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ESRF

Experiment Report Form

The double page inside this form is to be filled in by all users or groups of users who have had access to beam time for measurements at the ESRF.

Once completed, the report should be submitted electronically to the User Office using the **Electronic Report Submission Application:**

http://193.49.43.2:8080/smis/servlet/UserUtils?start

Reports supporting requests for additional beam time

Reports can now be submitted independently of new proposals – it is necessary simply to indicate the number of the report(s) supporting a new proposal on the proposal form.

The Review Committees reserve the right to reject new proposals from groups who have not reported on the use of beam time allocated previously.

Reports on experiments relating to long term projects

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Should you wish to make more general comments on the experiment, please note them on the User Evaluation Form, and send both the Report and the Evaluation Form to the User Office.

Deadlines for submission of Experimental Reports

- 1st March for experiments carried out up until June of the previous year;
- 1st September for experiments carried out up until January of the same year.

Instructions for preparing your Report

- fill in a separate form for each project or series of measurements.
- type your report, in English.
- include the reference number of the proposal to which the report refers.
- make sure that the text, tables and figures fit into the space available.
- if your work is published or is in press, you may prefer to paste in the abstract, and add full reference details. If the abstract is in a language other than English, please include an English translation.

ESRF	**Experiment title:** Characterization of the Gd cellular uptake and radiosensitization with Gd contrast agents	**Experiment number**: MD-1022
Beamline: ID16-A-NI	**Date of experiment**: from: 09/02/2017 to: 14/02/2017	**Date of report**: 07/2017
Shifts: 15	**Local contact(s)**: Sylvain Bohic, Peter Cloetens	*Received at ESRF:* 29/02/2020

Names and affiliations of applicants (* indicates experimentalists):

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Report:

Objective of the experiment:

The experiment was carried out on the ID16 - A line, which makes it possible to produce XRF images of very high resolution for the quantification of specific elements at the cellular scale. The local contact and internal collaborator of the project, Sylvain Bohic, produced the images and the vitrification of the samples. The main objectives were to observe, locate and quantify the internalization of the gadolinium contrast agents (GdCA) Magnevist (Gd-DTPA linear complex) vs Dotarem (Gd-DOTA cyclic complex) in F98 cells after 24, 48 or 72 hours of incubation time with different concentrations of gadolinium from 0.5 mg Gd / mL (close concentrations to those of an injection for an MRI image) to 5 and 10 mg Gd / mL (concentrations necessary for photo-activation therapy to be effective).

These measurements have an interest in terms of:

- Diagnostics: these contrast agents are used in clinical MRI and have shown, for repeated MRI, an accumulation of Gd in the brain and kidneys, which can cause damage to patients, in particular that of renal failure, the clinical consequences of brain retention having not yet determined. The fact that GdCA can internalize cells when there is retention can be a cause of increased toxicity of these agents.
- Dose-Enhanced therapy: the interest is to study the impact of cellular internalization of gadolinium in terms of Sentitization Enhancement Ratio (SER). This will allow to assess the role of this internalization in global radiosensitization, compared to Gd nanoparticles (cf. previous experiments, Taupin *et al.* PMB 2015, 60, 4449–4464, Delorme *et al.* Phys. Med. 44(11), 5949-5960). The next experiments on ID17 from June 30 to July 3, 2017 is dedicated to measure the SER produced at different irradiation energies for the same incubation conditions.

Contrary to what was indicated in the proposal, Motexafin was not used and we therefore focused rather on the prospect of use in photo-activation rather than gadolinium neutron capture therapy (GdNCT). In addition, imaging experience has not confirmed nuclear internalization of the contrast agents, contrary to what De Stasio *et al.* 2006 had mentioned. However, GdNCT requires nuclear internalisation to be effective because it only uses the "Auger effect" subsequent to neutron captures.

Sample preparation and image acquisition:

The “fine” spectral images are acquired with a very high spatial resolution (50 nm) and take around 3h per cell. The sample is irradiated pixel by pixel on an average scanned area of 20 x 20 µm, using a very intense X-ray beam, with 50 ms/pixel (high-dose mode). The spectral ray emitted by each element in the sample following their ionization are detected and analyzed. The final signal intensity maps are obtained for each element individually. The advantage over fluorescence microscopy is that the atoms of interest (here Gd) are imaged directly, and not through an intermediate molecule marked with a fluorofore that could bias the results. The samples are prepared on very thin silicon membranes (200µm on 1.5x1.5 mm² surface) on which the cells are cultured. After incubation with the contrast agent studied, the samples are rinsed, dried and then directly immersed in liquid nitrogen for vitrification. The rapid cooling and the critical drying step (blotting) allow the formation of a thin layer of ice and cells are thus kept in their incubation state without modifying their morphology and the location of the internal GdCA. The image is made in 2 dimensions, accumulated over the entire thickness of the ice, which does not allow precise location of elements in case of superimposed structures.

These preparation steps are very delicate, the thickness and quality of the ice for example can be variable and some samples couldn’t be used. The 72h incubated samples were not imagined due to lack of time. The samples incubated at 10 mg / mL for Magnevist showed significant toxicity and could not be used. Those at 5 mg / ml were preferred because samples were well prepared and cells weren’t suffering. Only two cells were imaged for the control condition, but we kept all prepared samples frozen for further acquisitions if necessary for publication.

The Table 1 is a summary of the prepared samples and cell-image finally acquired in high-resolution:

Table 1: summary of prepared sample conditions (Gd concentraion, GdCA type and incubation time), usable samples for imaging and number of cells imaged and exploitable for Gd-internalization comparison.

Gd Concentration	Dotarem		Magnevist		Control (no Gd)
Incubation time	24h	48h	24h	48h	2 cells
C1 (0.5 mg/mL)	Non-imaged	4 cells	Non-imaged	4 cells	
C2 (10 mg/mL)	Non-usable	5 cells	Non-usable	Non-usable	
C3 (5 mg/mL)	3 cells	Non-imaged	4 cells	Non-usable	

Preliminar results:

Images: qualitative results

Qualitative comparison could be done between Manevist and Dotarem in same incubation conditions: at 48h with 0.5 mg/mL or at 24h with 5 mg/mL. We compared signal maps obtained for Zn element, highlighting in particular the cell nucleus structure, and signal maps of Gd element to determine if Gd is located inside or outside the cell nucleus. However, as there are 2D cumulated images, we cannot really differentiate what is in the nucleus or in the cytoplasm parts that cover, or hide behind the cell nucleus from the detector’s view. No indication of cellular suffering was observed by microscopy on imaged samples.

The internalization of Gd takes place for both agents and internalized Gd-atoms appear in the form of aggregates, rather distributed around the nucleus and in the cytoplasm. Especially for the high Gd concentration (5 mg/mL), intracellular accumulation was very important for both agents and gadolinium seems to be included in vesicles such as endosomes or lysosomes. No clear differences appears between the two GdCA agents on these images.

Without comparing with Magnevist (too much toxic for cells at those conditions), we have imaged F98 cells incubated with Dotarem agent at higher concentration (10 mg/mL) and longer incubation time (48h). A very large accumulation of Gd in cell cytoplasms, forming vesicules with larger sizes as for the condition 5 mg/mL

and 24h. It is likely that the Gd is mainly confined in lysosomes in that case, but further co-localisation studies with specific markers would be needed to support this way.
For both conditions of high-concentration (5 or 10 mg/mL) of Dotarem, either at 24h or 48h of incubation time, the formation of Zinc vesicles were observed, called "Zincosomes" due to the similar aspect of these structures with the endosomes. This accumulation in the vesicle was not expected and not observed in the control conditions, and very little marked in the presence of Magnevist. This could mean that DOTA cyclic chelates, containing the Gd atoms, potentially interact with the Zinc present in the cell (or outside) to accentuate the formation of Zincosomes. There is also no co-localization between the vesicles of Zinc and those of Gd, which raise questions about the possible replacement of the atoms of Gd by those of Zn and therefore of the potential presence of free (toxic) gadolinium in the cell. A situation of sudden stress on the cell is also possible as some studies have shown the appearance of such zincosomes in tumor cells under stress. This would be a sign of higher toxicity induced by Dotarem compared to Magnevist under long incubation conditions, although clinically this molecule seems more stable and safe. The role of these structures is poorly understood yet and further specific studies would be required to better determine the reasons and conditions of zincosome formations, their impact at the cell level and the interaction with the DOTA chelate.

Quantification of intracellular Gd accumulation:
We also performed quantification studies of intracellular Gd accumulation for both agents, by means of ICP-MS to measure the kinetics of accumulation from 30 min to 24h of incubation times, and by means of imaging quantification analysis measuring Gd quantity in ROI after correction of background and imaged-cell surface.
The ICP-MS measurements were only performed at 10 mg/mL. Similar increase of internalization was observed for both GdCA, but the maximal quantity of Gd per cell achieved at 24h was superior for Dotarem (2.1±0.3 pg/cell) than for Magnevist (1.5±0.05 pg/cell). In contrast, on image analysis, Gd internalization was superior with Magnevist than with Dotarem at similar concentrations (for C1 and C3). The Gd measured quantities seem about twice less important than those measured with ICP-MS. The preparation sample conditions was not fully equivalent in both approaches and it is possible that a release of internalized Gd has occurred if the samples have been waiting for a long time after rinsing and before freezing for analysis. Further additional points would be needed to complete data in ICP-MS, with a closer preparation protocol (cf. second report of MD-1038 experiment).

Conclusions:
We performed high-resolution (50nm) spectral images of F98 glioma cells treated with two gadolinium contrast agents (Magnevist (linear chelate) and Dotarem (cyclic chelate)) used in clinical routine for MR imaging, in order to observe their ability to incorporate Gd after incubation time of 24h, 48h and 72h at concentrations of 0.5, 5 and 10 mg/mL. All conditions showed significant internalization of Gd in the cells, preferentially located in the cytoplasm compartment rather than in cell nucleus, for both Dotarem and Magnevist. This internalization is found localized in vesicles (endosomes or lysosomes), of larger sizes (lysosomes) for the longest incubations and the highest concentrations. Systematic formation of "Zincosomes" was observed in conditions of high-concentration (5 and 10 mg/mL) of Dotarem incubated with cells, either during 24 or 48h. Further investigations would be needed to explore the reason and impact on cells of these struture formation in presence of Dotarem. Concentrations higher than 5 mg/mL and longer than 72h shown significant toxicity (cell suffering observed on microscopic images of samples after treatment), especially for Magnevist.
To our knowledge, it is the first time that a cellular internalization of these Gd contrast agents has been imagined with such precision. These results will constitute the basis of an article to be combined with the results in cell survival (see experimental report of complementary experiment MD-1032) corresponding to the imaged and comparable conditions between Magnevist and Dotarem, namely 48h of incubation time at 0.5 mg / mL and 24h at 5 mg / mL.