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# **Radiosensitizing Fe-Au Nanocapsules (Hybridosomes®) increase survival of GL261 brain tumor-bearing mice treated by radiotherapy**

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**Keywords:** Glioblastoma, Nanoparticles, Hybridosomes®, Combined therapies, Radiotherapy, Chemotherapy; Mouse Models, MRI and Bioluminescence

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## **Author contributions**

CG, UJ, PAE, MB and SC performed most experiments and analyses. MK synthesized the iron oxide nanoparticles. CG, UJ, TG, FG and SC designed and supervised the study. CG, UJ, PAE, FG and SC wrote and edited the manuscript.

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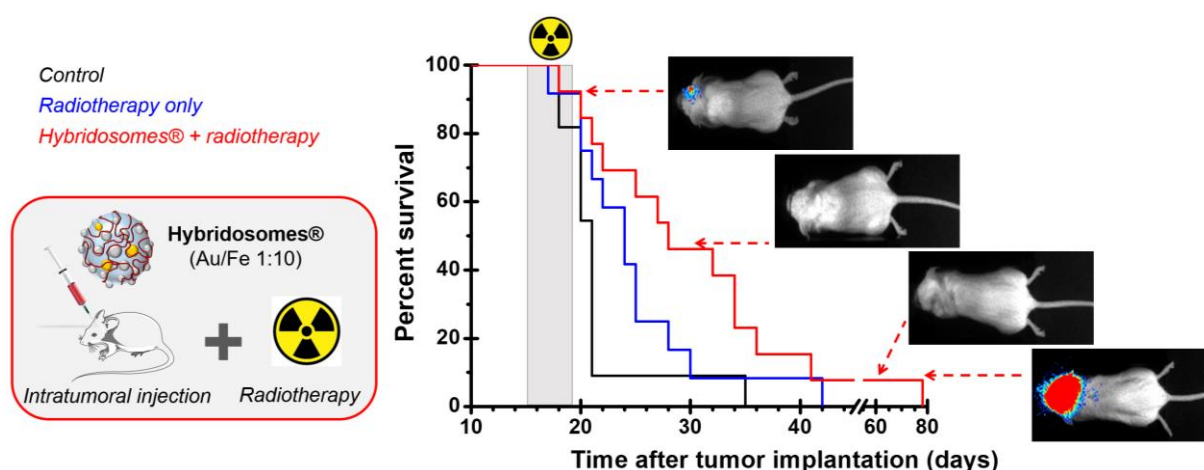
**Conflicts of Interest:** The authors disclose no potential conflicts of interest

**Abbreviations:** BBB, Blood Brain Barrier; GBM, Glioblastoma; H®, Hybridosome®; H(Fe), Iron oxide Hybridosome®; H(Fe;Au), Iron Oxide and Gold Hybridosome®; IR; Irradiation; MRI, Magnetic Resonance Imaging; NP, nanoparticle(s); NTA, Nanoparticle Tracking Analysis; ROS, Reactive Oxygen Species; SPION, iron oxide superparamagnetic nanoparticles; TEM, Transmission Electron Microscopy.

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## Graphical Abstract



**Legend:** Intratumoral injection of Iron Oxide – Gold nanocapsules (Hybridosomes®) with radiotherapy increase the survival of brain tumor-bearing mice. The bioluminescence can be monitored as the tumoral cells express luciferase.

## Abstract

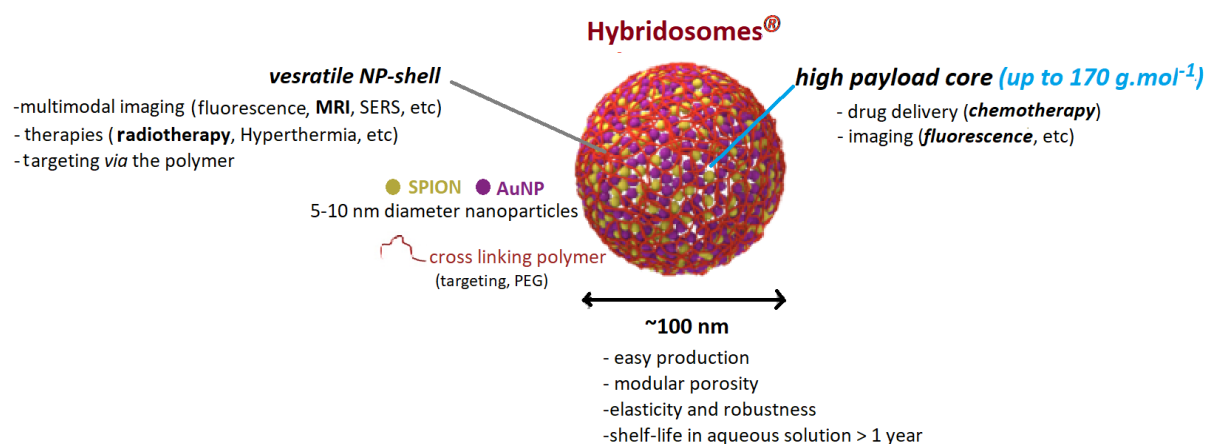
Glioblastoma remains a cancer for which the effectiveness of treatments has shown little improvement over the last decades. For this pathology, multiple therapies combining resection, chemotherapy and radiotherapy remain the norm. In this context, the use of high-Z nanoparticles such as gold or hafnium to potentiate radiotherapy is attracting more and more attention. Here, we evaluate the potentiating effect of hollow shells made of gold and iron oxide nanoparticles (hybridosomes®) on the radiotherapy of glioblastoma, using murine GL261-Luc<sup>+</sup> brain tumor model. While iron oxide seems to have no beneficial effect for radiotherapy, we observe a real effect of gold nanoparticles —despite their low amount— with a median survival increase of almost 20% compared to radiotherapy only and even 33% compared to the control group. Cellular and *in vivo* studies show that a molecule of interest nano-precipitated in

the core of the hybridosomes® is released and internalized by the surrounding brain cells. Finally, *in vivo* studies show that hybridosomes® injected intra-tumorally are still present in the vicinity of the brain tumor more than 5 days after injection (duration of the Stupp protocol's radiation treatment). Interestingly, one mouse treated with radiotherapy in the presence of gold-containing hybridosomes® survived 78 days. Monitoring of the tumoral growth of this long-term survivor using both MRI and bioluminescence revealed a decrease of the tumor size after treatment. These very encouraging results are a proof-of-concept that hybridosomes®, are really effective tools for combined therapies development (chemo-radiotherapy).

## Introduction

Glioblastoma multiforme (GBM: WHO grade IV), with an incidence of 5 per 100,000 people, is the most common type of brain tumor. It is highly aggressive and often deeply infiltrates brain tissues<sup>1</sup>. Today, the standard treatment, defined as the Stupp protocol, is based on surgery followed by radiotherapy (5 times 2 Gy per week for 6 weeks; total 60 Gy) with concomitant (75 mg per square meter of body-surface area per day, 7 days per week from the first to the last day of radiotherapy) chemotherapy with temozolomide as adjuvant (150 to 200 mg per square meter for 5 days during each 28-day cycle)<sup>2</sup>. Despite this severe treatment, the median survival remains poor (< 18 months) with a rate of 5-year survival less than 5%<sup>3,4</sup>. GBM is one of the few cancers for which there has been little increase in life expectancy over the past 30 years. In addition to the infiltrating nature of the tumor, which makes total resection of the tumor by surgery almost impossible, one of the major obstacles to the treatment of GBM is the Blood Brain Barrier (BBB). Indeed, the BBB severely limits the penetration of chemotherapeutic molecules and radiosensibilizing agents into brain tumor tissues. Brain tumor development partly disturbs the permeability of this BBB but often at its latest stage and not uniformly, which leads to an inefficient drug delivery<sup>5</sup>. To overcome this limitation, one strategy is the use of local administration<sup>6</sup>. Thus, implantable polymer devices delivering a chemotherapy such as Gliadel® are presently used in the clinics. Direct intracranial delivery of drug-loaded vehicles, notably lipid nanocapsules, or of cellular vehicles such as stem cells producing growth factor inhibitors, has yielded promising results in orthotopic GBM models.<sup>7</sup> Alternatively, intracranial infusion of drugs through convection-enhanced delivery devices enables the administration of drugs over longer period and has shown encouraging results in small animal models (see reference<sup>8</sup> for review).

Among the numerous therapeutic strategies which have been explored<sup>9,10</sup>, an attractive lead with the promise of a breakthrough in cancer therapies is the improvement of radiotherapy through the use of radiosensitizing nanoparticles, in particular high-Z nanoparticles (Z: atomic number)<sup>11–14</sup>. Indeed, the use of conventional radiotherapy is limited by the damages caused to healthy tissues in the path of irradiation. In the presence of high-Z nanoparticles, the energy is absorbed and “deposited” much more locally than in normal tissues, due both to the strong photon absorption and to the conversion of the initially high energy radiation into several low energy Auger electrons. First, photon-X are absorbed by atoms *via* the photoelectric effect, with an efficiency roughly proportional to  $Z^4$ , thus favoring high-Z atoms in comparison to soft tissues. Following the absorption of a single photon of high energy, a high-Z atom may expel a dozen of Auger electrons of lower energy, hence of low penetration (~10 nm). Furthermore, the generated Auger electrons and secondary X-rays induce the production of hydroxyl radicals (HO·) *via* water radiolysis. These, and other reactive oxygen species (ROS) that are further generated readily react with biological molecules, including DNA, proteins and lipids inducing cell death<sup>15</sup>. Gold (Z=79) is a material of choice, since it combines the advantages of biocompatibility and strong photoelectric effect<sup>12</sup>. The first *in vivo* experimental evidences of the radiosensitization effect of gold nanoparticles (AuNP) dates back to the 2000s<sup>16,15</sup> and the number of reports has kept increasing since then. The location of the gold nanoparticles at the tumor site can be verified by computed tomography (CT). Among the other metal-based materials that have been considered for radiosensitization, gadolinium and superparamagnetic iron oxide have the advantage of being easily tracked by MR-imaging (MR: magnetic resonance)<sup>17</sup>.



**Scheme:** Schematic representation of a hybridosome® summarizing its main physicochemical characteristics and its multiple and general theranostic potentialities in terms of therapies, imaging and targeting. The potentialities used in the present work are highlighted in bold.

Recently we have introduced a new type of multimodal nanocapsules, named hybridosomes® (H®), whose shell consists of inorganic nanoparticles (NP) crosslinked with a polymer<sup>18,19</sup> (Scheme). Interestingly, these nanocapsules are produced by a simple nanoprecipitation method, which allows at the same time the formation of the NP-shell and the encapsulation of a hydrophobic active ingredient. A previous study demonstrated that the storage capacity in the core of these nanocapsules is extremely high (over 170 g.L<sup>-1</sup>), the hydrophobic ingredient being condensed in a nanoprecipitated form<sup>20</sup>. Hybridosomes® containing iron oxide superparamagnetic nanoparticles (SPION), named hereafter H(Fe), can be used as MRI T<sub>2</sub> contrast agents, with an efficiency comparable to commercial iron oxide based contrast agents such as Feraspin XXL<sup>TM</sup><sup>18</sup>. In addition, a stealth-providing poly(ethylene glycol) block (PEG) can be added to the polymer holding the shell, to limit uptake by monocytes and delay their sequestration in the specialized organs of the immune system. Based on these results, hybridosomes® appear to be prime candidates for the treatment of cancers requiring multimodal therapies, such as glioblastoma.

The main aim of the present work is to evaluate the radiosensitizing effect of H(Fe;Au) hybridosomes® composed of superparamagnetic iron oxide and a small amount of gold nanoparticles (10%) on GBM after intratumoral injection in mice implanted with the GL261 brain tumor model. We first evaluated the distribution of hybridosomes® *in vitro* and *in vivo*. We took advantage of iron oxide NP to follow the *in vivo* biodistribution of H(Fe;Au) using MRI and to evaluate their presence in post-mortem tissues by histology (Perls staining). Then, we performed a survival study to evaluate the increased therapeutic effect of H(Fe;Au) in combination with irradiation. Six groups of mice were examined, including 3 groups not treated with radiotherapy (injected with H(Fe), H(Fe;Au) or none) and 3 similar groups receiving radiotherapy. Tumor growth was monitored in all groups by both bioluminescence and MRI. We have also characterized the transfer of an encapsulated charge from hybridosomes® to tumor cells, *in vivo* and *in vitro*, using a fluorescent dye BODIPY as a model. All these works and the good results obtained, allow us to consider the use of hybridosomes® as a unique theranostic nanoplatform to efficiently combine chemo- and radiotherapy, offering the possibility of a follow-up by imaging.

## Materials and methods

### *Elaboration and characterization of hybridosomes®*

Poly(ethylene glycol)-*b*-Poly(acrylic acid) (PEG-PAA) (M<sub>PEG</sub> = 2 kg.mol<sup>-1</sup>; M<sub>PAA</sub> = 7.2 kg.mol<sup>-1</sup>) was kindly provided by Dr G. Casterou. Tetrahydrofuran (THF, GPR Rectapur ≥99%

stabilized) was purchased from VWR and used as received. Samples were made using MilliQ water ( $18.2 \text{ M}\Omega\cdot\text{cm}^{-1}$ ). The fluorescent dye 4,4-difluoro-8-(4-trimethylsilylethynylphenyl)-1,3,5,7-tetramethyl-2,6-diethyl-4-bora-3a,4a-diaza-s-indacene (BODIPY,  $M = 476.2 \text{ g}\cdot\text{mol}^{-1}$ ) was kindly provided by Dr O. Mongin (Univ Rennes, CNRS, ISCR-UMR6226, F-35000 Rennes, France.)<sup>21</sup>.

The iron oxide ( $\gamma\text{-Fe}_2\text{O}_3$ ) nanoparticles (SPION) were synthesized following a reported procedure with slight modifications<sup>22</sup>. Briefly, the SPION are obtained *via* hydrolysis of an organometallic Fe(II) precursor in the presence of a stabilizing ligand (here 2 eq. of octylamine and 1 eq. of oleic acid, relative to Fe), in the absence of any other solvent. Oleylamine-coated gold NP were synthesized following the procedure reported by Liu *et al.*<sup>23</sup>. Briefly, AuNP were synthesized in oleylamine, which acts both as a reductor of the gold salt ( $\text{HAuCl}_4$ , 3  $\text{H}_2\text{O}$ ) at  $150^\circ\text{C}$  and as a capping agent to stabilize the newly synthesized NP. After 1h30 of reaction, AuNP are readily dispersed in THF after precipitation in ethanol and centrifugation. Note that it is important for the elaboration of hybridosomes® to use alkyl-coated (hydrophobic) NP.

The magnetic hybridosomes® H(Fe) and H(Fe;Au) were prepared by rapidly adding water to a THF solution containing the iron oxide NP (plus the AuNP for H(Fe;Au)), in order to reach a water/THF composition of 75/25 v/v. Mixing was promptly followed by a very quick vortexing. After ca 7 h, PEG-PAA ([acrylic acid units] =  $2.4 \text{ mmol}\cdot\text{L}^{-1}$ ) was added to the mixture and the solvent was slowly evaporated overnight at  $40^\circ\text{C}$ . Two 24 h magnetic separations were performed using a permanent magnet, and the pellets were dispersed in fresh water to recover the purified hybridosomes®. To elaborate dye-loaded hybridosomes®, the BODIPY was dissolved in the THF solution in order to reach a final dye concentration in the water/THF mixture of  $50 \mu\text{mol}\cdot\text{L}^{-1}$ . After encapsulation, the BODIPY-loaded hybridosomes® can be excited at 488 nm, which is close to their maximum of absorbance, to produce a broad band emission around 550 nm<sup>20</sup>. The distribution of hydrodynamic diameters and H® concentration was determined by Nanoparticle Tracking Analysis (NTA), using a Nanosight LM10 device system (Malvern Panalytical) equipped with a 40 mW laser working at  $\lambda = 638 \text{ nm}$ . Video sequences were recorded *via* a CCD camera operating at 30 frames per second and evaluated via the NANOSIGHT NTA 2.0 Analytical Software Suite. For each sample, 3 acquisitions with an acquisition time of 60 s were performed at  $25^\circ\text{C}$ . TEM analysis was performed using a Jeol 1400 electron microscope equipped with a Gatan Orius 1000 camera. The elemental concentrations of iron ( $18.7 \mu\text{g}/\text{mL}$ ) and gold ( $2 \mu\text{g}/\text{mL}$ ; in the case of H(Fe;Au)) in the purified H® suspensions were determined by atomic spectroscopy. Typically, the H® were dissolved in nitric acid for one week under heating at  $40^\circ\text{C}$ . After appropriate dilution with milliQ water,

the concentrations were determined using an iCAP 7000Series ICP-OES spectrometer (Thermo Scientific) under an argon flow.

#### *Tumoral cells, mice and brain implantation*

GL261-Luc<sup>+</sup> cells were kindly provided by R. Hashizume (University of California, San Francisco, CA).<sup>24</sup> Cells were cultured in DMEM medium (Dutscher, Brumath, France) supplemented with 10 % heat inactivated FBS (Dutscher) and 2 mM L-Glutamine (PanBiotech, Aidenbach, Bavaria, Germany).

C57BL/6 (B6/Rj) and C57BL/6 albino (B6/Rj-Tyrc/c) mice were purchased from Janvier Labs (Saint Berthevin, France) and bred in the animal facility of the University of Rennes (ARCHE, Biosit, SFR UMS CNRS 3480-INSERM 018, Université de Rennes 1) under specific pathogen-free status. Mice were used at 6–12 weeks of age and manipulated according to institutional guidelines.

For stereotaxic implantation, mice were anesthetized with an intraperitoneal injection of ketamine (10 µg/g) and xylazine (1 µg/g) and were placed in a stereotactic frame (Stoelting, Dublin, Ireland). The animals underwent an injection (0.5 µL/min) of 10.10<sup>3</sup> GL261-Luc<sup>+</sup> cells in 2 µL sterile PBS with a Hamilton syringe, at the level of the bregma 3 mm on the right of the medial suture and at a depth of 2.5 mm. The syringe was held in place for an additional minute and was slowly removed to avoid backfilling of the solution.

The protocols were approved by the French Ministère de l'Enseignement Supérieur et de la Recherche and by the local Ethic Committee (Comité Rennais d'Ethique en matière d'Expérimentation Animale (CREEA) (France) (Agreement # 10972)). In particular, the number of animals per study group was validated and therefore allowing not to multiply the number of tumor models.

The mice were daily observed and were euthanized when showing specific clinical signs, following standard procedures. Among the conventional signs, those that were observed are the following:

**Notable Clinical Signs** (limit point reached if 3 simultaneous signs are detected): agitation/aggressiveness; hypomotility/hyporeactivity; moderate incoordination; stereotypes; generalized muscle flaccidity; abdominal swelling; arched back; weight loss less than 10 %, no food intake for less than 2 days.

**Severe Clinical Signs** (limit point reached if 1 sign is detected): akinesia; decubitus; difficulty/impossibility of mobilization; marked incoordination; generalized tremors; convulsions; weight loss more than 10%; no food intake for more than 2 days.

## Hybridosomes® injection and irradiation protocol

For *in vivo* distribution, dye-loaded H(Fe;Au) were injected in the brain tumor of mice 14 days after implantation of the GL261-Luc<sup>+</sup> tumor cells. For assessment of the irradiation procedure, H(Fe) were injected in the tumor to groups 2 & 5 and H(Fe;Au) to groups 3 & 6, at day 14 or 16 (albino mice) after tumor cells injection, and radiotherapy started the same day for groups 4, 5 & 6, as indicated in Table 1. The albino (a) and non-albino (na) mice were distributed within the groups as follows: group 1 = 6(a)/5(na); group 2 = 7(a); group 3 = 3(a)/6(na); group 4 = 6(a)/6(na); group 5 = 11(a); group 6 = 7(a)/6(na). The injected amount is 5  $\mu\text{L}$  of a concentrated dispersion of hybridosomes® in PBS (500  $\mu\text{g.mL}^{-1}$  Fe and 53  $\mu\text{g.mL}^{-1}$  Au in the case of H(Fe;Au), i.e. ca 125  $\mu\text{g}$  Fe & 13  $\mu\text{g}$  Au/mouse kg. The intratumoral injection is performed as described above at the rate of 1  $\mu\text{L.min}^{-1}$  at the exact same location as tumor implantation. For tumor irradiation mice were anesthetized with isoflurane. Irradiation was performed using an X-ray generator (CellRad, Faxitron Bioptics, Tucson, AZ)) on the head area at 2 Gy/day for 5 days.

## Confocal microscopy

Images were acquired using a LSM 880 confocal microscope (Zeiss, Oberkochen, Deutschland) equipped with a 63X oil-immersion objective (NA 1.4) and driven by ZEN software. The beam excitation laser was 488 nm and the emission channel detector was set at 517-544 nm. Images were analyzed and processed with ImageJ.<sup>25</sup>

## Tissue isolation and Histology

After collection, brains, livers, cervical lymph nodes and spleens were frozen in isopentane (Sigma-Aldrich, St Louis, MO) and serially sectioned at 10  $\mu\text{m}$  using a Leica cryostat (Leica, Solms, Germany) at -30 °C. Slides were firstly scanned using the NanoZoomer 2.0 HT (Hamamatsu Photonics K.K., Hamamatsu, Japan) for hybridosomes® fluorescence acquisition. Then, slices were stained either with DAPI (SouthernBiotech, Birmingham, AL), Hematoxylin and eosin (H&S) and/or Perls (specific iron staining) before second acquisition.

## In vivo Bioluminescence assays

Mice received an intraperitoneal injection of 150  $\mu\text{L}$  of 15  $\mu\text{g.}\mu\text{L}^{-1}$  D-Luciferin K<sup>+</sup> solution, (Interchim, Montluçon, France) while anesthetized via inhaled isoflurane and monitored using a Photon imager (Biospace Lab, Nesles la Vallée, France) equipped with a highly sensitive

cooled CCD camera. Data were analyzed using the cpm (count per minutes) per cm<sup>2</sup> focused on the head.

#### *In vivo Magnetic Resonance Imaging*

*In vivo* Magnetic Resonance Imaging (MRI) experiments were carried out at 4.7 Tesla with a horizontal Biospec 47/40 imaging system (Bruker Biospin, Wissembourg, France). After prior anesthesia with isoflurane, mice were placed in a prone position and held in a contention system equipped with a face mask for anesthesia with 1-2.5% isoflurane in air. Breathing was monitored with a pneumatic probe. The body temperature was maintained at 36.5-37.5°C by a feedback-regulated heating pad throughout the imaging protocol. Tumor size was monitored using axial and coronal T2-weighted images obtained using a Rapid Acquisition with Relaxation Enhancement (RARE) sequence <sup>26</sup>. Acquisition parameters were as follows: TR = 3500 ms; TE/TEeff = 9.35/28.5 ms; RARE factor = 8; 2 averages, in plane resolution = 100 μm x 100 μm, slices thickness = 1 mm. Acquisition time was 2 min 20 s for each acquisition.

Tumor volumes V (mm<sup>3</sup>) were calculated according to the formula,  $V = (L \times l \times h) \times \pi/6$ , with L, l and h the lengths of the 3 main axes of the tumor measured on axial and coronal MR Images.

#### *Statistical analysis*

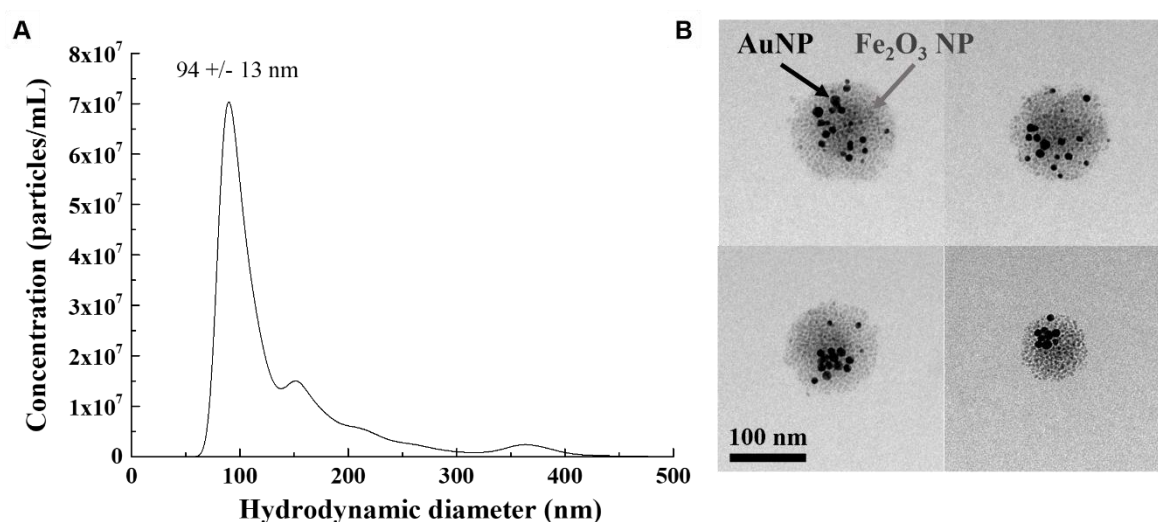
To avoid tumor size bias, the mice were distributed into 6 groups such that each mice group contains tumors of all sizes, based on MRI measurements at days 9/10 after tumor implantation (see Figure SI-1). Two mice (out of 54) with tumor size exceeding 30 mm<sup>3</sup> were excluded from the cohort, after which the mean diameter size was  $4.8 \pm 1.12$  mm<sup>3</sup>.

Data are expressed as median survival and were analyzed using GraphPad Prism 7.0 (GraphPad Software, Inc., San Diego, CA) or Origin softwares. The P-values were calculated using the Logrank (Mantel-Cox) test (\*p<0.05).

## Results

### Characterization of the gold containing hybridosomes® H(Fe;Au).

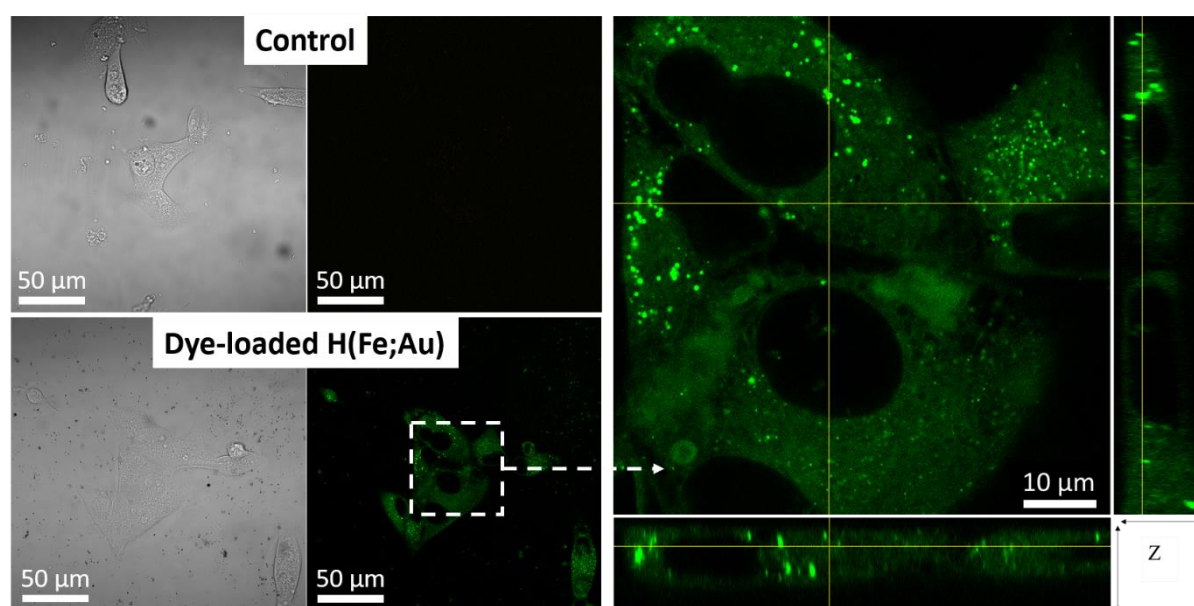
As already described in detail previously elsewhere<sup>18,27</sup>, our hybridosomes® are very original types of nanocapsules (Scheme). They are original both in *i*) their synthesis (simple and fast), *ii*) their composition (inorganic NP, organic polymer) and *iii*) their multifunctionality, coming from the NP shell (imaging, therapy) as well as from the core allowing the loading of a large number of molecules (efficient drug delivery). In this work, we have optimized the shell of our magnetic iron-oxide hybridosomes(R), usually only made of SPION, by adding a small number of gold nanoparticles (AuNP) in order to confer a radiosensitization property to our nanocapsules. The designed mixed iron-gold hybridosomes® H(Fe;Au) were characterized by transmission electron microscopy (TEM) and single Nanoparticle Tracking Analysis (NTA) (Figure 1A). The size distribution measured by NTA in aqueous suspensions shows a main mode of the hydrodynamic diameter at  $94 \pm 13$  nm. The TEM analysis of the suspensions dried on a TEM grid confirms the formation of capsules of ~100 nm, containing both gold and iron oxide NP. Single AuNP (diameter  $9.8 \pm 2.3$  nm) are larger than single iron oxide NP (diameter  $4.2 \pm 1.7$  nm) and appear with a darker contrast, since TEM contrast increases with electron density ( $Z_{\text{Au}} = 79$ ,  $Z_{\text{Fe}} = 26$ ,  $Z_{\text{O}} = 8$ ). A final Fe:Au composition of ca 10:1 was determined using ICP-EOS titration.



**Figure 1 :** Hybridosomes® characterization. (A) Single Nanoparticle Tracking Analysis of a water-suspension of H(Fe;Au). (B) Transmission electron micrographs of H(Fe;Au) with arrows indicating AuNP (black) and  $\text{Fe}_2\text{O}_3$  NP (grey). The mass ratio Fe:Au is ca 10:1.

### Intracellular delivery of encapsulated payload.

In a recent study, we developed the process of encapsulation in hybridosomes®, using a fluorescent model dye (BODIPY) and iron oxide hybridosomes®<sup>20</sup>. The dye is encapsulated in a solid form (nanoprecipitate) with two main emission bands centered at 560 nm (green) and 625 nm (red). Here, we investigated the release of the encapsulated dye from the gold containing H(Fe;Au) into GBM cells. To this aim, GL261-Luc<sup>+</sup> cells were incubated for 2 hours with BODIPY-loaded H(Fe;Au) and then analyzed by confocal microscopy. As seen in Figure 2, the green fluorescence of the dye was detected in the cytosol of incubated cells, but not in the nucleus.

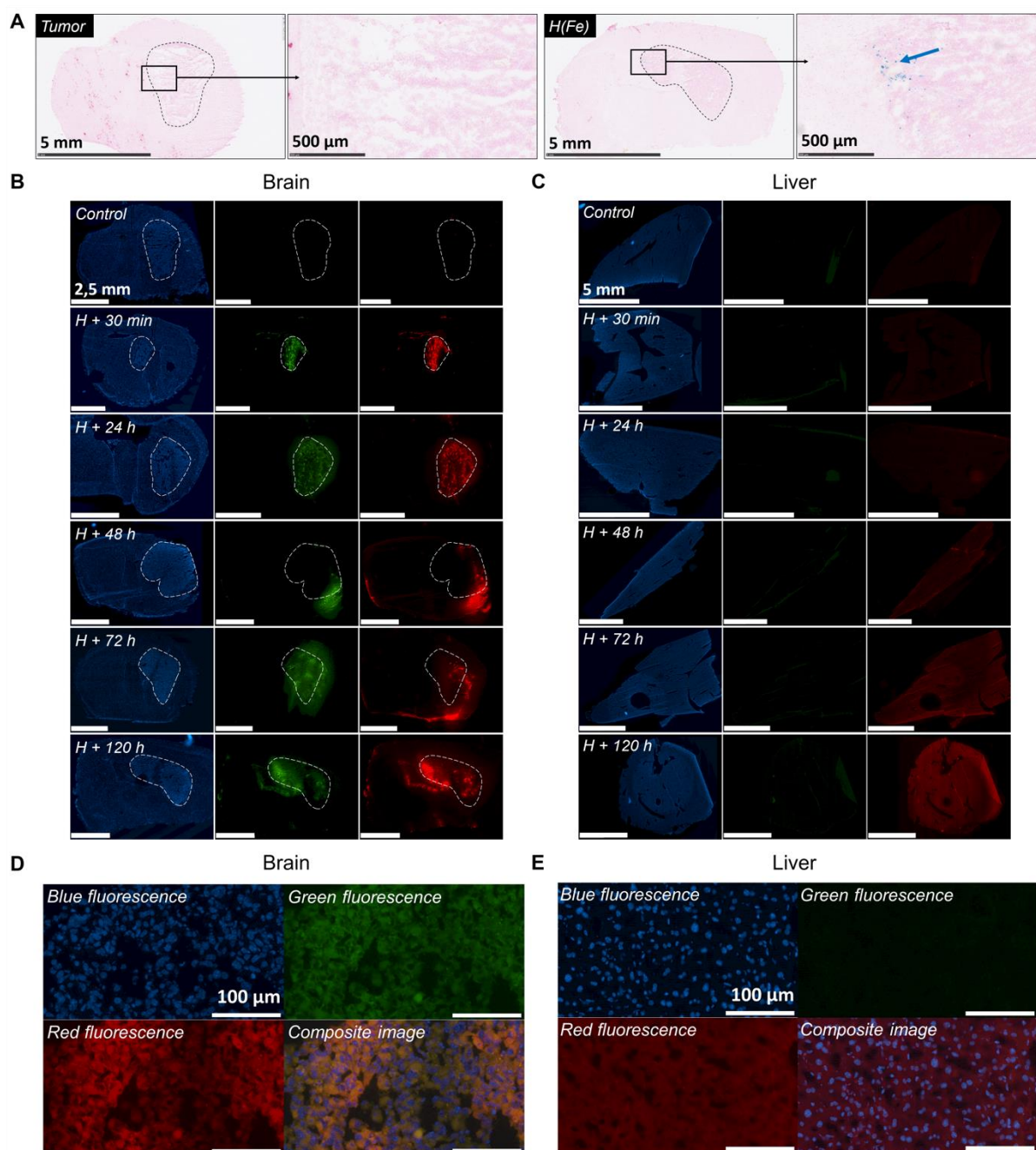


**Figure 2** – Left: Bright-field image and confocal fluorescence microscopy of unstained GL261 cells after incubation with dye-loaded H(Fe;Au), showing diffusion of the dye in the cytosol but not in the nucleus. Right: enlargement and Z-scan. Imaged for the BODIPY green fluorescence.

### Biodistribution of the hybridosomes® and of their cargo

We next sought to evaluate the *in vivo* biodistribution of the capsule and cargo (i.e. encapsulated load) after intra-tumoral injection of dye-loaded hybridosomes®. To this aim, BODIPY-loaded H(Fe), were injected in the brain tumors of 10 mice, 14 days after implantation of the GL261 - Luc<sup>+</sup> tumor cells. Two mice were sacrificed 30 min after injection, and then every day to study their main organs. The presence of iron (more precisely Fe<sup>3+</sup> ions) from the shells of the hybridosomes® was assessed by optical microscopy following Perls staining on slices of brains and livers at day 5 (Figure 3A). In brain slices, iron is irregularly present near the edges of the tumor. No significant accumulation of exogenous iron could be detected in the liver. The BODIPY fluorescence (green + red) from slices of brain, liver, spleen and cervical lymph nodes is displayed in Figure 3B & 3C (brain and liver) and Figure SI- 2 (spleen and cervical

lymph nodes). No signal was detected for non-injected mice (upper pictures). For injected mice, 30 min after injection, the fluorescence of the BODIPY remains localized in a limited area, most probably surrounding the site of injection (Figure 3B). Then, it spreads spatially over the 5 days of monitoring. Interestingly, the fluorescence signal seems to co-localize with the tumor (with the exception of the individual analyzed at 48 h). Some diffuse fluorescence also appears in the liver, but with a delay of a few days. A low amount of fluorescence is also significant in the cervical lymph nodes after a few days but remains non-significant in the spleen (Figure SI-2).



**Figure 3** – Presence of iron in the tumor, and release of encapsulated dye (BODIPY), emitting in the red and green, in the brain and liver after intratumoral injection of BODIPY-loaded H(Fe) in mice with

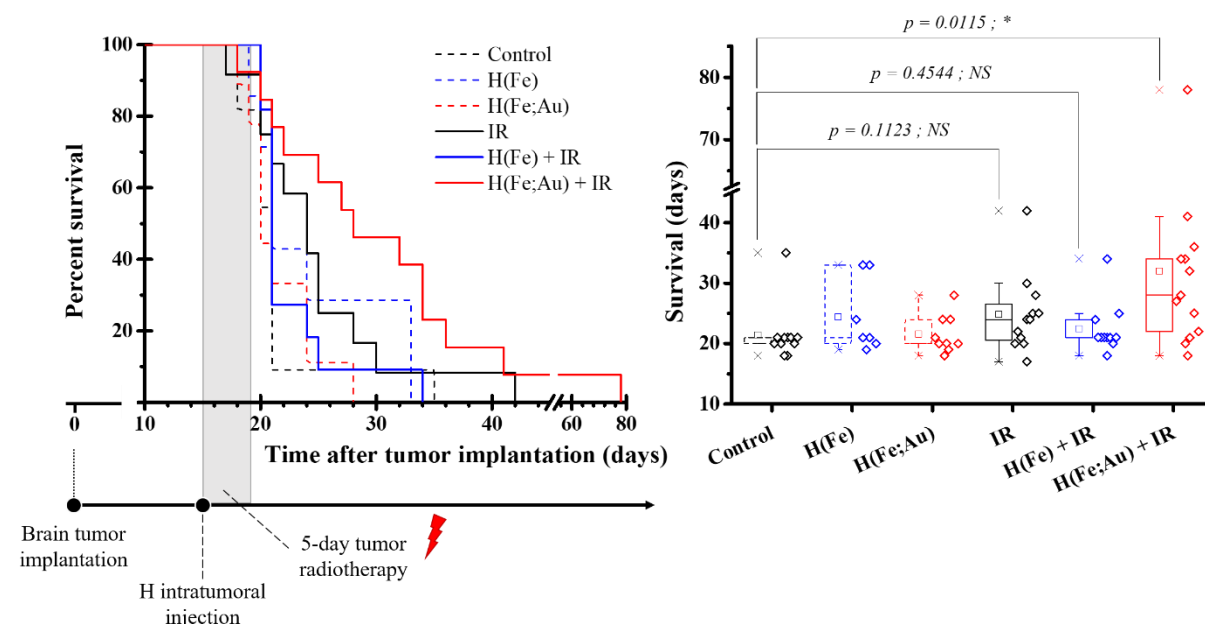
GL261 brain tumor. (A) Optical microscopy images with HES/Perls stained sections of tumoral brain 5 days after the injection, showing distribution of the iron from the hybridosomes® shell. Pictures show representative results (left: non-injected; right: injection of BODIPY loaded-H(Fe)). The blue arrow indicates the localization of iron (blue staining). (B) & (C) Fluorescence imaging: brain and liver slices were stained with DAPI and imaged for DAPI (blue) and BODIPY (green+red) fluorescence. Dash lines: tumor area. (D) & (E) Enlargements of (B) and (C), respectively, at day 5. The images show that the dye is released in the cytosol.

## Survival

We sought to assess whether hybridosomes® containing a small amount of AuNP (H 90% Fe; 10% Au) could potentiate the therapeutic effect of radiations on GL261-Luc<sup>+</sup> brain tumor model. To this aim, GL261-Luc<sup>+</sup> brain tumor-bearing mice were divided into six groups with similar tumor size distributions (Figure SI-1), as measured by MRI 9 or 10 days after tumor implantation. The mice received intratumoral injection of either H(Fe;Au), H(Fe) or none, depending on their group, as shown in Table 1. The same day, radiotherapy started for the groups concerned. Radiotherapy was applied for 5 days with 2 Gy / day. Figure 4 shows the Kaplan-Meier survival curves for all groups.

**Table 1** – Description of the different mice groups and the associated treatments.

Group treatment	Number of mice
<b>Group 1:</b> tumor – no treatment (control)	12
<b>Group 2:</b> tumor + H(Fe)	8
<b>Group 3:</b> tumor + H(Fe;Au)	9
<b>Group 4:</b> tumor + radiotherapy	12
<b>Group 5:</b> tumor + H(Fe) + radiotherapy	11
<b>Group 6:</b> tumor + H(Fe;Au) + radiotherapy	13



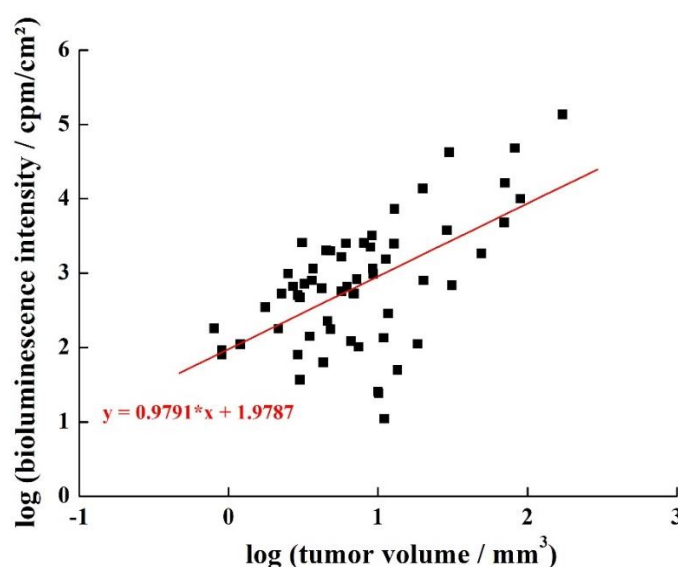
**Figure 4** – Kaplan-Meier curves (left) and box plot representation (right) of mice survival with GL261 gliomas after treatment with radiotherapy and/or hybridosomes®. Intratumoral injection of H(Fe) (blue), H(Fe;Au) (red) or none (black) was achieved two weeks after the implantation of GL261-Luc+ cells, and irradiation started immediately with 2 Gy per day for 5 days (full lines: irradiated; dashed lines: non-irradiated). P values were calculated by the log-rank test. IR=irradiation.

The Figure 4 shows that the group of mice treated with H(Fe;Au) combined with irradiation (red full line) exhibit the longest median survival (28 days), far ahead of other groups which have a median survival in between 21 days (untreated mice) and 24 days (radiotherapy only). More precisely, survival was significantly increased in the H(Fe;Au) + irradiation group compared to untreated mice (\* $p = 0.0115$ , logrank). In contrast, even if the number of mice used was almost the same (12 and 13 respectively), treatment with irradiation showed no significant benefit ( $p=0.1123$ ). The groups treated with H(Fe), H(Fe;Au) and even H(Fe)+irradiation showed no significant difference with the control group receiving no treatment.

### Monitoring of the tumoral growth by Bioluminescence and MRI

The first bioluminescence measurement was achieved on day 9 after tumor cells implantation, and days 10 or 11 for MRI. No measurements were achieved during the irradiation period (until day 21) to avoid repetitive anesthesia. Then, measurements were acquired every 4-5 days until death. Only albino mice could be followed by bioluminescence. All measurements are presented in Figure SI- 4. Whereas the survival of mice belonging to the 3 non-irradiated groups (#1-3) rarely exceeded the irradiation period (only 2 mice out of 18), it is clear from bioluminescence measurements that in the groups receiving irradiation (groups 4-6) several

individuals exhibited a reduction in signal intensity after the irradiation period. This is particularly true in group 6 (H(Fe;Au) + irradiation) where 3 out of 7 mice showed a significant decrease of bioluminescence after irradiation. The case of one particular mouse who survived for 78 days is detailed in the following section. In the control group 4, irradiated but receiving no hybridosomes®, only 1 out of 6 mice underwent such signal decrease. Two mice in the group irradiated in the presence of H(Fe) (group 5) had a temporary stop of tumor growth, but no decrease in size was observed.



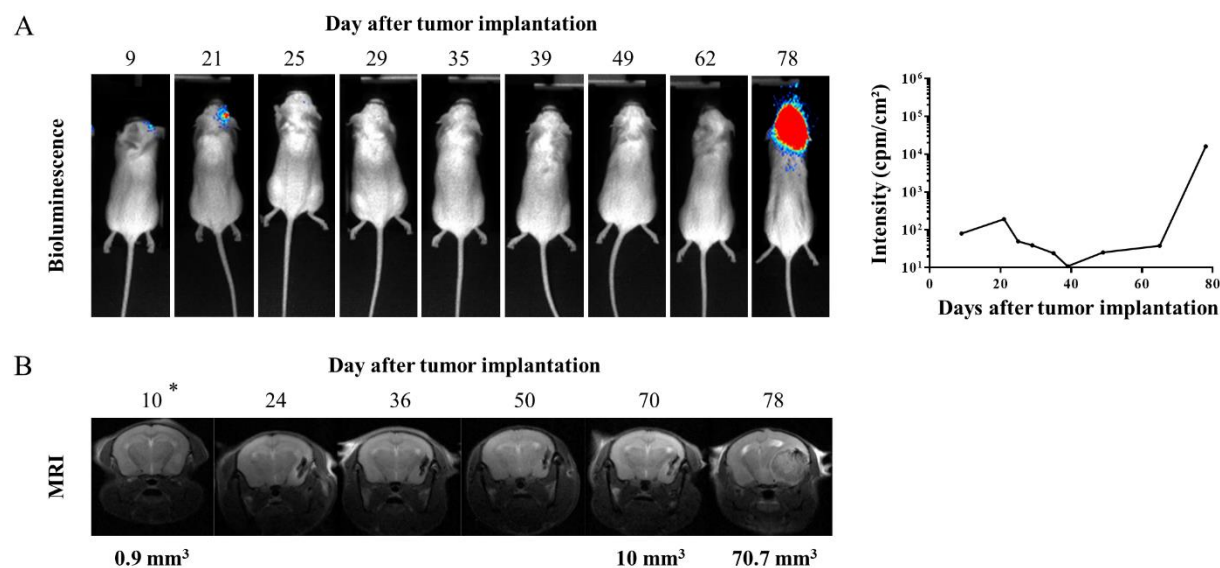
**Figure 5** – Log-Log representation of the bioluminescence intensity vs MRI measurement of tumor volume, for albino mice over the survey.

Bioluminescence and MRI are complementary techniques for the monitoring of tumor growth. Bioluminescence provides a measurement of the activity of the tumoral cells. It is rapid and cost-effective, but cannot be applied in the clinics. MRI is an imaging technique giving detailed morphological information on the tumor. However, the distinction between inflammation and tumor itself is not always straightforward. Since few studies present MRI and bioluminescence data simultaneously, we took advantage of this large set of data to check the correlation between MRI and bioluminescence measurements. Figure 5 shows a Log-Log representation of bioluminescence intensity vs tumor volume measured from MRI. It appears that the data follow roughly a straight line of slope 1, which indicates that the volume (from MRI) is positively correlated to bioluminescence intensity. The discrepancy is more important for small tumor volumes ( $< 10 \text{ mm}^3$ ). This might be due to the hyposignal of the iron oxide NP, masking the tumor contrast.

# Case study of a long term survivor treated by radiotherapy combined with H(Fe;Au)

Of note, one mouse (#1082) treated with H(Fe;Au) combined with irradiation survived until day 78 after tumor implantation (Figure 4). Its tumor size was monitored over all this period by both bioluminescence and MRI. The bioluminescence signal (Figure 6A) from the GL261-Luc+ implanted tumor clearly decreased after the irradiation treatment, over a period of almost three weeks (from day 21 to day 39). The decrease in intensity over this period is more than a factor of 20. From day 40, the bioluminescence increased again, first slowly, then abruptly. The tumor size was also monitored using MRI (Figure 6B). From day 24 to day 62 the tumor remained extremely small and hindered by the hypointense signal of the iron-oxide NP. A sudden burst of the tumor growth occurred in-between days 70 and 78.

To go further, the brain of the mouse # 1082 was collected after death, serially sectioned (every 100  $\mu\text{m}$ ) and analyzed by optical microscopy after HES and Perls staining (Figure SI-3). A large tumor is clearly visible on HES stained slices, consistently with MRI and bioluminescence observations. Moreover, at day 78, Perls staining confirms the localization of iron at the upper edge of the tumor, as seen by MRI. It also confirms that iron from the H(Fe;Au) is still present even two months after intratumoral injection.



**Figure 6** – Case study. At day 14 after brain implantation of GL261-Luc+ cells, the mouse 1082 underwent intratumoral injection of H(Fe;Au) and was treated by irradiation for one week. (A) Bioluminescence signal from the GL261-Luc+ tumor. (B) MRI of the brain. Day 10 is before injection of hybridosomes®, all other images are after injection of the hybridosomes® and show the hypointense signal from the magnetic iron-oxide NP.

## Discussion

The results presented here suggest that the H(Fe;Au) efficiently potentialize the radiosensitization of GBM.

For these investigations, we used the GL261 model in immunocompetent mice considered as the standard model for glioblastoma research<sup>28</sup>. Indeed, despite some distinct features from spontaneous glioblastoma, notably the lack of characteristic histological vascularity, it shows rapid development and excellent reproducibility<sup>28</sup>. The nano-objects that are under investigation here have the potential to provide radiosensitization and encapsulate a chemotherapy at the same time.

*Hybridosome® should be considered for the release of a chemotherapy.*

The standard treatment for glioblastoma is a combination of chemotherapy and external radiation therapy. In this context, we are interested in developing and characterizing the capacity of hybridosomes to combine concomitant radiotherapy and chemotherapy. Thus, in the work presented here, we wanted to characterize the ability of our hybridosomes to deliver a molecule of interest to the tumor site of injection and to evaluate its fate (residence time, circulation). For this proof of concept, we have first used hybridosomes® loaded with a fluorescent dye to model the release of a drug in the tumor environment.<sup>29</sup> The *in vivo* fluorescence analysis of the brain shows that the dye-loaded hybridosomes® release their payload throughout the tumor area after intratumoral injection. The dye is present at least for 5 days after injection. The model dye also accumulates into the liver, with some delay. A possible explanation for the emission observed in the liver, is that a fraction of dye-loaded hybridosomes® extravasate from the tumor site into the vascular system, where they are taken-up by the monocytes and conveyed to the liver. Indeed, since the hybridosomes® are ca 90 nm in diameter, they ought to be rapidly sequestered by the mononuclear phagocyte system (MPS) if they enter the bloodstream. Over this period of time, some dye also reaches the spleen and possibly the cervical lymph nodes. At the cellular level, we observed *in vitro* that the dye is able to diffuse into the cytosol of GL261 cells after incubation with dye-loaded H(Fe;Au), but also *in vivo* in the brain tumor area (cf. Figure 3D). The diffusion of the fluorescent BODIPY in the cytosol but not in the nucleus is consistent with previous fluorescence microscopy observations using a variety of encapsulated BODIPY dyes<sup>30</sup>. Since the capsule shell remains out of the cell<sup>20,30</sup>, the subcellular repartition of the encapsulated cargo depends only on its molecular properties. Thus, a specific drug may be able to enter the nucleus whereas our model dye does not. Altogether, these results are consistent with previous results, both *in vitro* and *in*

*vivo*, showing that the iron-oxide hybridosomes® have a porous structure enabling the release of their payload in lipophilic compartments<sup>20,27</sup>.

*H(Fe;Au)* significantly increase survival of brain tumor bearing mice treated by irradiation, at very low amount of gold content. Our main objective was to evaluate the *in vivo* radiosensitization effect of hybridosomes®, containing gold in small amounts. Our results show that the nanocapsules H(Fe) or H(Fe;Au) do not increase survival nor cause a tumor growth inhibition, when they are not associated with irradiation. Importantly, they also did not induce any toxicity. Very interestingly, the group receiving H(Fe;Au) combined with radiotherapy shows a significantly prolonged survival (28 days compared to 21 for the control group, \*p=0.0115), whereas all other groups did not. In addition, it is also the only group for which bioluminescence intensity show a clear decay, for 3 mice over 7. One mouse of this group even showed a 78-days survival. This shows that the presence of gold is indispensable to achieve a beneficial effect.

Interestingly enough, the increase in survival rate was achieved here with very low amounts of gold. An early work<sup>31</sup>, based on numerical simulations, reported that an intratumoral concentration of 0.7% by weight wt/wt (i.e. 7 mg Au/g tumor) is required for a twofold dose enhancement using 140 kVp X-rays. Another numerical study suggested that a gold nanoparticle concentration of at least 0.1% is necessary to generate radiosensitization using low-energy X-rays (~100 keV)<sup>32</sup>. Most *in vivo* experimental studies obtained significant improvements in the survival of animals using gold nanoparticles in the range of 0.1-75 mg/g tumor<sup>11,15,31</sup>. It is important to note that in the case of intravenous injection, these quantities are determined by postmortem gold titration in the tissues. However, the proportion of nanoparticles having reached the tumor is generally less than 1%, which may lead to injecting doses that are beyond reasonable for clinical translation<sup>33</sup>. Roux *et al.* estimated that an injection of 8 µg/g (body weight) or less is relevant for clinical translation. Since then, some significant radiosensitizing effects were observed with gold nanoparticles at less than 5 µg/g<sup>34-36</sup>. In the present study, mice were injected 0.25 µg of Au, or approximately 0.01 µg/g (body weight) associated with irradiation in the range ~ 10-100 keV, as in most reported preclinical studies. Therefore, there is an opportunity to increase the amount of gold to increase the radiotherapeutic benefit. This can be easily achieved without increasing the total amount of hybridosomes® by increasing the ratio of gold to iron oxide NP in their shell or even using pure gold hybridosomes®. Indeed, iron oxide was used here as a mean to assess the presence of the hybridosomes® at the site of injection. However, in the case of surgical application, this might

not be necessary. Note that despite the fact that the radiation enhancement is expected to be much lower at clinical radiation energies (MeV), many reports show that high-Z materials such as gold and gadolinium still have a significant dose deposition effect <sup>11,13,37</sup>.

*Iron oxide show no radiosensitization effect.* It is important to note that H(Fe) did not potentiate the radiotherapy, and even possibly induced a slight deleterious effect compared to radiotherapy alone. Indeed, it contrasts with a few previous studies reporting a radiosensitization effect of iron oxide nanoparticles.<sup>38,39</sup> This effect is not primarily related to the increase in dose deposition, as in the case of gold, but results from the iron-catalyzed production of reactive oxygen species (ROS) *via* the Fenton or Haber-Weiss reactions <sup>14</sup>. More recently, it has been demonstrated that irradiated SPION target the immunosuppressive tumor environment, and re-activate the immune defense at the tumor site <sup>40</sup>. Even if iron oxide alone shows no effect, a synergy may occur between gold and iron by combining the effect of strong dose deposition and ROS catalysis. This was observed before in the case of particles made of gold and manganese oxide <sup>41</sup>.

*H(Fe;Au) associated with irradiation can induce long-term survival of brain tumor-bearing mice: a case study.* A careful investigation of the long-term surviving mouse treated with both radiotherapy and H(Fe;Au) was particularly interesting. After radiotherapy, the bioluminescence intensity decreased continuously during 20 days. Then, from day 39, it started increasing again, first slowly then bursting, leading finally to the death on day 78. One can reasonably hope that an additional radiotherapy approximately at day 40, that is 20 days after the end of the first treatment, would have further improved the survival of this individual. This would have not required a new injection of radiosensitizing materials, since MRI observations confirmed by histology show that the hybridosomes® were still at the tumor site.

*Intratumoral administration after surgery is appropriate for hybridosomes®-based therapy of GBM.* We believe that these results are encouraging in the context of postoperative radiotherapy combined with chemotherapy, which is the case for standard of care for GBM. Indeed, the blood brain barrier (BBB) has remained an impassable obstacle for most chemotherapies and radiosensitizing agents. Therefore, alternative options are convection assisted delivery (CVD) and local administration of sustained released chemotherapy, embedded in polymer matrixes such as Gliadel®. Deposition of a radiosensitization agent during surgery is a particularly advantageous option. Our results show that the nanoparticles constituting the nanocapsule shell

are present and visible *in vivo* at the tumor site for at least two months, allowing enough time for one or even several cycles of radiotherapy. In addition, we demonstrate that an encapsulated cargo could be delivered from the hybridosomes® specifically to the tumor cells.

*MRI vs bioluminescence.* In the clinic, the diagnosis is made by MRI. For preclinical studies, bioluminescence is much more convenient, provided that a Luciferase-expressing tumor model is at hand. Importantly, our comparative results show that both techniques are reasonably correlated. Some discrepancy may arise from the highly scattering nature of the brain tissues, resulting in intensity loss at the detector for the more profound tumors. In addition, when present, the SPION hinders the measurements of small tumors. The position of the mouse relatively to the bioluminescence detector is also an important factor<sup>42</sup>. In addition, in contrast to bioluminescence, MRI may not account for isolated tumor cells which in the case of GBM, may extend several centimeters away from the main tumor.

## Conclusion

The current study demonstrates that mixed gold – iron oxide hybridosomes® have a real potentiating effect on radiotherapy treatment of GBM, with a significant increase of survival even for a very low amount of gold. Numerous studies (especially simulations and theoretical calculations) tend to show that the potentiating effect of radiosensitizing nanoparticles is drastically impacted by their state of aggregation and organization.<sup>43,44</sup> In hybridosomes®, the assembly of gold nanoparticles into a robust nanocapsule is certainly the key to their performance in terms of potentiating applied radiation. We are currently investigating this hypothesis in the laboratory. The decrease of tumoral activity observed for a number of mice well-responding to this treatment is extremely encouraging, as well as the long-term survival (78 days) of one individual. Since nanoparticles were still at the tumor site at this time, additional radiotherapy cycles can be considered for an improved benefit.

Moreover, while iron oxide nanoparticles were not effective to enhance the radiotherapy, they enabled particle tracking and could further be used in the clinics for image-guided radiotherapy. In addition, cellular and *in vivo* studies have shown that a molecule of interest, concentrated under its nanoprecipitated form in hybridosomes®, is internalized by the cells and remains durably in the tumor.

Based on these results, we believe that gold-containing hybridosomes® have a great potential for treatments requiring combined-modality therapy, including surgery, radiotherapy and chemotherapy, as it is the case for glioblastoma. Few objects present such potentialities.

Optimization of the gold content and combination with an encapsulated chemotherapy should further improve the benefits and enable translation of this innovative strategy into the clinics.

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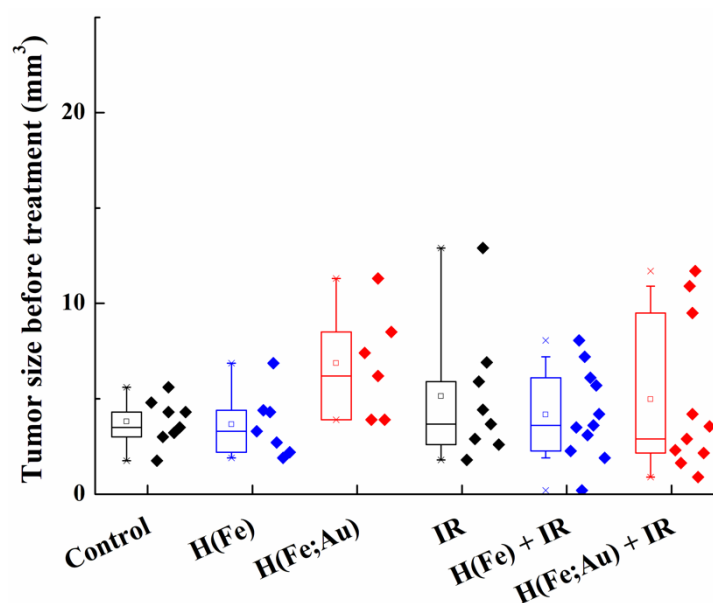
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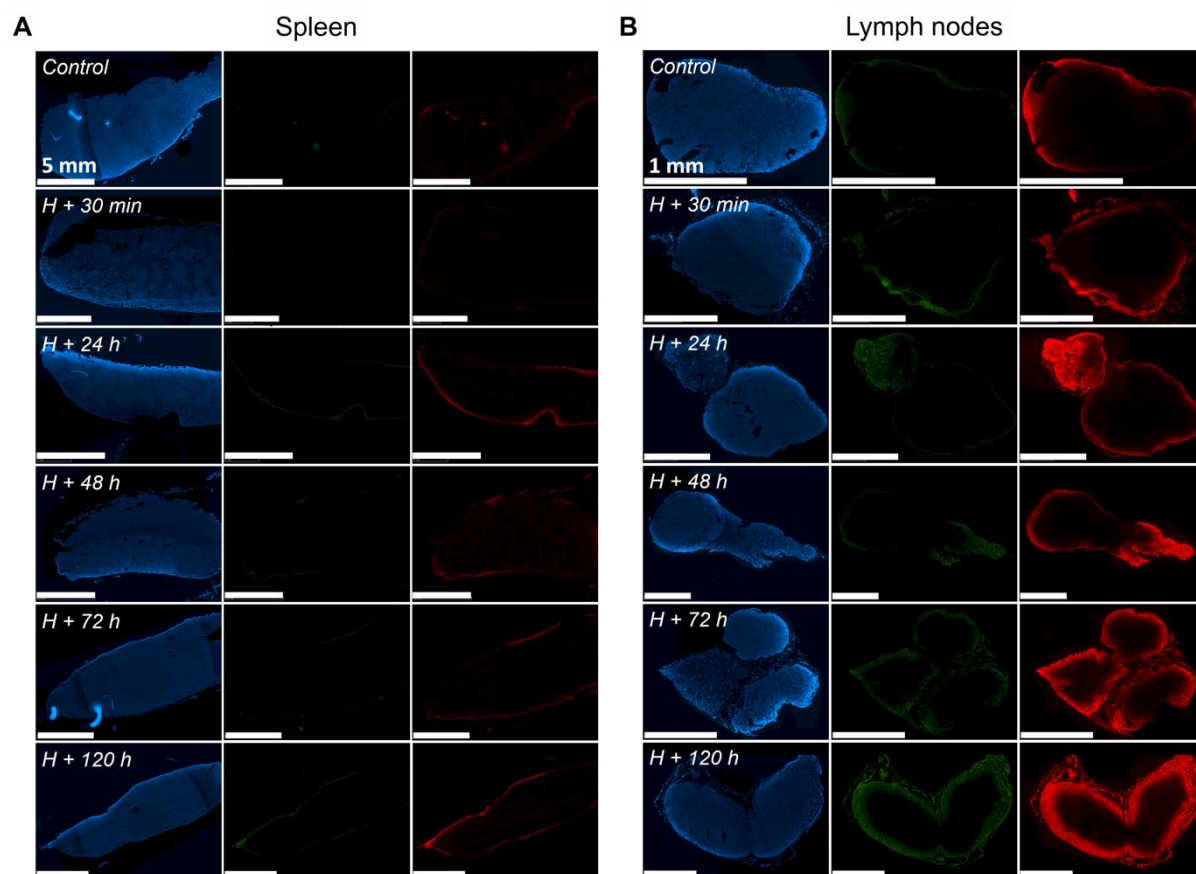
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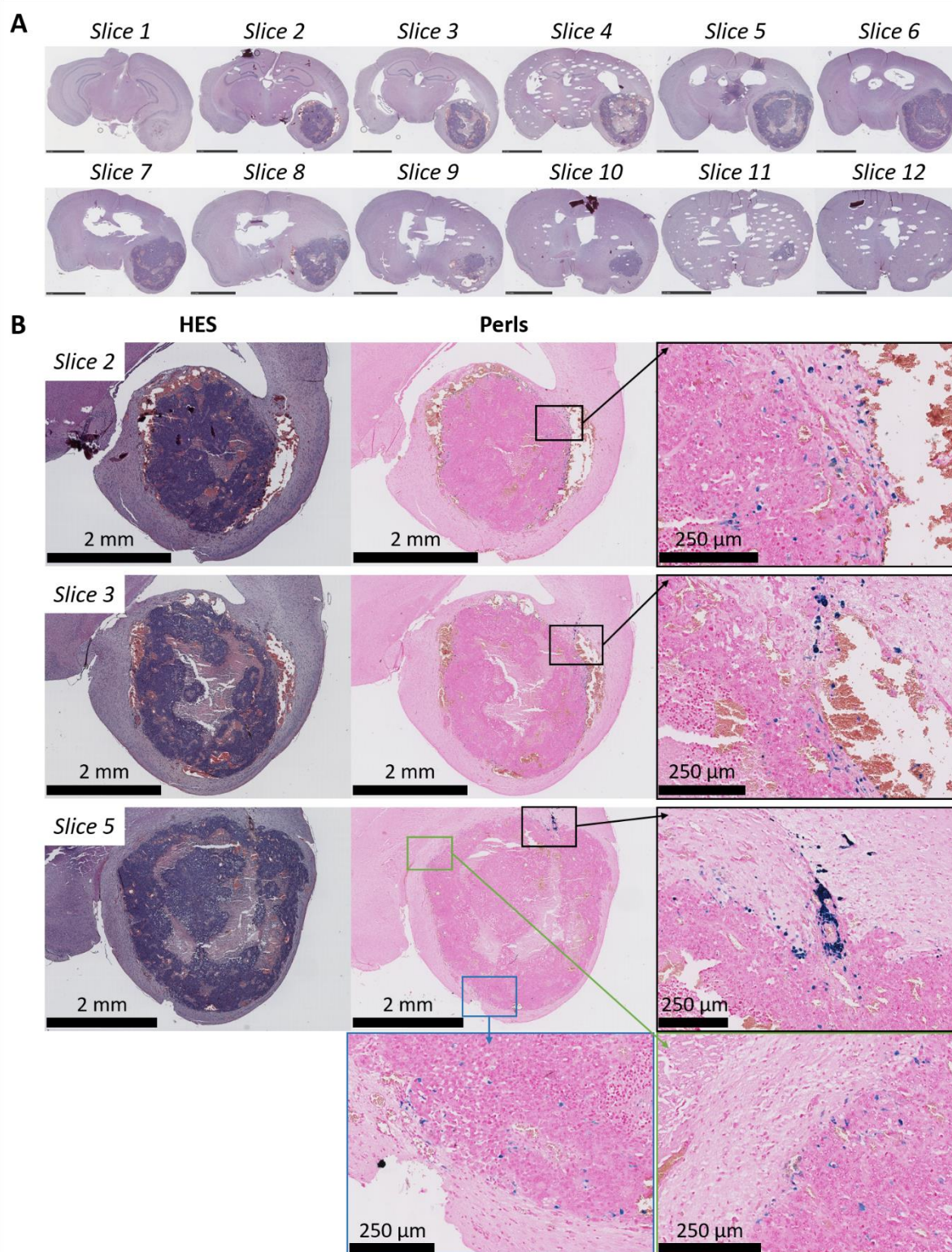
## Supplemental Figures



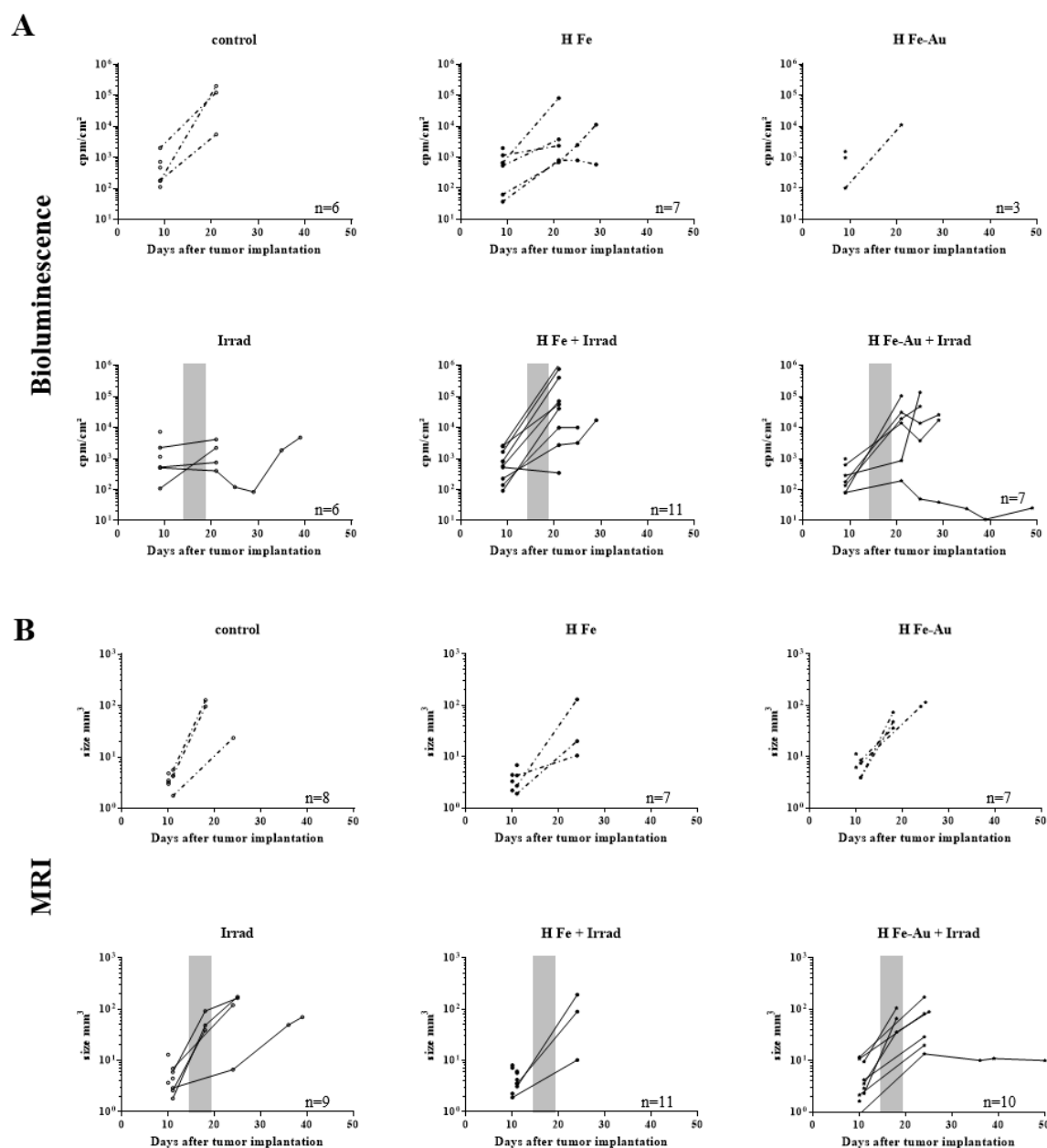
**Figure SI-1** – Initial tumor size distribution (day 9/10) within the 6 groups of mice. The inner walls in the boxes represent the median. The lower and upper walls of the boxes represent respectively the 1<sup>st</sup> and 3<sup>rd</sup> quartiles. Lower and upper ticks represent the 1<sup>st</sup> and 9<sup>th</sup> deciles.



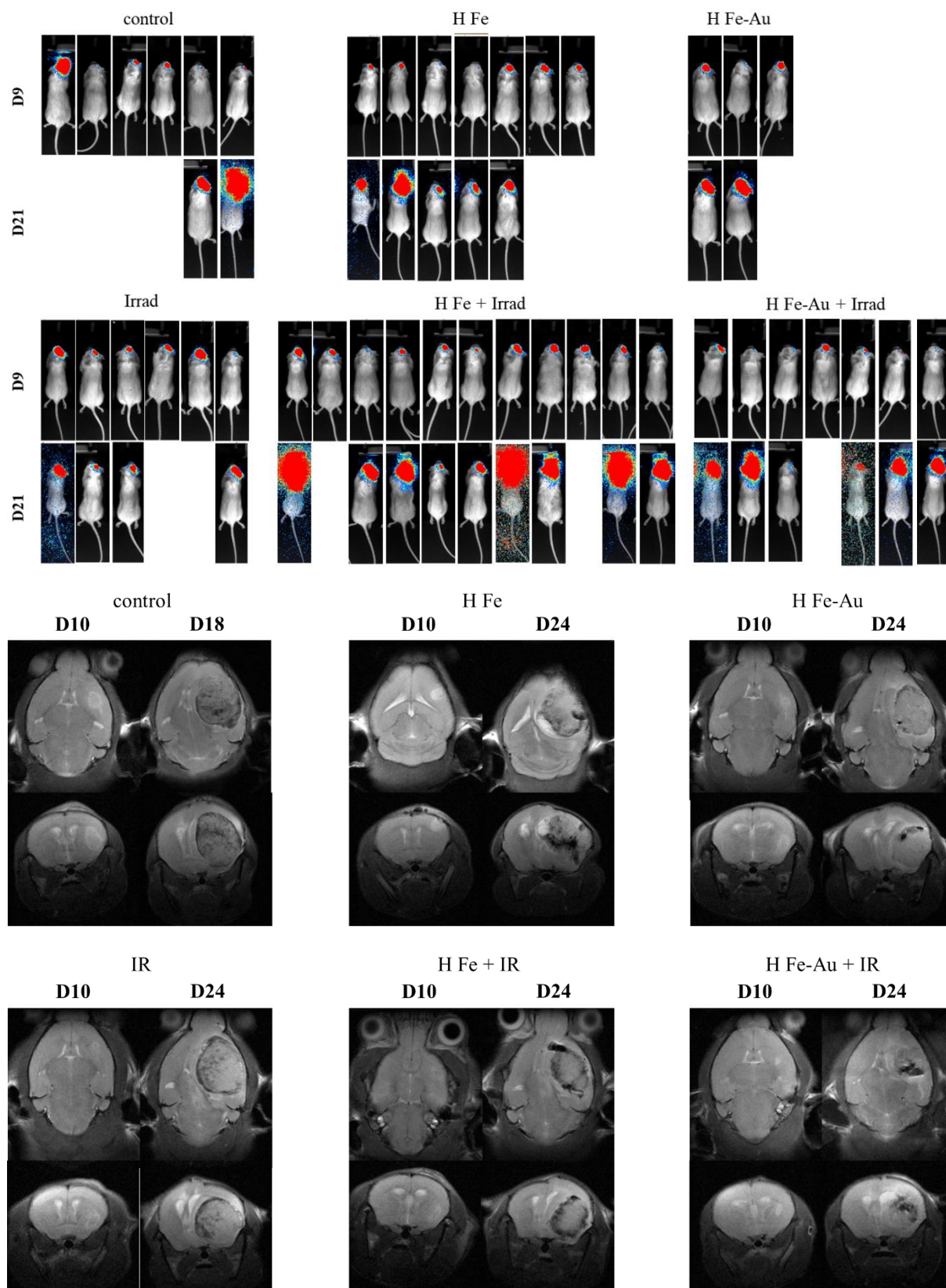
**Figure SI- 2** – Confocal microscopy fluorescence of slices of (A) spleen and (B) cervical lymph nodes of mice at different times after intratumoral injection (t=0) of BODIPY-loaded H(Fe). Tissues were stained with DAPI and imaged for DAPI (blue) and BODIPY (green+red) fluorescence.



**Figure SI-3** – Microscopy observations of slices of the brain of mouse #1082, after HES and Perls staining. (A) Slices (1 to 12) were performed every 100  $\mu$ m. (B) Enlargement centered on the tumor, of slices 2; 3 and 5, with HES and Perls staining. Iron (blue) is clearly present at the edges of the tumor.



**Figure SI- 4** Bioluminescence and MRI follow-up. The hybridosomes® H(Fe) or H(Fe;Au) were intratumorally injected at day 14 or 16 after GL261-Luc+ cells brain implantation. Irradiation started at the time of injection, for 5 days. Mice were monitored by (A) Bioluminescence (intensity) and (B) MRI (tumor volume). The grey zone represents the radiotherapy sessions.



**Figure SI- 5** Bioluminescence and MRI follow-up. The hybridosomes® H(Fe) or H(Fe;Au) were intratumorally injected at day 14 or 16 after GL261-Luc+ cells brain implantation. Irradiation started at the time of injection, for 5 days. (A) Bioluminescence recording of all albino mice before and after the period of radiotherapy. (B) Selection of MRI images of mice brain before and after the period of radiotherapy.