercial ELISA kit are collected in Table 3 (age and gender characteristics of each subject are indicated in the Table). As there was limited volume for some samples, CT5 and CT6 could not be measured with the IrNCs@LA-based immunoassay. For the same reason, not all the POAG samples were validated with the ELISA kit (only APOE concentration in P3 and P4 samples were determined with the two methodologies). The APOE concentration values determined with the immunoassay were in accordance with those measured with the commercial ELISA kit. A paired sample T-test was applied to the samples that were measured with both methodologies (CT1, CT2, CT3, CT4, CT7, P3, and P4) and no significant differences were obtained with a 95% confidence. Therefore, no matrix effects were present in the IrNCs based methodology for the determination of APOE in aqueous humor samples. The experimental values obtained in the current study were higher than those reported by Nowak et al. [12] and Inoue et al. [6]. Limited publications tackled the determination of APOE levels in aqueous humor showing high heterogeneity. This heterogeneity may be related with sampling methods, or with the analytical platform used where the proper selection of antibodies with high specificity is mandatory.

It has been previously reported that POAG patients show a higher APOE concentration compared to CT subjects [5,12]. The average APOE concentration observed in our experiments for all controls was 774 ± 361 ng mL^{-1}, while the average APOE concentration for POAG patients was 807 ± 180 ng mL^{-1}. No significant differences were found between control and POAG groups when applying a T-test at 95% confidence assuming equal variances. Nevertheless, the number of analyzed samples is limited and a larger cohort should be studied to correctly determine differences in APOE expression between CT and POAG patients. However, it could be worth to note the particular high concentration of APOE (1388 ± 59 ng mL^{-1}) obtained for the older CT subject (CT3, 93 years old). The average of all controls, except for this 93 years old CT which concentration is well above the average, is 621 ± 130 ng mL^{-1}. Therefore, the removal of this older individual allows some increase of the differences observed between both cohorts.

Table 4
Comparison of different analytical methodologies based on elemental-tagged immunoassays with ICP-MS detection for the determination of proteins in biological fluid samples.

Elemental tag	Analyte (A) & sample (S)	Brief description of the analytical method & analytical performance characteristics	Ref.
Progesterone labelled with CdSe/ZnS QDs	A: Progesterone S: Milk	Indirect immunoassay with ICP-MS detection of ^{114}Cd. LOD 0.028 ng mL^{-1}.	[35]
Anti-Ferritin Ab labelled with $[Ru(bpy)_3]^{2+}$	A: Ferritin S: Human serum	Sandwich immunoassay employing magnetic beads with isotope dilution ICP-MS detection of Ru. LOD 0.48 ng mL^{-1}; 23 mol of Ru per mole of Ab.	[36]
Biotinylated secondary Ab bioconjugated to streptavidin modified CdSe/ZnS QDs	A: Tf, C3, APOA1, TTR and APOA4 proteins S: Human serum	Sandwich immunoassay with ICP-MS detection of Cd. LOD 0.18 ng mL^{-1} for Transferrin. Amplification 153 Cd atoms per QD.	[37]
Anti IgG secondary Ab labelled with AuNPs	A: Caspase 3 S: HepG2 cell lysates	Non-competitive immunoassay with ICP-MS detection of Au. LOD 0.42 ng mL^{-1}.	[38]
AuNPs conjugated with mAb-HA	A: H9N2 virions S: Chicken dung	Sandwich immunoassay employing magnetic beads with ICP-MS detection of Au. LOD 0.63 ng mL^{-1}.	[39]
Iodinated anti-transferrin Ab	A: Ferritin S: Breast cancer cells	Sandwich immunoassay with ICP-MS detection of I. LOD 140 ng mL^{-1} 27.3 mol I per mole of Ab.	[40]
Anti-CFH labelled with AgNCs	A: CFH S: Human Serum	Competitive immunoassay with ICP-MS detection of Ag. LOD 0.4 ng mL^{-1} of CFH. 753 Ag atoms per Ab.	[26]
Anti-IgE Ab labelled with PtNCs	A: IgE S: Human serum	Competitive immunoassay with ICP-MS detection of ^{195}Pt. LOD 0.08 ng mL^{-1} of IgE. 1264 Pt atoms per Ab.	[27]
Anti-PGI and Anti-PGII labelled with Eu^{3+} and Sm^{3+} respectively.	A: PGI/PGII S: Human serum	Sandwich immunoassay employing magnetic beads with ICP-MS detection of Eu and Sm. LOD 1.8 ng mL^{-1} for PGI and 0.3 ng mL^{-1} for PGII.	[41]
Anti-IgE Ab labelled with IrNCs	A: IgE S: Aqueous humor	Competitive immunoassay with ICP-MS detection of ^{193}Ir. **IrNC:Ab molar ratio of 5:1; LOD 0.02 ng mL^{-1}** for IgE; 956 Ir atoms per Ab. **IrNC:Ab molar ratio of 10:1;** LOD 0.03 ng mL^{-1} for IgE; **1760 Ir atoms per Ab**	This work

4. Conclusions

IrNCs@LA (1.89 nm diameter) ready for bioconjugation through their carboxylic groups have been synthesized. The IrNCs@LA were bioconjugated by EDC chemistry with antibodies which specifically recognize a protein of interest. The selected IrNCs@LA-based immunoprobe (offering an average amplification of 1760 iridium atoms per detected protein molecule) was successfully applied to the determination of APOE and IgE in human aqueous humor through competitive immunoassays and ICP-MS detection of iridium. The number of samples analyzed was small; however, the results point out towards the interest to analyze larger cohorts, as well as to investigate the age-dependency of APOE levels in aqueous humor and for other neurodegenerative diseases.

The Ab recognition capabilities and amplification power of the immunoprobe strongly depends on the IrNC:Ab molar ratio employed in the bioconjugation reaction. Thus, LODs and sensitivity can be tunable to the particular analytical requirements. LODs obtained with the proposed approach are much lower than those reported with metal NCs such as AuNCs [25] or AgNCs [26] for the determination of proteins by fluorescence. In addition, the selected immunoprobe offers low limits of detection compared to the representative references collected in Table 4 (i.e. five times better LODs than the obtained by ICP-MS using PtNCs [27]). Therefore, the low LODs achieved point out to a further use of IrNCs@LA as labels for the determination of very low concentrations of specific proteins and for other types of microsamples, such as nasal exudate or single cells.

CRediT author statement

Paula Menero-Valdés: Investigation, Methodology, Writing - Original Draft. **Ana Lores-Padín**: Methodology, Validation, Review. **Beatriz Fernández**: Supervision, Conceptulization, Writing - Review & Editing. **Héctor González-Iglesias**: Visualization, Conceptualization. **Rosario Pereiro**: Supervision, Funding acquisition, Review.

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

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