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Cell Temperature Gradients Report

First Version

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Abbreviations and Acronyms

ABCP	Amphiphilic Block Copolymer
AC	Alternating Current
AMF	Alternating Magnetic Fields
ABCP	Amphiphilic block copolymer
CSIC	Agencia Estatal Consejo Superior de Investigaciones Cientificas
Eu	Europium
LMP	Lanthanide Molecular Probe(s)
Ln	Lanthanide
MNPs	Magnetic Nanoparticles
NP	Nanoparticle(s)
Sm	Samarium
TMNP	Thermometric Magnetic Nanoparticle(s) (MNPs loaded with LMPs)
TNP	Thermometric Nanoparticle(s) (NPs loaded with LMPs)
UAVR	Universidade de Aveiro
WP	Work package(s)

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Table of Contents

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D5.2 CELL TEMPERATURE GRADIENTS REPORT

Foreword

Work package 5 (WP5) of the project is titled:" Magnetothermal microscope for 2D thermal cellular images and its use for the development of localized intracellular Hyperthermia therapy".

The main objective of WP5 is the construction of a new device for simultaneous ac magnetic field application, luminescent thermal imaging and optical microscopy imaging of cell cultures. The device will be used for the assessment of punctual intracellular magnetic hyperthermia therapies for cancer in combination with chemotherapy and immunotherapy.

The first task (Task 5.1) in WP5 is "Instrument development". This task is described in the project as the construction of an instrument for temperature and luminescence imaging of cells cultures under application of an ac magnetic field. The instrument includes an optical microscope equipped with: i) a temperature system to obtain pixel-to-pixel temperature images and temperature time profiles; ii) a magnetic induction system. This task has already been fulfilled and it is described in deliverable D5.1 "Instruction manuals first edition"

The second task (Task 5.2) is the monitoring of intracellular temperature gradients of magnetic nanoparticles under radiation with radiofrequencies generated by an ac magnetic field generator. The report of the first trial in this direction is the object of this deliverable (D5.2). This report is mainly given by a lead beneficiary of the WP5: Agencia Estatal Consejo Superior de Investigaciones Científicas (CSIC), along with Universidade de Aveiro (UAVR).

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1. Introduction

The first task of WP5 (Task 5.1) was the construction of a new device for simultaneous ac magnetic field application, luminescent thermal imaging and optical microscopy imaging of cell cultures. Within this task, two instruments have been built. The first one, referred as Instrument I is able to apply alternating magnetic fields to cell cultures, and at the same time to perform temperature imaging of cells in the culture. A description of this instrument was included in deliverable D5.1 consisting on a first edition of the instruction manuals for both instruments and presented at month 9.

The second task of WP5 (Task 5.2) was the realization of cell experiments, in which targeted magnetic-induced hyperthermia in breast-cancer cells would be followed by high spatiotemporal resolution luminescence thermometry using Instrument 1 and magnetic nanoparticles produced in tasks 1.4 and 4.1.

This deliverable (D5.2), due for month 12, was meant to describe the first cell temperature imaging experiments during intracellular hyperthermia. Apart from the construction of Instrument 1, which was accomplished on month 12, the realization of these experiments involved the following points:

i) The synthesis of thermometric and magnetic NPs. In this set of thermometric experiments two types of TNPs were contemplated: 1) TNPs consisting of molecular lanthanide complexes embedded in polymer NPs; and 2) TMNPs that are TNPs with a magnetic core in the interior.

ii) The realization of cell cultures, incubation of NPs in the cell cultures, and testing the internalization of these NPs in the cells.

iii) Tests of temperature imaging of TNPs internalized in cells using Instrument 1, and detection of temperature at different temperatures. As described in D5.1, an essential prerequisite for this operation is to ensure that the thermometric probes stand the complex chemical environment of cell cultures, and preserve their luminescence properties in these conditions.

iv) Application of ac magnetic fields to cells cultures and monitoring of intracellular temperature under the action of such fields.

Such an intense activities program was very ambitious for a 3 months period after the delivery of Instrument 1, and the risk of delays due to unexpected troubles was high. As a matter of fact, some trouble appeared with the design of the thermostatic sample holder in Instrument 1, which forced us to redesign the sample holder and caused a delay in the realization of the scheduled program. At the present moment the progress of the proposed activities reached point iii), and we estimate the realization of point iv) in the next 3 months. Therefore, this report describes activities i) to iii), the unexpected problem with the sample holder and its solution, and the readjustment of the project schedule.

Nevertheless, this delay is not compromising at the moment the achievement of the delivering of the next deliverables in due time.

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2. Synthesis of TNPs and TMNPs

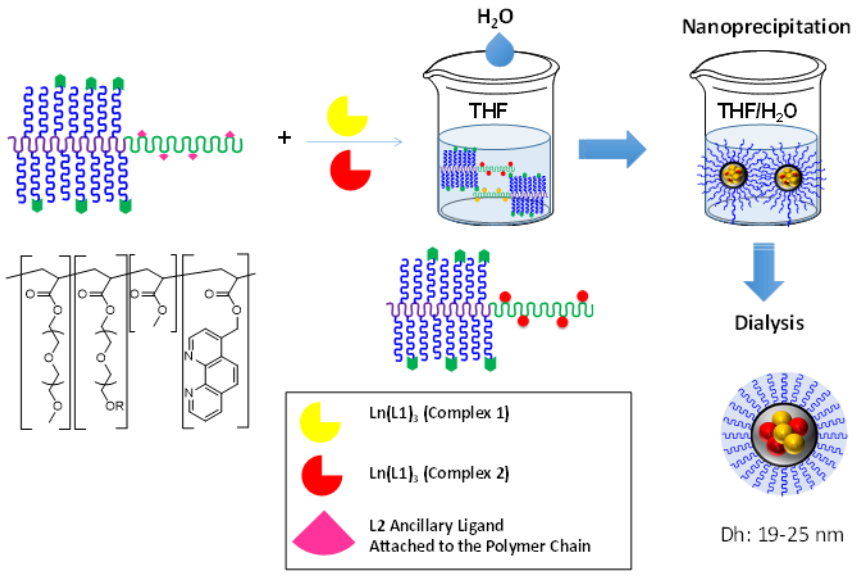
The basic thermometric units in this type of nanothermometers are lanthanide a complexes with light harvesting organic ligands. These complexes are encapsulated in a polymer micelle (TNPs) or in the polymer shell of a magnetic NP (TMNPs). A detailed description of the synthetic procedures and optical properties of these materials was already given in deliverable D1.1.

Molecular lanthanide complexes were designed according to the special requirements for intracellular thermometry: 1) high brilliance; 2) high temperature sensitivity; 3) capacity for cell internalization; and 4) durability under cell chemical environment. In order to achieve these features, several compositions and structures have been tested, and the final choice was DNPD complexes of Eu and Sm [Ln(DNPD)3(H2O)2]. These complexes were prepared adapting the method described in [McGehee *et al*. Adv. Mater. 1999, **11**, 1349-1354 and Reddy *et al.* Dalton Trans., 2013, **42**, 15249-15262].

2.1. Synthesis of TNPs

TNP polymeric micelles were designed to obtain a robust linking of the lanthanide complexes in the micelle structure. For that, phenanthroline ligand groups were incorporated into the P(MPEGA-co-PEGA)-b-P(MA-b-PhenA) amphiphilic block copolymer (ABCP) structure to fix the complexes on the polymer chains. To make the nanothermometers ratiometric, a binary mixture of Eu and Sm complexes were used with 1,3-di(naphthalen-2-yl)propane-1,3-dione (DNPD) ligands (McGehee et al. Adv. Mater. 1999, 11, 1349-1354) Peng et al. Adv. Mater. 2010, 22, 716–719). These complexes were incorporated into the copolymer chain and then, the micelles were formed by self-assembly of the resulting luminescent block copolymer (ABCP) in water.

The process of coordination and micellar formation is shown in Scheme 1



Scheme 1. Synthesis of TNPs.

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2.2. Synthesis of TMNPs

Iron oxide magnetic cores with a high heating power were used. Their synthesis is described in [D. Bonvin *et al*. Nanomaterials (Basel) 2017, 7, 225 (1-18)]. The coating of the iron oxide cores with a polymer coating embedding the molecular thermometric probes to obtain the heater/thermometer nanoplatforms was carried out as reported previously [R. Piñol et al. ACS Nano 2015, 9, 3134-3142]. *Europium and Samarium [Ln(btfa)$_3$(H$_2$O)$_2$] complexes* were prepared following the synthetic procedure reported by Binnemans et al. (*J. Mater. Chem.* 2004, *14*, 191-195).

2.3. Optical Properties of TNPs and TMNPs

TNPs showed an excitation spectrum peaked at 440 nm that, interestingly, is in the visible range allowing a possible application in conventional confocal microscopes. The emission spectra of TNPs under this excitation wavelength showed significant changes depending of the temperature (in the physiological range) as shown in Fig. 1a. The emission of Sm^{3+} decreases much faster (4 times from RT to 55 ^{o}C) than that of Eu^{3+} decreases increasing temperature (18% in the same range). These results suggest a different behavior of Sm^{3+} luminescence in materials with BTFA and DNPD ligands

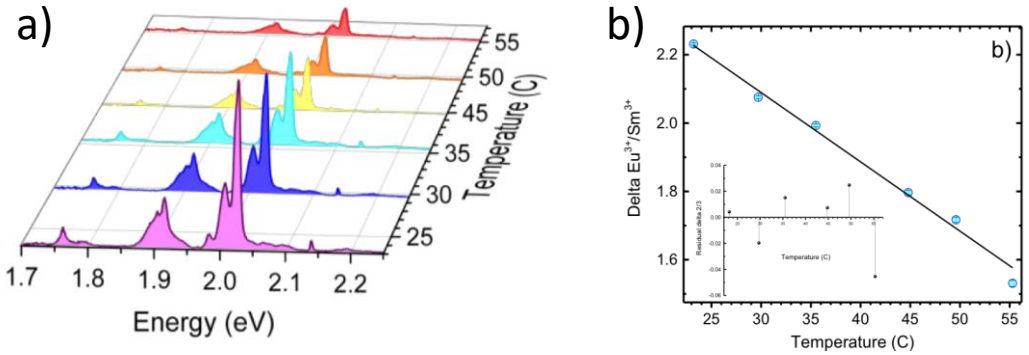

Figure 1. Thermometric performance of TNPs. (a) temperature dependence emission spectra under 436 nm excitation and (b) variation of delta parameter (I_{Eu}/I_{Sm}) with the temperature and fitting to a linear equation

TMNPs showed an excitation spectrum peaked at 370 nm. The emission spectra of TMNPs under this excitation wavelength showed clearly the emission of both Eu and Sm with relative intensities (Fig. 2) that varied with the temperature (in the physiological range).

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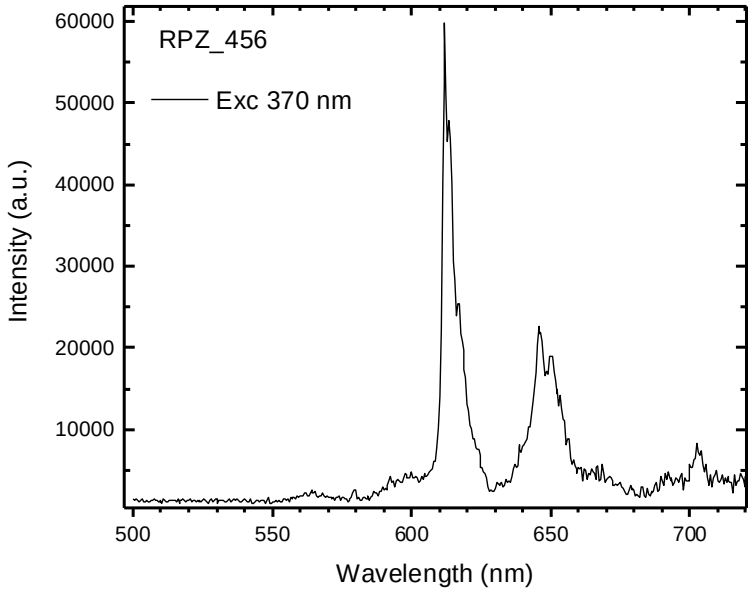

Figure 2. Emission spectrum of TMNP under 370 nm excitation.

3. Cell Cultures

MDA-MB-468 breast cancer cell lines were cultured in commercial DMEM medium without phosphate and glucose, supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM GlutaMax, 100 U/mL penicillin and 100 µg/mL streptomycin and kept in a thermostatic incubator (Heraeus Cell) in saturated humid air with 5% CO_2 at 37 °C. Successive subcultures were done every 2 or 3 days. Cells, in absence of manipulation, were kept at 37 ºC in a thermostatic incubator (Heraeus Cell) in saturated humid air with 5% CO2. Cells were refed every 2 or 3 days and their viability was calculated by the Trypan blue (Sigma) exclusion technique. Manipulations of cell cultures were carried out inside a vertical laminar flow cabinet (Telstar) in sterility conditions.

3.1. Preparation of cell cultures for temperature imaging

The cells were made quiescent for 24 hours previous to the treatments with ferrofluids by incubating them in the same medium containing only 0.5 % FCS. Then, the cells were incubated with TNPs for 24 hours, with different concentrations of TNPs (50, 100, 200 and 400 mg Fe_2O_3/mL). In order to eliminate the presence of non-internalized NPs in the sample, the supernatants were aspirated out and the cells were washed 3 times with cold PBS, fixed with 3% (w/v) paraformaldehyde for 10 minutes, washed again three times and mounted for microscopy. Cell samples previously to incubation with TNPs were also prepared as a control.

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4. Tests of temperature imaging

Cultured MDA-MB-468 cells before and after incubation with TNPs were observed with Instrument 1 (see D5.1). Phase contrast and fluorescence images (Fig.3) were taken with both Leica color camera and Hamamatsu camera of Instrument 1 (see D5.1). Phase contrast images showed that the cells were well formed and uniformly spread. Color images showed the fluorescence of internalized TNPs, whereas images from control cells previous to incubation with TNPs were totally dark. Then, the cells were observed with the Hamamatsu camera after passing the emission beam through the beam splitter. Luminescence was observed in both the Sm and the Eu channels in cells incubated with TNPs, and the images of control cells were totally dark. After separation of each channel, images of intensity ratio were obtained pixel by pixel and then were transformed into temperature images by application of the calibration equation. The obtained temperature images showed a uniform temperature across the sample. Then the variation of the temperature with time in selected areas was followed, showing a flat profile as expected.

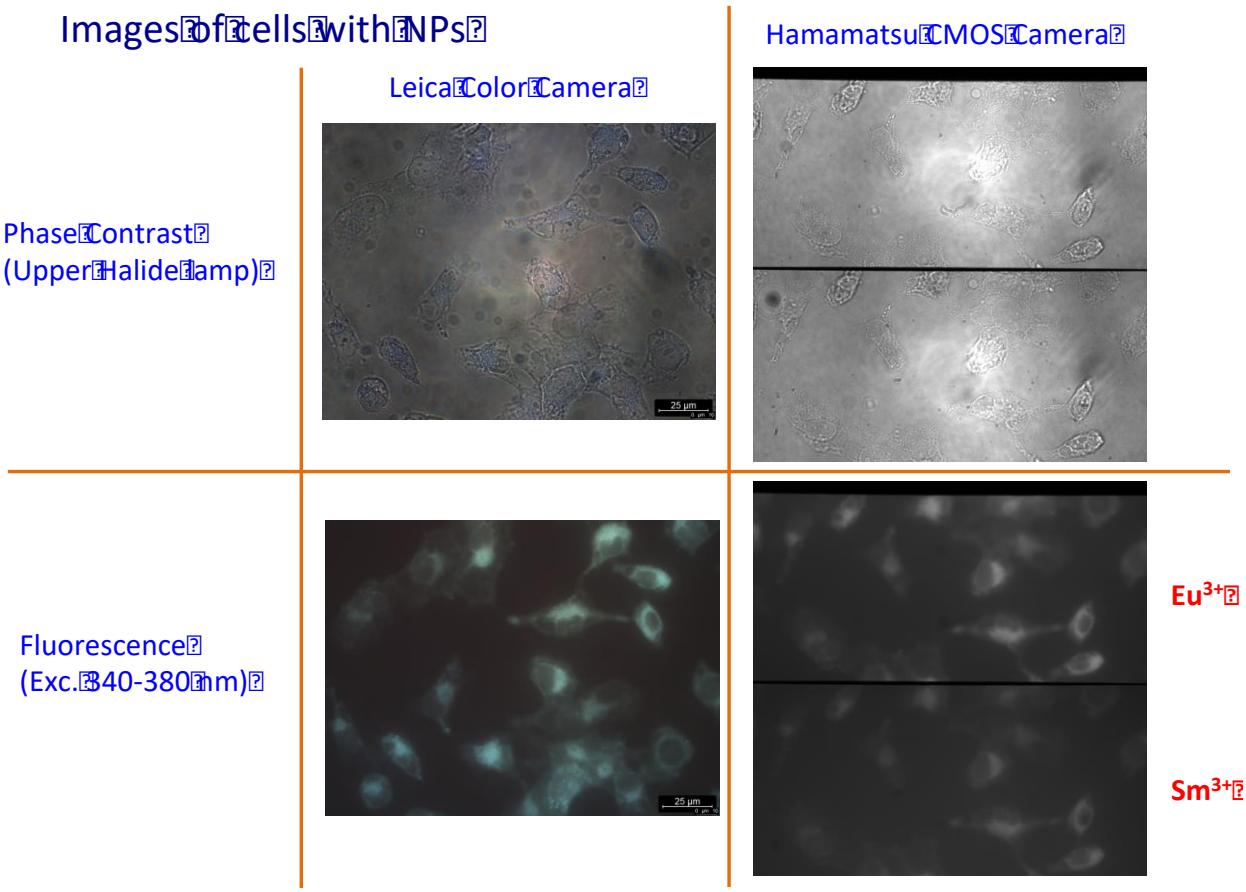

Figure 3. Phase contrast and fluorescence images of cell cultures after incubation with TNPs obtained with the Leica color camera and Hamamatsu CMOS camera

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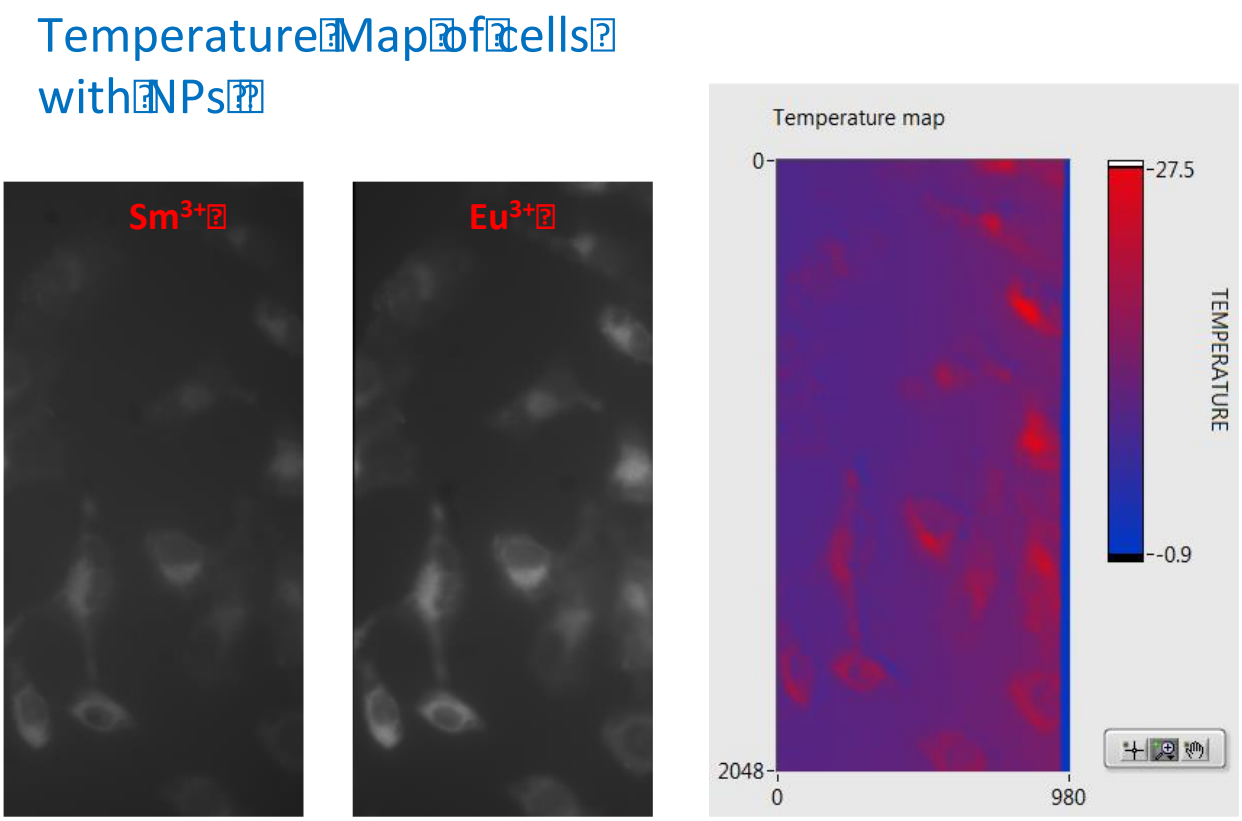

Figure 4. Images of Sm and Eu channels in Hamamatsu CMOS camera and corresponding temperature image of cell cultures after incubation with TNPs.

5. Monitoring of intracellular temperature gradients during magnetic hyperthermia

Before the realization of hyperthermia experiments, the functioning of the temperature imaging system was tested in gels simulating the intracellular conditions. The gels were formed in cell culture wells and placed in the thermostatic sample holder as shown in D5.1, section 2.1.4. The temperature in the gel was followed with an optical fiber thermometer (see section 2.4.1 in D5.1). Then, the temperature of the thermostatic cell holder was set to higher values and the changes in temperature images captured by the T imaging system, and on selected areas within these images were followed. It was observed that the T imaging system was able to detect the temperature changes. However, the time for stabilization of temperature of the culture was too long (hours). Therefore, it was concluded that the thermostatic holder was not sufficiently efficient, and it was decided to redesign it.

Actually, a new thermostatic holder has been built replacing some of the plastic parts in contact with the cell culture were by pieces made of copper for a more efficient heat transfer and rapid heating. The new holder is now being tested and the experiments of intracellular temperature determination under ac magnetic fields will be resume in a few weeks.

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6. Future developments

As explained above, hyperthermia experiments and temperature gradient determination will start in a few weeks, after the test of temperature control unit are completed. In a first instance, temperature-imaging tests on TMNPs will be performed, and then the magnetic field applicator will also be tested. It is expected that the first measurements of temperature increase on intracellular TMNPs under magnetic field applications can be possible in 2-3 months.

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