PO-IM-01 : DEVELOPMENT OF NEW BIOIMAGING METHODOLOGIES BY LA-ICP-MS TO STUDY THE ROLE OF ZINC IN AGE-RELATED MACULAR DEGENERATION

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SUMMARY

In this contribution it is presented our research with laser ablation - ICP-MS addressed towards the development of new methodologies for simultaneous elemental and molecular bioimaging. The final aim of this work is to develop improved analytical tools to study the role of zinc in age-related macular degeneration, an ocular pathology which is one of the leading causes of progressive and irreversible central vision loss.

Keywords: Laser ablation; ICP; mass spectrometry, bioimaging, biological tissues.
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INTRODUCTION

Nowadays, bioimaging studies are of crucial interest in biomedical research because the investigation of elemental and molecular distribution along biological tissues and cells could help to understand complex cellular processes and could bring some light into dramatic diseases such as cancer, neurodegenerative processes, etc. Currently, the combination of laser ablation (LA) with inductively coupled plasma – mass spectrometry (ICP-MS) is established as a multi-elemental direct-solid analysis technique at trace and ultratrace levels that allows obtaining high spatially-resolved images of micrometer scale structures. In addition, one of the main advantages of LA-ICP-MS for the analysis of biological samples is that the measurements are carried out directly on the tissue surface preserved in paraffin or cryogenic conditions, so reducing the time consumed during sample preparation [1,2]. Moreover, combination of both, elemental and molecular information can be achieved with LA-ICP-MS.

In our case, we employ LA-ICP-MS aiming to a better understanding of the processes involved in age-related macular degeneration (AMD). The main hallmark of this eye disease, one of the leading causes of progressive central vision loss and irreversible blindness worldwide, is the formation of extracellular deposits located between the retinal pigment epithelium (RPE) and Bruch´s membrane, where the accumulation of Zn can reach millimolar levels [3]. Metallothioneins (MTs) are the main cytosolic proteins that serve as Zn-ion sensors, and are involved in neuroprotection and defense mechanisms against oxidative damage. In previous studies we proposed the redox system Zn-MTs as a potential therapeutic target in AMD [4]. Within this context, there is a need for simultaneous co-localization of metals such as Zn and Cu and proteins like MTs, superoxide dismutase and amyloid precursor protein, among others.

In our research we are developing complementary elemental and molecular methodologies, based on LA-ICP-MS, for a better understanding of the role of the system Zn-MTs in the pathophysiology of AMD.

MOLECULAR BIOIMAGING

Bioimaging of Specific Proteins in Human Ocular Tissues

Metal-tagged immunoprobes allow protein bioimaging with LA-ICP-MS. As it is collected in *Figure 1*, interesting advantages could be expected when using LA-ICP-MS as compared to traditional detection modes, such as no problems with the autofluorescence signals typical observed in conventional immunohistochemistry, higher multiplexing capabilities (because there is less risk of spectral overlapping and there is a wide variety of metal isotopes which could be used as tags), and easier quantification procedures. Also, for metalloproteins it can be measured with LA-ICP-MS simultaneously the distribution of the protein and its coordinated metals.

In this context, single metal chelates like DOTA coordinated with lanthanide ions have been proposed for elemental tagging. Polymeric tags containing several metal chelates (such as the commercial MaxPar) have been also proposed because they provide signal amplification; however, these labels have an important non-metallic part, giving rise to a low amplification when small tags are

being used. Such inconvenient could be overcome by tagging the antibody with metal nanoclusters (0.2 – 3 nm of diameter). As compared to metal-containing polymeric tags, the "number of metal atoms/tag size" ratio is higher, so higher amplification can be achieved with a smaller size.

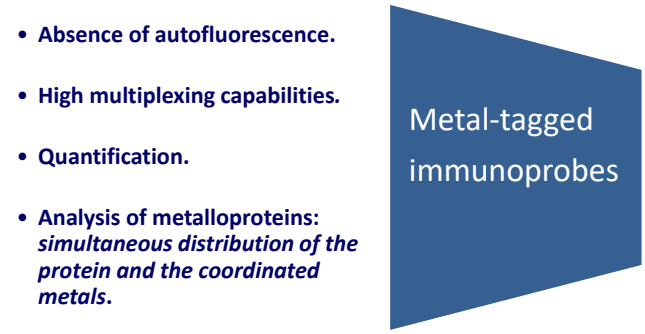

Figure 1. *Expected advantages of using metal-tagged immunoprobes for specific protein bioimaging using LA-ICP-MS in comparison with conventional immunohistochemistry detection.*

We are investigating the use of metal nanoclusters for specific protein binding. In a first approach, we are using 2.7 nm gold nanoclusters (AuNCs) containing about 580 gold atoms each [5]. The nanoclusters are bioconjugated with an appropriate antigen *via* carbodiimide chemistry and the optimized protocol for 5 µm thick tissue sections is very simple (see *Figure 2*) because it does not use a secondary antibody (as it is typically required in conventional immunohistochemistry).

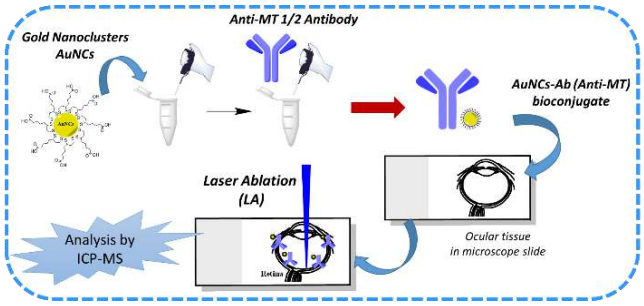

Figure 2. *Scheme followed for the immunoassay in human ocular tissue with antibodies bioconjugated with AuNCs.*

ELEMENTAL BIOIMAGING

Our studies related with elemental distribution are being carried out in two types of samples: human ocular tissues and human RPE cells, both maintained in cryogenic conditions. For such purpose, a home-made cryogenic ablation cell is used [6]. This ablation cell has a small internal volume and a reliable on-sample temperature control. The use of a flexible temperature sensor, directly located on the sample surface, ensures a rigorous sample temperature control throughout the entire analysis time and allows instant response to any possible fluctuation. In this way, reproducibility can be guaranteed during the ablation. The refrigeration of the proposed cryogenic cell combines an internal refrigeration system (controlled by a sensitive thermocouple) with an external refrigeration system. Cooling of the sample is directly carried out by Peltier elements placed below a copper plate where the sample is placed.

Quantitative distribution of Zn in human ocular tissues

Human ocular tissue cryosections of 35 µm thickness were analyzed by LA-ICP-MS to study the distribution of Zn in the regions of the RPE, retina and sclera. The analysis conditions were optimized to get the higher sensitivity and lateral resolution as possible, removing all the sample material. The quantification of Zn was carried out by using matrix-matched standards of gelatin prepared in our laboratory following the protocol schematized in *Figure 3.*

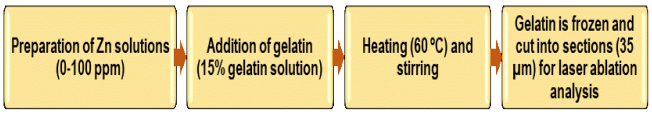

Figure 3. *Protocol followed for the preparation of matrix-matched gelatin standards*

Figure 4 shows an example of the quantitative distribution of Zn obtained by LA-ICP-MS analysis for a human ocular tissue section.

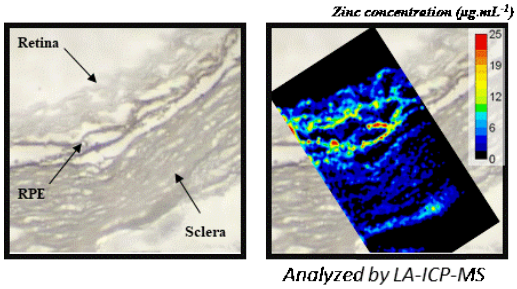

Figure 4. *Example of quantitative image of ^{64}Zn (µg mL^{-1}) in cryogenic human ocular tissue (35 µm thick) obtained by LA-ICP-MS analysis.*

As can be seen in *Figure 4*, there is a higher accumulation of Zn in the region of RPE compared to retina and the sclera. Results were validated determining total concentrations in dissected tissues of RPE, retina and sclera by conventional nebulization ICP-MS after the acidic digestion of the samples [7].

Bioimaging of Zn in human RPE cells

In previous studies, carried out by HPLC-ICP-MS with isotopic dilution analysis in an *in vitro* model of human RPE cells, it was observed that Zn is a potent inductor of MTs. In order to better understand the role of Zn, a novel procedure based on the treatment of RPE cells with

different isotopically enriched isotopes of Zn is being carried out and a quantitative Zn bioimaging methodology by LA-ICP-MS has been developed.

The protocol followed for the induction of MTs in the *in vitro* model of human RPE cells with isotopically-enriched Zn inductors is shown in *Figure 5.*

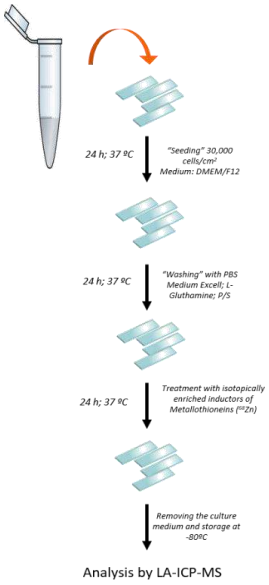

Figure 5. *Protocol followed for the induction of MTs in an in vitro model of human RPE cells.*

Preliminary results show a good lateral resolution that allows distinguishing each cell individually with high sensitivity of signal.

OUTLOOK

Both molecular and elemental bioimaging methodologies based in LA-ICP-MS offer useful information for a better understanding of the processes involved in AMD.

The high signal amplification provided by the use of AuNCs as labels allows to obtaining high-resolution images of proteins by LA-ICP-MS. Moreover, the use metal nanoclusters of other metals (e.g. Pt and Ag) bioconjugated to specific antigens will allow protein multiplexing bioimaging.

On the other hand, quantitative images of Zn obtained by LA-ICP-MS in human ocular tissue sections showed a preferential distribution of Zn in the RPE region compared to the retina and the sclera. Further work will address the quantitative measurement of bioimages of other elements such as Cu and Ca.

Finally, preliminary results of Zn bioimaging in human RPE cells by LA-ICP-MS show 2D-images of cell cultures with a high lateral resolution that allows distinguishing individual cells easily, making feasible to differentiate between natural abundance and isotopically-enriched Zn isotopes inside RPE cells, aiming at a better understanding of the role of zinc in biological and pathophysiological processes, including AMD.

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