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Comparison of Gadolinium Nanoparticles and Molecular Contrast Agents for Radiation Therapy-Enhancement

DELORME Rachel^{1,7}, TAUPIN Florence^{2,3,6,*}, FLAENDER Mélanie^{2,3,6,*}, RAVANAT Jean-Luc^{5,6},
CHAMPION Christophe⁴, AGELOU Mathieu¹, ELLEAUME Hélène^{2,3}

¹ CEA, LIST, F-91191 Gif-sur-Yvette, France

² Université Grenoble Alpes, EA-7442 Rayonnement Synchrotron et Recherche Médicale, F-38058 Grenoble cedex 9,
France

³ European Synchrotron Radiation Facility, F-38000, Grenoble, France

⁴ Centre d'Études Nucléaires de Bordeaux Gradignan, CENBG, CNRS/IN2P3, Université de Bordeaux, France

⁵ Laboratoire "Lésions des Acides Nucléiques", Univ. Grenoble Alpes, INAC-SCIB, F-38000 Grenoble, France

⁶ CEA, INAC-SCIB, F-38000 Grenoble, France

⁷ IMNC Laboratory, UMR 8165-CNRS/IN2P3, Paris-Saclay university, 91405 Orsay, France

** Authors equally contributed to this study*

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Corresponding author: delorme@imnc.in2p3.fr

32 **Abstract**

33 **Purpose:** Nanoparticles appear as a novel tool to enhance the effectiveness of radiotherapy in
34 cancer treatments. Many parameters influence their efficacy, such as their size, concentration,
35 composition, their cellular localization, as well as the photon source energy. The current Monte
36 Carlo study aims at comparing the dose-enhancement in presence of gadolinium (Gd), either as
37 isolated atoms or atoms clustered in nanoparticles (NPs), by investigating the role played by these
38 physical parameters at the cellular and the nanometer scale. In parallel, *in vitro* assays were
39 performed in presence of either the gadolinium contrast agent (GdCA) Magnevist® or ultras-small
40 gadolinium NPs (GdNPs, 3 nm) for comparison with the simulations.

41 **Methods:** PENELOPE Monte Carlo Code was used for *in silico* dose calculations. Monochromatic
42 photon beams were used to calculate dose-enhancements in different cell compartments and
43 low-energy secondary electron spectra dependence with energy. Particular attention has been
44 placed on the interplay between the X-ray beam energy, the Gd localization and its distance from
45 cellular targets. Clonogenic assays were used to quantify F98 rat glioma cell survival after
46 irradiation in the presence of GdNPs or GdCA, using monochromatic X-rays with energies in the
47 30 keV-80 keV range from a synchrotron and 1.25 MeV gamma photons from a cobalt-60 source.
48 The simulations that correspond to the experimental conditions were compared with the
49 experimental results.

50 **Results:** *In silico*, a highly heterogeneous and clustered Gd-atom distribution, a massive
51 production of low energy electrons around GdNPs and an optimal X-ray beam energy, above the
52 Gd K-edge, were key factors found to increase microscopic doses, which could potentially induce
53 cell death. The different Gd localizations studied all resulted in a lower dose enhancement for the
54 nucleus component than for cytoplasm or membrane compartments, with a maximum dose-
55 enhancement factor (DEF) found at 65 keV and 58 keV, respectively. *In vitro*, radiosensitization
56 was observed with GdNPs incubated 5h with the cells (2.1 mg Gd/mL) at all energies. Experimental

57 DEFs were found to be greater than computational DEFs but follow a similar trend with irradiation
58 energy. However, an important radiosensitivity was observed experimentally with GdNPs at high
59 energy (1.25 MeV), whereas no effect was expected from modeling. This effect was correlated
60 with GdNPs incubation time. *In vitro*, GdCA provided no dose-enhancement at 1.25 MeV energies,
61 in agreement with computed data.

62 **Conclusions:** These results provide a foundation on which to base optimizations of the physical
63 parameters in Gd radiation-enhanced therapy. Strong evidence was provided that GdCA or GdNPs
64 could both be used for radiation dose-enhancement therapy. Their *in vivo* biological distribution,
65 in the tumor volume and at the cellular scale, will be the key factor for providing large dose-
66 enhancements and determine their therapeutic efficacy.

67 Introduction

68 An innovative therapeutic approach using heavy elements (i.e. high atomic number) in conjunction
69 with low-energy radiation of the order of tens to hundreds of keV, seems to offer a promising
70 approach for the treatment of resistant cancers. Indeed, loading tumors with heavy elements results
71 in a differential effect between the tumor and the surrounding unloaded healthy tissue due to the
72 large increase in low energy X-ray absorption in these elements. This effect is defined as “radiation
73 dose-enhancement”. Many studies have been published, with a particular emphasis in the last
74 decade, on the use of nanoparticles (NP) replacing contrast agents (CA) as radiation dose-enhancers.
75 Numerous *in vivo* and *in vitro* experiments have shown a significant increased efficacy in the
76 presence of gold ($Z_{Au} = 79$)¹⁻⁵ or gadolinium ($Z_{Gd} = 64$)⁶⁻⁸ NPs, but the underlying mechanisms
77 leading to increased cell kill are still unclear. This efficacy can be attributed in part to physical
78 aspects, such as the macro- and nano-scale radiation dose-enhancement, but also to additional
79 chemical and biological mechanisms, here defined as radiosensitization, such as high-density
80 reactive oxygen species (ROS) generation, cell cycle effects², DNA reparation impairment and
81 cytoplasmic events due to damage to the lysosomal system⁹ or to mitochondria¹⁰. Numerical
82 investigations showed the importance of considering the heterogeneous distribution of high-Z
83 atoms at the micrometer scale^{11,12} as well as the influence of the NP structure at the nanometer
84 scale¹³⁻²¹ to improve the description of gold NP (AuNP) radiation interactions. To better understand
85 and optimize the dose-enhancement and toxicity effects of high-Z NPs, it is of great importance to
86 study in detail the influence of beam energy. The main advantage of using low energy photon beams
87 is the large cross sections of photoelectric (PE) interactions on the K and L shells of heavy materials,
88 whereas high-energy beams predominantly induce Compton interactions. PE interaction is followed
89 by an atomic reorganization that leads to the emission of a large number of Low-Energy Electrons
90 (LEE: photoelectrons, Auger and Coster-Kroenig electrons) whose relative biological efficiency has
91 been noted in the past and place LEE as a major responsible for the effectiveness of NP (see, e.g.

92 the review of Nikjoo *et al.*²²). The prior work of McMahon *et al.*^{19,23} has demonstrated very high
93 dose heterogeneities in the near vicinity (a few hundred nanometers) of an AuNP and attributed it
94 to the large number of LEE produced. Assuming the AuNP location in the cell nuclei, their biological
95 efficiency have been well correlated to *in vitro* results by combining these nanometric doses and
96 the Local Effect Model^{17,19}. However, the NPs currently used in *in vivo* and in clonogenic assays are
97 often located outside the cell nucleus^{7,9,24,25}. The question of other cellular targets then arises with
98 the need of modelling realistic distributions of NPs within a cell to take into account these distances.
99 The impact on dose distribution of Au-clusters located in the cytoplasm¹⁵ or AuNPs located around
100 mitochondria^{18,26} have been investigated with a compartmentalized cell model. Although most of
101 the literature is focused on AuNP dose-enhancement, recent experimental studies have promoted
102 the use of gadolinium nanoparticles (GdNP), by demonstrating both cellular and *in vivo* increased
103 efficacy^{7-9,24,27}. Some of GdNPs (AGuIX® NPs) have proved to be of interest for *in vivo* imaging and
104 for image-guided radiation therapy, because of their paramagnetic properties used in MRI
105 techniques^{6,28}. Verry *et al.*²⁹ in a recent study announced their use in an ongoing Phase I clinical trial
106 (Grenoble University hospital, France). It is therefore especially important to better characterize
107 GdNPs behavior under irradiation. In a previous study⁷, we reported large sensitization-
108 enhancement ratio (SER) measured by clonogenic assays, when F98 cells (rodent glioma cells) were
109 irradiated after incubation with ultra-small GdNPs, both in the kilo-voltage energy range (31-80 keV)
110 and at high energy (1.25 MeV). In the same study, we evaluated the radio-sensitizing effect of
111 gadolinium contrast agent (GdCA) using various concentrations (2.1, 5 and 10 mg/mL) of Gd and we
112 observed radiosensitization only in the kilo-voltage energy range. For cells irradiated in presence of
113 GdCA, the SER profile *versus* x-ray energy was in good agreement with the macroscopic dose-
114 enhancement calculated by Monte Carlo simulations. These simulations however could not describe
115 the SER profile *versus* x-ray energy obtained when the cells were irradiated in presence of GdNPs⁷.

116 In the present study, complementary clonogenic experiments were performed with GdNPs
117 and GdCA. The F98 cells were incubated during 5h with GdNPs and then rinsed before irradiation,
118 for removing the contribution of GdNPs in the culture medium. This treatment condition differs
119 from the previous study, where the GdNPs remained in the culture medium during the irradiation.
120 Cell survival was measured after irradiation at various monochromatic X-ray beam energies (from
121 31 keV to 80 keV) at the European Synchrotron Radiation Facility (ESRF, Grenoble, France) and using
122 a Cobalt-60 source (mean energy 1.25 MeV). Radiation produced by synchrotron source provides a
123 unique tool for evaluating the mechanisms by which radiosensitization and radiation dose-
124 enhancement occur since it is possible to tune monochromatic X-ray beams over a broad energy
125 range. The dose-enhancement factor (DEF) was defined as the ratio of the dose in presence of Gd
126 relative to the dose without Gd. In the Monte Carlo simulations, the role of the Gd micro-distribution
127 (homogeneously distributed Gd-atoms or clustered Gd-atoms in nanospheres), as well as that of the
128 primary beam energy, were investigated at the sub-cellular scale, in terms of secondary particles
129 generated from Gd ionization and DEF within different cell compartments (membrane, cytoplasm
130 and nucleus). The homogeneously distributed Gd-atoms configuration was used to represent GdCA
131 while clustered Gd-atoms in nanospheres were used to represent isolated GdNP or accumulation of
132 GdNP in Lysosomes. At the nanometer scale, the dose distribution around a single GdNP varying in
133 size was studied for various primary beam energies. The objectives of this study were, firstly, to
134 evaluate by Monte Carlo simulations the dose-enhancement produced by Gd at the sub-cellular and
135 nanometer scale and secondly, to compare selected simulations with the corresponding
136 experimental data.

137

138 **I. Materials and Methods**

139 ***1.1. Experimental parameters***

140 Experimental dose-enhancements induced by ultra-small GdNPs and the GdCA Magnevist® were
141 evaluated *in vitro* by clonogenic assays performed at different energies.

142 *Gd compounds:* The GdNPs were provided by the laboratory of O. Tillement (Institut Lumière
143 Matière, univ Lyon, 69622 Villeurbanne cedex, France). These NPs were made of gadolinium
144 chelates (diethylenetriaminepentaacetic acid (DTPA)) covalently grafted to a polysiloxane inorganic
145 matrix. These nanoparticles had a mean hydrodynamic diameter of $3 \text{ nm} \pm 1.0 \text{ nm}$ (full description
146 of their synthesis is given in Di Corato *et al.*³⁰). The GdCA Magnevist®, is a complex of Gd with the
147 same chelating agent, DTPA.

148 *Cell irradiations:* F98 rat glioma cells (American Type Culture Collection, Manassas, VA (ATCC, # CRL-
149 2397)) were irradiated in suspension in a volume of 500 μL of DMEM in 1.5 mL Eppendorf tubes.
150 Low energy irradiations (31 to 80 keV) were performed at the ESRF medical beamline (ESRF-
151 biomedical ID17 beamline - $\Delta E/E \approx 0.1\%$). The dosimetry was performed using an ionization
152 chamber (PTW Semiflex ion chamber 31010 – 0.125 cm^3) placed into an Eppendorf tube. The
153 ionization chamber was scanned vertically through the beam (2 mm in height and 50 mm in width)
154 at a speed of 2.5 mm/s to measure the dose rate. Taking into account, the ring current and the dose
155 rate, the number of scans to deliver 4 Gy to the cells was calculated. High-energy irradiations were
156 performed at the NUCLEART facility (CEA, Grenoble, France), using a cobalt-60 source, whose
157 gamma emissions are 1.17 and 1.33 MeV (1.25 MeV mean energy). For all conditions, the cells were
158 irradiated at a single dose (D) of 4 Gy evaluated in water.

159 *Cell survival study versus X-ray energy after 5h incubation with GdNPs:* in a first experiment, F98
160 cells were incubated for 5 hours in culture medium (DMEM) containing GdNPs at a concentration
161 of 2.1 mg Gd/mL and then rinsed before irradiation with beam energies from 31 keV to 1.25 MeV.
162 The GdNPs uptake by the cells was determined by means of inductively coupled plasma-mass

163 spectrometry (ICP-MS). The “control” conditions correspond to cells irradiated at 4 Gy at each of
164 the above mentioned energies without GdNPs.

165 *Cell survival study at high energy (1.25 MeV)*: A second experiment was performed to evaluate the
166 influence of incubation time and Gd molecular shape on the cell radiosensitization at high energy.
167 Five treatment conditions were evaluated: (1) control cells (i.e. 4 Gy irradiation alone); (2) cells
168 irradiated in the presence of 2.1 mg Gd/mL Magnevist® (*GdCA*); (3) cells irradiated in the presence
169 of 2.1 mg Gd/mL of GdNPs (*GdNP*); (4) cells incubated for 5h with 2.1 mg Gd/mL GdNPs, rinsed and
170 irradiated (*GdNP-5h-rinsed*); and (5) cells incubated for 5h with 2.1 mg Gd/mL GdNPs and irradiated
171 (*GdNP-5h*).

172 *Clonogenic assay*: Three different cells concentrations were seeded in triplicate into Petri dishes
173 (100 mm diameter) containing 8 mL of complete DMEM, and they were incubated at 37 °C in an
174 atmosphere containing 95% air and 5% CO₂ for 11 days. All experiments were repeated three times.
175 Following staining with crystal violet, colonies of greater than 50 cells were enumerated⁷. The
176 surviving fractions (SF) were determined as the ratio of the number of colonies counted divided by
177 the number of cells plated, normalized to non-irradiated controls. The survival fraction (SF_{controls}) of
178 cells irradiated without Gd *versus* x-ray dose provided the alpha and beta parameters of the linear
179 quadratic (LQ) model used to fit the survival plots (Eq. 1). These parameters were evaluated at three
180 energies: 33 keV, 50 keV and 1.25 MeV.

$$181 \quad SF_{control} = \exp(-\alpha D - \beta D^2) \quad \text{Equation 1}$$

182 *Sensitization-enhancement ratio and experimental DEF*: The Sensitizing Enhancement Ratio *versus*
183 energy (SER) was defined as the ratio of the SF for control cells to that of cells irradiated with
184 gadolinium either as contrast agent or in the form of GdNP, (SF_{Gd}) (Eq. 2).

$$185 \quad \mathbf{SER} = \frac{SF_{control}}{SF_{Gd}} \quad \text{Equation 2}$$

186 Assuming that the sensitizing enhancement ratio measured in presence of Gd was uniquely induced
187 by “physical dose-enhancement” (DEF), we could estimate an “experimental dose-enhancement
188 factor” (DEF_{exp}) (Eq. 3 to 5).

$$189 \quad SF_{Gd} = \exp(-\alpha D \times DEF_{exp} - \beta(D \times DEF_{exp})^2) \quad \text{Equation 3}$$

$$190 \quad SER = \exp(\alpha D \times (DEF_{exp} - 1) + \beta D^2 (DEF_{exp}^2 - 1)) \quad \text{Equation 4}$$

191 From this expression, one can calculate the DEF_{exp} by resolving equation 4:

$$192 \quad \mathbf{DEF}_{exp} = \frac{-\alpha D + \sqrt{(\alpha D)^2 + 4\beta D^2 \times (\alpha D + \beta D^2 + l(SER))}}{2\beta D^2} \quad \text{Equation 5}$$

193 Three energy ranges were defined: low (31 - 40 keV), intermediate (50-80 keV) and high (1.25 MeV)
194 energy range. The α and β parameters obtained at 33 keV, 50 keV and 1.25 MeV were used to
195 calculate the SER in these three energy ranges, respectively.

196 *Simulation parameters:* Gd distributions were chosen to model the GdCA and GdNPs as
197 homogeneously distributed Gd-atoms or clustered Gd-atoms in nanospheres, respectively. Images
198 taken by confocal microscopy showed that, when incubated 2h and 5h with F98 cells, the GdNPs
199 agglomerate in clusters around the cell membrane (*see Supplemental Material Data*). To take into
200 account these clustered conditions and compare the computational DEF with experimental DEF
201 *versus* photon energy, the GdNPs were modeled as Gd-nanospheres of 50 nm radius randomly
202 distributed around the cell membrane. This geometry was also used for the experimental
203 comparison at high-energy (*GdNP-5h-rinsed*). The three other conditions at high energy were
204 modeled using a homogeneous mixture of water and free Gd-atoms in an extracellular medium for
205 the *GdCA* condition, 50 nm radius GdNPs randomly distributed in an extracellular medium for the
206 *GdNPs* condition and also distributed in the cell cytoplasm for the *GdNPs-5h* condition. The cell
207 geometry is described in Section 1.2. As the energy bandwidth of the synchrotron beam on the ID17
208 beamline is very small ($\Delta E/E=0.1\%$), monochromatic photon beams were used in the simulations.

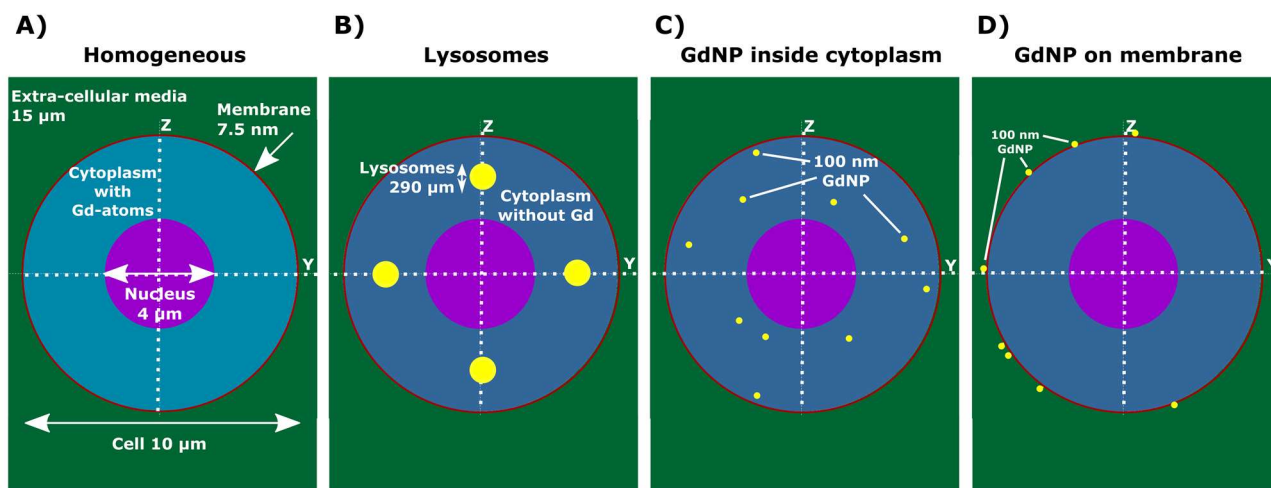
209 Additional configurations were considered using Monte Carlo simulations to simulate geometries
210 described in the literature^{9,24,25,31}.

211 ***1.2. Monte Carlo Simulations at the Sub-Cellular Scale***

212 A modified and parallelized version of the PENELOPE code was used to take advantage of its variance
213 reduction tool that is well adapted to problems of low probability radiation interactions with
214 nanoparticles³². We did not use the default PENELOPE variance reduction technique for "interaction
215 forcing", but a technique developed in the laboratory. For each photon entering the volume of
216 interest, the photon is split into two parts: one that undergoes interaction and one that continues
217 without interacting until the next volume on the trajectory. In this way, the dose is calculated with
218 much lower statistical uncertainty in the volume of interest and, weighted by the adapted
219 probabilities, remain consistent with actual physical cross-sections. The PENELOPE code generates
220 electron and positron histories based on a mixed procedure. The electron transport level of detail
221 is controlled in PENELOPE by specifying the values of several parameters, viz. C_1 , C_2 , W_{CC} and W_{CR} .
222 The C_1 and C_2 parameters are associated with the gathering of elastic scattering processes for
223 electrons and positrons. W_{CC} and W_{CR} , represent the cut-off energy loss for, respectively, hard
224 inelastic collisions and hard Bremsstrahlung emission. A detailed description of the algorithms used
225 in PENELOPE can be found in Salvat *et al.*³³. The present simulations were done with detailed event-
226 by-event transport setting $C_1 = C_2 = 0$, $W_{CC} = 50$ eV, $W_{CR} = 50$ eV and using 50 eV as the lowest
227 absorption energy, PENELOPE's low energy threshold.

228 We used a compartmentalized cell model^{15,26,34}, that consists of a single spherical cell of 10 μm
229 diameter (the mean diameter of F98 cells was estimated from the fluoroscopy images, see
230 *supplementary data* file) with a 7.5 nm thick membrane and a 4 μm diameter spherical and centered
231 nucleus. The cell was included in a cube of 15 μm side which represents the extracellular medium.
232 All compartments were filled with water by default. The primary X-rays were monochromatic and

233 came from a non-divergent square source with 15 μm width, placed before the cube. Three-
 234 dimensional dose-enhancement maps (voxels of 150 nm) and mean DEFs in nucleus, cytoplasm and
 235 membrane were calculated for various intracellular gadolinium distributions: A) Gd-atoms uniformly
 236 distributed in the cytoplasm; B) Gd-atoms agglomerated in 290 nm diameter spheres simulating
 237 lysosomes; C) 50 nm radius GdNP spheres randomly distributed in the cytoplasm; D) 50 nm radius
 238 GdNP spheres randomly distributed over the cell membrane. The geometries are shown in Figure 1.
 239 Configuration A simulates GdCA cytoplasmic internalization, as observed *in vitro* by *De Stasio et al.*³¹.
 240 Configuration D simulates the experimental study previously described with GdNPs. The B and C
 241 configurations correspond to other internalization of nanoparticles in the cytoplasm, for example
 242 NPs aggregating in clusters or accumulating in vesicles, as described in the literature^{9,24,25,35}. For all
 243 conditions, the gadolinium mass was 0.6 pg/cell, to simulate the measured GdNPs cellular uptake.
 244 The monochromatic photon energies range from 25 to 1250 keV, with a special focus above/below
 245 the Gd K-edge (50.24 keV).



246

247 *Figure 1: Geometries taken into account in the simulations for the different gadolinium distributions: A) homogeneous distribution of*
 248 *Gd-atoms in cytoplasm, B) accumulation in lysosomes, C) 50 nm-nanoparticles randomly distributed in the cytoplasm and D) 50 nm-*
 249 *nanoparticles randomly distributed on the membrane. The gadolinium mass was 0.6 pg/cell in all conditions.*

250 The secondary electron spectra coming from Gd interactions were also computed. The electrons
 251 passing through the nucleus were analyzed and relative spectra were obtained as the ratio of

252 electrons collected in the presence of gadolinium *versus* the electrons collected in the control
253 condition.

254 ***1.3. Monte Carlo Simulations around a single nanoparticle***

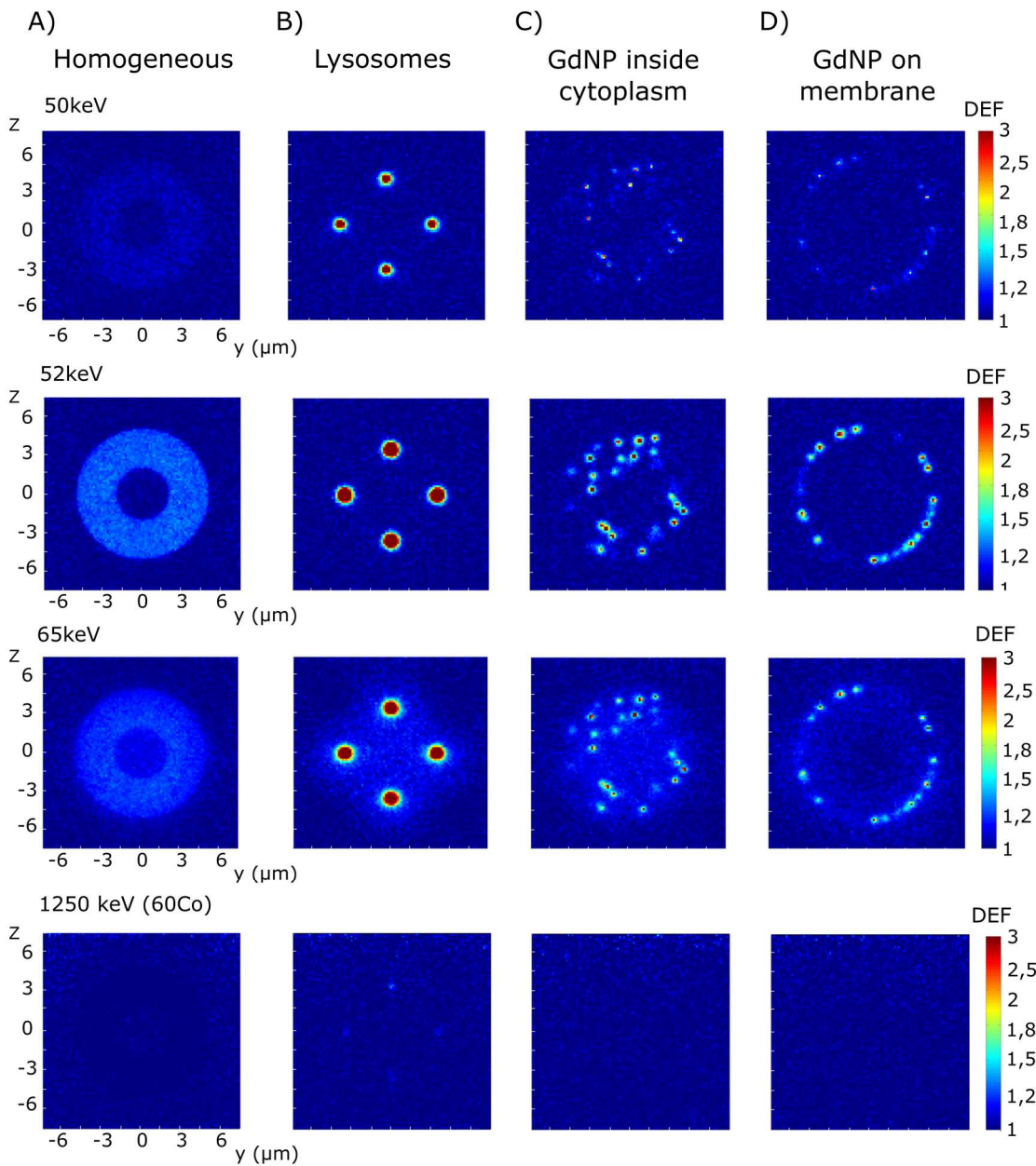
255 In order to quantify the dose-enhancement due to low-energy secondary particles in the immediate
256 vicinity of the nanoparticles, a study was carried out at a smaller scale using a geometry approaching
257 those of previous works^{14,16,19}. It consisted in the calculation of DEF in a 1 μm diameter water sphere
258 in whose center a single GdNP is placed. Simulations were performed for GdNPs of different sizes
259 (radius from 1 to 50 nm) and for different beam energies (from 25 keV to 1.25 MeV). The source
260 was circular with the same radius as the nanoparticle and located just in front of it. Note that all the
261 photons emitted from the source intercept the nanoparticle. This geometry was chosen to keep a
262 reasonable computation time (about 12 h per beam energy and per GdNP size using 24 CPUs) with
263 acceptable statistical errors (< 3%).

264 **II. Results**

265 ***II.1 Monte Carlo Simulations: Intracellular Gd Distribution***

266 The dose-enhancements calculated at the sub-cellular scale are shown in Figures 2 and 3 for the
267 various gadolinium distributions described in section 1.2. Figure 2 shows the dose-enhancement
268 maps for the beam energies bracketing the gadolinium K-edge (50 and 52 keV), the energy for which
269 the maximum radiosensitization effect (65 keV) was observed⁷, and the cobalt-source mean energy
270 (1250 keV). The color scale maximum (Figure 2) was set to DEF = 3 for better readability. However,
271 the dose-enhancements were much larger in the close vicinity of the NPs and the “lysosomes”. The
272 maxima observed with the homogeneous Gd-atom distribution are much smaller than those
273 observed with nanoparticles. In all cases, the DEFs at high energy are close to 1. For kilovolt energies,
274 Gd strongly enhances the doses and the DEF is found to be larger above the Gd K-edge than below
275 it (Figure 2).

276 At the surface of the “lysosomes”, the dose is increased by factors up to 60 and 25, for 52 and
 277 65 keV beam energies respectively. This tremendous dose increase may be explained by the high
 278 production of LEE (< 2 keV) following the photoelectric interactions that occur in the gadolinium
 279 clusters. These are essentially K photoelectrons as well as Auger and Coster-Krönig electrons
 280 resulting from atomic relaxation and secondary photoelectric interaction cascades.



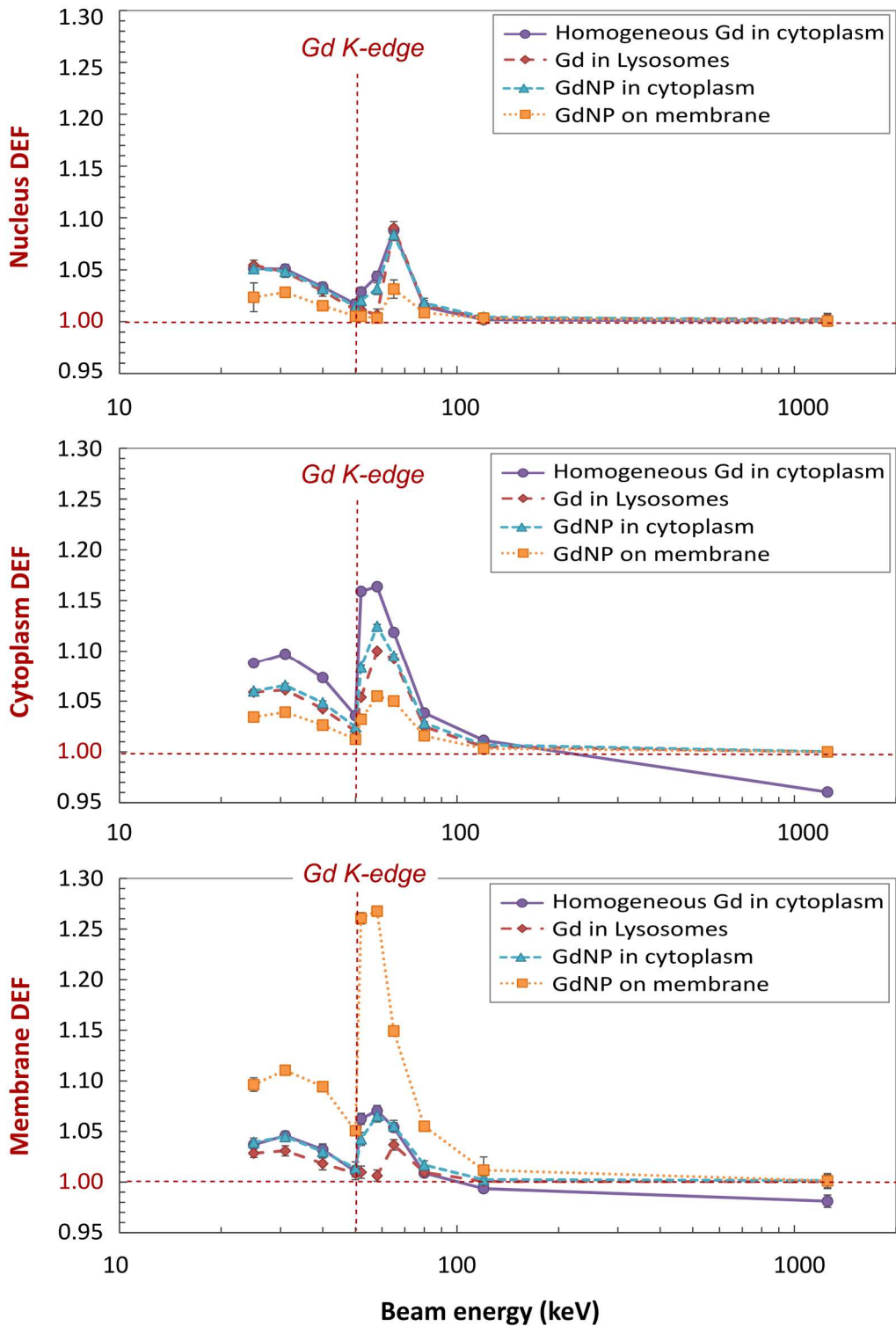
281
 282 *Figure 2: DEFs obtained at 50, 52, 65 and 1250 keV for the different gadolinium distributions: A) homogeneous Gd-atom distribution*
 283 *in the cytoplasm, B) accumulation of Gd-atoms in lysosomes, C) 50 nm radius nanoparticles distributed in cytoplasm and D) 50 nm*
 284 *radius nanoparticles distributed on the membrane. The gadolinium mass was 0.6 pg/cell in all conditions. Y and Z scales are in*
 285 *micrometers. The pixel resolution is 150 nm.*

286

The mean DEF to the nucleus, cytoplasm and membrane are compared in Figure 3 for the Gd

287

micro-distributions and for photon energies ranging from 25 keV to 1250 keV.



288

289 *Figure 3: Mean DEFs calculated to the nucleus, cytoplasm and membrane as a function of the beam energy (from 25 keV to 1250 keV)*
 290 *for four gadolinium distributions with 0.6 pg Gd/cell: homogeneous Gd-atom distribution in cytoplasm (●), accumulation of Gd-atoms*
 291 *in "lysosomes" (◆), 50 nm radius GdNPs distributed in cytoplasm (▲) and 50 nm radius GdNPs distributed on the membrane (■).*
 292 *Uncertainties of confidence interval 3 sigma are close to 1%.*

293 In all the cases, the nucleus exhibits the lowest mean DEF (< 1.10) with a maximum value at
 294 65 keV regardless of the Gd distribution as long as Gd is in the cytoplasm. For the cytoplasm and the
 295 membrane, the K-edge is much sharper with a maximum around 58 keV. The mean DEF values are
 296 larger, up to 1.16 for the cytoplasm in the case of homogeneous Gd-atoms (representing GdCA) and
 297 1.27 for the membrane when the GdNPs are distributed around the membrane. It is worth
 298 mentioning that in all cases the DEF is very close to 1 for energies above 100 keV.

299

300 ***II.2 Experimental results and comparison with the simulations***

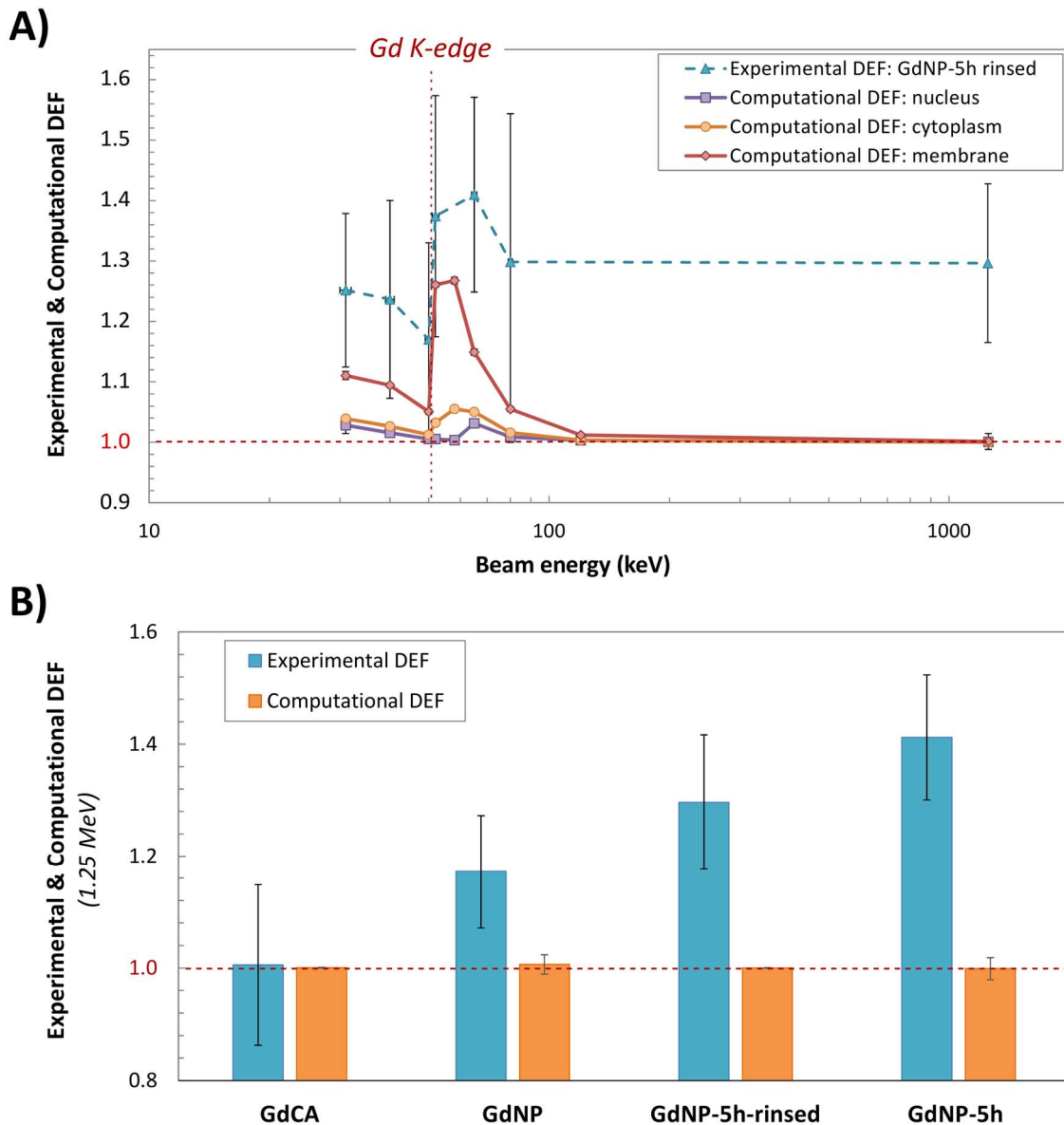
301 The alpha and beta parameters calculated from the linear quadratic fits of the dose-survival plots
 302 without Gd are given Table 1. They were measured at 33 keV, 50 keV and 1.25 MeV.

Beam Energy (keV)	$\alpha \pm \sigma_\alpha$ (Gy ⁻¹)	$\beta \pm \sigma_\beta$ (Gy ⁻²)
33 keV	0.134 (0.015)	0.030 (0.013)
50 keV	0.014 (0.013)	0.045 (0.018)
1.25 MeV	0.221 (0.020)	0.009 (0.003)

303 *Table 1: Results of the fit of the linear-quadratic model (Eq. 1): parameters α and β and their standard deviation in parenthesis. The*
 304 *survival curves versus x-ray dose were measured for irradiations without Gd at 33 keV, 50 keV and 1.25 MeV, i.e. low, intermediate*
 305 *and high-energy range, respectively.*

306 The Gd uptake of the F98 cells in presence of GdNPs was measured by means of ICP-MS. The Gd
 307 uptake in the rinsed cells, measured after 5 h incubation with GdNPs, was 0.6 ± 0.05 pg Gd/cell. The
 308 remaining Gd concentration in the culture medium at the end of the incubation period was
 309 1.8 ± 0.05 mg Gd/mL (un-rinsed case). The kinetics for the uptake of GdNPs by F98 cells was
 310 reported in our previous publication⁷. No toxicity was observed when cells were incubated with
 311 GdNPs without irradiation. The experimental DEFs *versus* x-ray energy are shown in Figure 4-A, they
 312 were calculated using Eq. 5 and the corresponding α and β parameters. Figure 4 – A shows the mean
 313 computational DEFs calculated in the nucleus, cytoplasm and membrane in comparison with the
 314 experimental DEF (DEF_{exp}) measured after 5h of GdNP incubation with F98 cells (rinsed before

315 irradiation), as a function of photon beam energy. Note that the mean DEF calculated on the entire
 316 cell (nucleus + cytoplasm + membrane) would be similar to the cytoplasm DEF.



317
 318 *Figure 4: A) mean nucleus, cytoplasm and membrane DEF versus experimental DEF (DEF_{exp}) as a function of photon beam energy (from*
 319 *31 keV to 1.25 MeV). Numerical calculations correspond to 50 nm – GdNPs randomly distributed at the surface of the cell membrane.*
 320 *B) Computational DEF versus experimental DEF obtained with various Gd-treatment conditions: in presence of 2.1 mg Gd/mL GdCA*
 321 *during irradiation (GdCA); in presence of 2.1 mg Gd/mL GdNPs during irradiation (GdNP); 5 h incubation with 2.1 mg Gd/mL GdNPs*
 322 *and cells rinsed before irradiation (GdNP-5h rinsed); 5 h incubation with 2.1 mg Gd/mL GdNPs and cells not rinsed before irradiation*
 323 *(GdNP-5h). The mean relative errors on the survival data was about 10%. Uncertainties of confidence interval 3σ on computational*
 324 *DEF range from 1% to 3% (increasing with energy).*

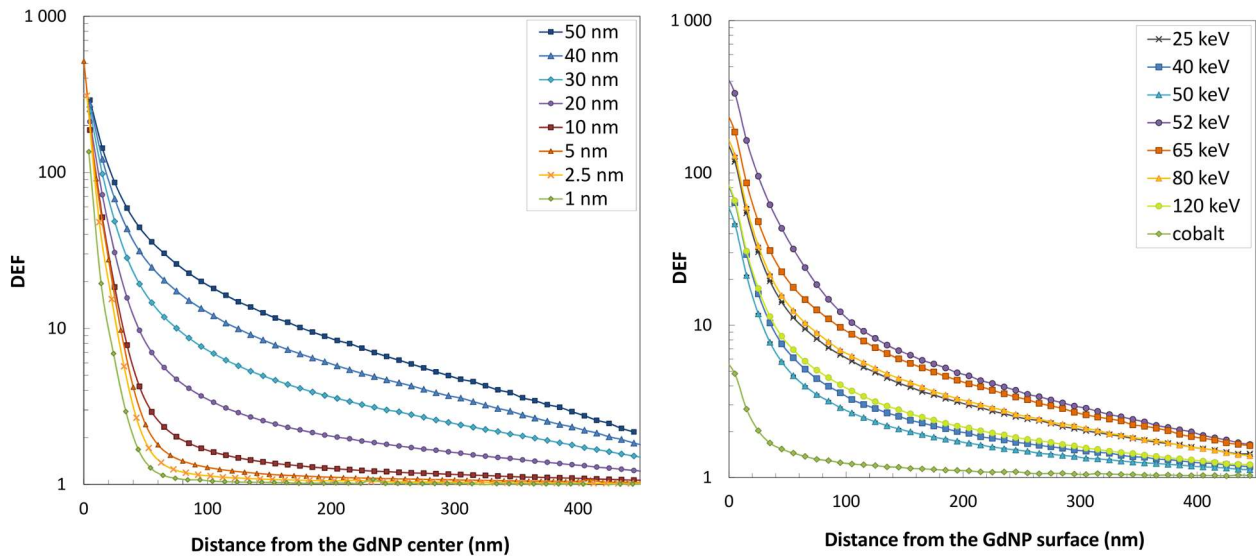
325 At all energies, experimental DEFs were found to be larger than the physical DEFs, but follow a
 326 similar trend *versus* the irradiation energy. Experimentally, a maximum is observed at 65 keV (DEF_{exp}
 327 = 1.41 ± 0.16). The Membrane DEF represents, the strongest gradient across the Gd K-edge, with
 328 DEF = 1.05 ± 0.004 and 1.26 ± 0.006 calculated at 50 and 52 keV, respectively.

329 In Figure 4 – B is reported the comparison at high-energy of mean cell computational DEF *versus*
330 experimental DEF with various incubation conditions (no incubation and 5 h incubation, rinsed or
331 not before irradiation) and molecular shape (GdCA or GdNPs). An important radio-sensitivity is
332 observed at high energy (1.25 MeV) with GdNPs whereas no effect was observed in presence of
333 Magnevist[®], as expected from modeling for all irradiation conditions. The maximum DEF_{exp} ($1.41 \pm$
334 0.11) is found for the cells incubated 5h with GdNPs and irradiated with the remaining GdNPs in the
335 medium. The presence of GdNPs inside the cells (*GdNP-5h rinsed*) induces higher DEF_{exp} (1.3 ± 0.12)
336 observed when cells are irradiated in presence of GdNPs but without incubation (*GdNP*,
337 DEF_{exp} = 1.17 ± 0.1), although the amount of Gd is far less in the first condition.

338

339 ***II.3 Nanoparticle Size and Beam Energy at the Nanometer Level***

340 Figure 5 reports the computational DEF profiles calculated for a single GdNP. The aim was to
341 evaluate the relative influence of the GdNP size and the beam energy at the nanometer scale. The
342 first set of simulations was performed at 55 keV with the GdNP radius varying from 1 to 50 nm (left
343 panel). The second set of simulations was performed with a 50 nm GdNP radius and with an X-ray
344 beam energy ranging from 25 keV to 1.25 MeV (cobalt source).



345

346 *Figure 5: Left panel: DEFs calculated along the beam direction as a function of the distance from the GdNP surface, beam energy*
 347 *55 keV and GdNP radius from 1 to 50 nm. Right panel: DEFs calculated along the beam direction as a function of the distance from*
 348 *the GdNP surface, 50 nm radius GdNP and beam energy from 25 keV to 1250 keV (cobalt source). Voxel size: 10 nm.*

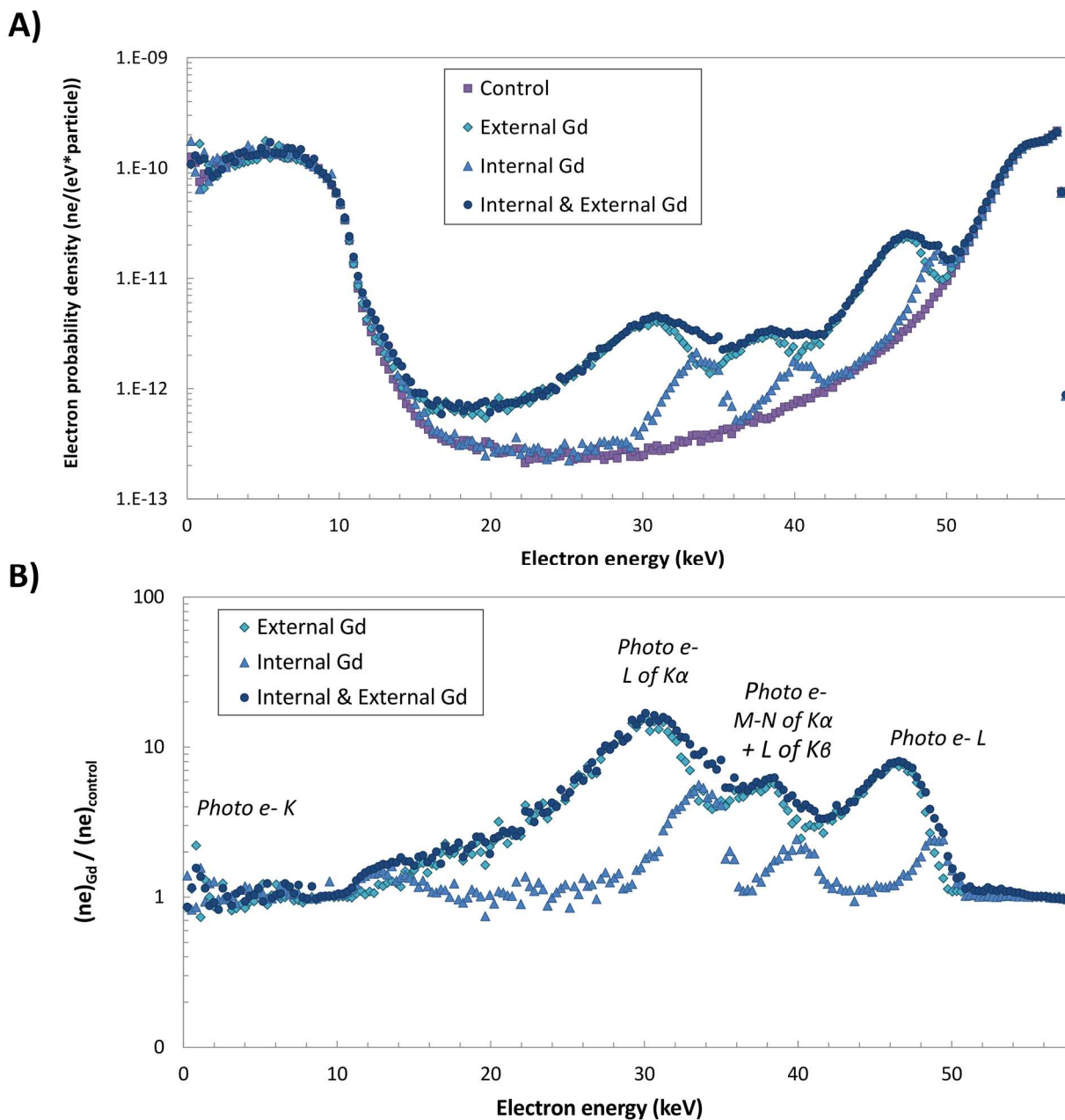
349 All DEF profiles show a strong decrease with the distance from the NP surface. Their slopes greatly
 350 vary with the NP radius (left panel), being inversely related with it. The DEF maxima at the NP surface
 351 extend from 200 to 500. For small NP (radius less or equal to 5 nm), the DEF is less than 2 beyond
 352 60 nm from the NP surface, while, for the largest ones, it is still greater than 2 at 500 nm from their
 353 surface. Looking at the beam energy influence (right panel), the DEF profiles all demonstrate a rapid
 354 decrease over the first 100 nm (from the surface of the NP) and a softening slope beyond, with DEFs
 355 less than 10 for all energies. DEFs from 5 to 400 are obtained at the surface of the NP, with a
 356 minimum for the cobalt source energy and a maximum at 52 keV.

357

358 **II.4 Electron Spectra Reaching the Cell Nucleus**

359 Figure 6 - A shows the probability density of electrons crossing the nucleus when the cell contains
 360 gadolinium (n_{Gd}) and in the absence of gadolinium (n_{control}). Figure 6 - B is the ratio of the two,
 361 representing only electrons due to Gd-interactions. Three homogeneous Gd-atom distributions
 362 were considered: in the extracellular medium (2.1 mg Gd /mL), in the cytoplasm (0.6 pg Gd /cell)
 363 and the combination of the two with a Gd-atom quantity of 0.6 pg Gd in the cytoplasm and a

364 concentration of 1.8 mg Gd /mL in the extracellular medium (in reference to the experimental
 365 conditions). The beam energy was set to 58 keV, *i.e.* 7.8 keV above the Gd K-edge.



366

367 Figure 6: A: electron probability density versus the electron energy, where $(ne)_{Gd}$ refers to the number of electrons crossing the nucleus
 368 when Gd is distributed within extra-nucleus areas (cytoplasm (\blacktriangle), external (\blacklozenge) and both (\bullet)) whereas $(ne)_{control}$ refers to the
 369 corresponding number in the absence of Gd (\blacksquare). The X-ray beam energy is 58 keV. B: Ratio of the number of electrons crossing the
 370 nucleus in presence of Gd relative to the number of electrons crossing the nucleus without Gd: $(ne)_{Gd} / (ne)_{control}$ ratio.

371 The amplitudes of the characteristic peaks vary from 3 to 15, for electron energies between 20 and
 372 50 keV (Figure 6B). This increase number of electrons reaching the nucleus due to Gd-interactions
 373 could be a cause of additional damages to the DNA, they are however not predominant in the
 374 electron spectra (Figure 6A). When the electrons are generated from Gd in the external medium,

375 they have to travel through more material before reaching the nucleus, inducing a spread of the
376 spectrum and wider energy bandwidths compared to the internal condition. The origin of the peaks
377 was identified by correlating their position with the atomic relaxation tables and the gadolinium
378 binding energies (50.24 keV, 7.24 – 8.38 keV and 1.2 – 1.9 keV for K, L1-L3 and M1-M5 layers
379 respectively). The peaks observed around 30 and 40 keV are due to the electrons originated from
380 the second photoelectric interactions of K_{α} and K_{β} fluorescence photons. Those coming from the
381 primary interactions on the K and L shells are found around 0.5 and 48 keV respectively. The great
382 majority of potential high-LET electrons²², K photoelectrons, Auger and Coster-Krönig electrons, are
383 not able to reach the cell nucleus because of their very low energies (< 10 keV), even in the case of
384 internal gadolinium.

385

386 **III. Discussion**

387 LEE and more particularly Auger electrons, were suggested in the literature as a key agent in the
388 radiobiological effects of NPs, since they are able to induce lethal DNA breaks *via* direct or indirect
389 ionization²². However, the NPs usually remain in the cytoplasm or on the cell membrane and do not
390 reach the cell nucleus^{7,9,24,25}. We have shown in this study that the LEE produced in the cytoplasm
391 or in the extracellular area are mostly absorbed before reaching the cell nucleus, even with
392 intracellular gadolinium (Figure 6). In addition, we have shown that the very strong dose gradients
393 observed around the NPs extend only a few tens of nanometers from small NPs (Figure 5). Since the
394 NPs are mostly located at a few micrometers from the nucleus, other components like the
395 organelles in the cytoplasm or the cell membrane may be potential targets.

396 When the NPs are clustered on the cell membrane, the highest DEF (1.27) was found on the
397 membrane with beam energy above the Gd K-edge (Figure 4 – A). The increase of dose can induce,
398 by direct or indirect effects, a membrane degradation and lead to cell death³⁶. Additional Gd
399 distributions were simulated to model the case where the GdNPs accumulate in vesicles such as

400 endosomes or lysosomes, such as reported by others^{25,37}. Large clusters of gadolinium distributed
401 into the cell cytoplasm were modeled and called “lysosomes” configuration. Dose-enhancement
402 factors up to 60 in the lysosomes were calculated (cf. Figure 2). These DEFs may also induce lethal
403 damage to the cell since the endosomal and lysosomal systems have important functions for cell
404 survival^{9,37,38}. Mitochondrial damage can also be a prevalent cause of cell death if NPs accumulate
405 there^{10,18,24,26} and our geometry with GdNPs distributed in the cytoplasm (Figure 2) can represents
406 the order of magnitude of achievable DEFs close to these structures. In good agreement with our
407 results, Douglass *et al.*, who modeled similar cellular geometry, suggested that LEE like Auger
408 electrons coming from the NP have a negligible effect on the overall dose increase due to their small
409 ranges¹⁵. The average dose-enhancement on the entire cell can be attributed mainly to higher
410 energy photoelectrons. Note that our simulated cell model is spherical and implies a bias because
411 of its symmetry and size. Indeed, cells actually have a more complex shape and the nucleus is
412 sometimes very close to the cell membrane. The overall biological effect may be due to cumulative
413 damage on the entire cell (nucleus, membrane and cytoplasm organelles). In a recent publication,
414 Sung *et al.*³⁴ have evaluated the influence of the cell shape and nucleus location on dose-
415 enhancement produced by AuNPs (2% Au in mass, distributed on the membrane of cells). They
416 demonstrated that a small increase could be observed at kV energies (51 keV and 150 kVp) when
417 the nucleus was shifted toward the membrane (up to 1.2 for F98 cell modelling), but no dose-
418 enhancement was obtained at high energies (6 MV). In addition, the shape of the cell did not
419 significantly affect their results when the nucleus was placed in the cell center for the F98 cells.

420 Various characteristic variations of the DEFs with beam energy were observed for the different
421 intracellular Gd distributions and the cell compartment considered. The results clearly demonstrate
422 that the larger heterogeneities and clustering behavior in Gd distribution lead to larger local DEFs
423 (Figure 2). This is due to a significant production of electrons by atomic relaxation cascades and to
424 secondary photoelectric interactions of fluorescence photons, the latter being more probable for a

425 higher density of Gd atoms. By contrast, homogeneously distributed Gd-atoms (representing GdCA)
426 led to larger DEFs in the cytoplasm (Figure 3 – *Cytoplasm*) in comparison with GdNPs. This is mainly
427 due to the auto-absorption of LEE in the Gd-clustered structures. This suggests that if the GdCA
428 could be internalized in the cytoplasm, the radiosensitizing effect on the cells could be potentially
429 important. This warrants further experimental studies to compare internalized GdCA with GdNPs.

430 In all conditions, the interactions beyond the Gd K-edge produce the higher DEFs (Figures 2
431 and 3). The DEF gradient as a function of the distance from Gd is very sharp at 52 keV (Figures 2 and
432 5) because of the low-energy photoelectrons (< 2 keV). At 65 keV, maximum DEFs in the Gd clusters
433 (lysosomes or GdNPs) are slightly lower than at 52 keV but with a more spatially extended DEF above
434 1.5 (Figure 2). Such a DEF halo could reach various critical cellular targets and create potential cell
435 death, as already mentioned.

436 The experimental DEFs (DEF_{exp}) in presence of GdNPs incubated 5h with cells showed higher
437 values than computational DEFs at all energies, with a maximum enhancement ratio at 65 keV
438 ($DEF_{exp} = 1.41 \pm 0.16$) and a sharp K-edge transition. As the membrane DEF attained the highest
439 calculated value ($DEF = 1.27$) and also showed a sharp K-edge transition with beam energy,
440 membrane damage could partly explain the observed biological effect. Interestingly, the DEF_{exp} at
441 1.25 MeV (Figure 4 – B) obtained in presence of pre-incubated GdNPs ($DEF_{exp} = 1.3 \pm 0.13$) was found
442 to be much larger than the computational DEF obtained with the “GdNP on Membrane” condition
443 ($DEF_{MC} = 1.0 \pm 0.004$). On the other hand, in presence of GdCA, no dose-enhancement was observed
444 at high energy ($DEF_{exp} = 1.01 \pm 0.14$), as predicted by the simulations. The increased biological
445 efficacy observed with GdNPs at high energy appears to be correlated with the degree of Gd
446 agglomeration in the cells and the incubation time (Figure 4 - B). Indeed, the internalized GdNPs
447 seem to have a much higher impact on the radiosensitivity than those remaining outside the cells,
448 given the differences in Gd concentrations. The experimental sensitivity reported after incubation
449 with GdNPs seemed to have two origins: one that is energy dependent and related to the physical

450 dose-enhancement; and the second that is independent of the energy. In comparison with the
451 condition of cells incubated during 5 h with GdNPs and irradiated without rinsing (reported in Taupin
452 *et al.*⁷), the experimental DEF with the rinsed cells (Figure 4-A) follows the same trend *versus* the
453 energy.

454 At the nanoscale level, the DEF at significant distances from the NP surface (> 100 nm)
455 increases with the size of the NP (Figure 5). This is explained by an increase of gadolinium mass with
456 NP size and consequently of the number of secondary LEE released. A hardening of the spectrum is
457 observed (auto-absorption within the NP of the LEE generated deeply) inducing a spreading of the
458 dose. One should note that these profiles have not been normalized by the NP mass in order to
459 focus on the effect of an isolated Gd cluster. By doing so and additionally normalizing the photon
460 flux passing through the GdNP (method used in the publication of Chow *et al.*³⁹), the average DEFs
461 in the water sphere were found to be larger for the smallest radii (data not shown). The NPs of small
462 radius therefore seem the most "efficient" at the nanoscale level due to the larger amount of LEE
463 that can be extracted from them. By extension, for an equal Gd concentration in the entire volume
464 considered, isolated heavy atoms (as GdCA) should be the most efficient Gd form in terms of LEE
465 production and local DEF. Our results confirm this idea on Figure 3 – *Cytoplasm* as slightly larger
466 DEFs are obtained in the homogeneous conditions.

467 In terms of radiosensitization efficiency, if the cell survival is linked to the mean dose, it would
468 be more appropriate to consider isolated atoms; on the other hand, if there is a specific biological
469 target, large NPs close to it could be of particular interest to increase cell damage by inducing local
470 hot spot of dose. However, to improve our understanding it would be crucial to consider the
471 biological and chemical consequences of the presence of NP in a cell environment, using more
472 specific radiobiological models (e.g. with Geant4-DNA and the LEM model).

473 It is noteworthy that monochromatic energies were used in the present study. The linear
474 accelerators conventionally used in clinics produce broad photon spectra including a large

475 contribution of photons around hundreds of keV. Polychromatic radiations might thus produce
476 more significant radiation dose-enhancement effects than those obtained with cobalt 60 in the
477 presence of NP, as discussed by McMahon et al.^{40,41}. Anyhow, kilovolt energies remain the most
478 adapted to this new therapy involving heavy atoms photo-activation since the dose-enhancements
479 are always found to be much higher (factor > 10-100) in this energy range. In clinical situations, a
480 compromise must be found in terms of low energy between the attenuation of the primary beam
481 in the patient and the number of interactions in the heavy material injected within the tumor. The
482 optimal X-ray beam energy seems to be around 80 keV according to the study by Edouard *et al.*¹¹.
483 In the perspective of patient's treatment, tumor accumulation of the GdCA and GdNPs should be
484 taken into account and preclinical data indicated quite different *in vivo* results. One should note that
485 the pharmacokinetics of GdCA is quite rapid, limiting their *in vivo* tumor uptake in quantity and
486 residence-time. On the contrary, the GdNPs might present a more favorable pharmacokinetic
487 leading to much higher tumor uptake and potentially to tumor cell internalization, and therefore a
488 higher radiosensitization effect.

489

490 **Conclusions**

491 The parameters which influence the local dose-enhancement were studied with Monte Carlo
492 simulations at a sub-cellular and nano-scale, in presence of GdNPs or GdCA and were compared
493 with experimental *in vitro* measurements. A high heterogeneity of the Gd distribution, a massive
494 production of low energy electrons around NPs and an optimal X-ray beam energy, above the Gd K-
495 edge, were shown to be key factors to increase both microscopic doses and cells radio-sensitivity.
496 The dose-enhancement calculated by Monte Carlo simulations at the sub-cellular scale account only
497 for part of the biological response observed in presence of GdNPs. Higher experimental DEFs were
498 observed at all energies, even at 1.25 MeV although no dose-enhancement was predicted by the
499 simulations. To understand these discrepancies, it would be worth refining the simulations by using

500 more realistic geometries (such as the source and the cell model) and other codes adapted to
501 nanodosimetry and chemistry modeling, such as Geant4-DNA. Performing further biological studies
502 would also help to decipher the bio-mechanisms induced by the presence of GdNPs in the cells. On
503 the other hand, the Monte Carlo simulations reflect the absence of dose-enhancement observed
504 experimentally in presence of GdCA at high energy but suggest that high DEF would be obtained
505 using GdCA in the energy range 50-65 keV. In conclusion, this study provides strong evidence that
506 GdCA or GdNPs could both be used for radiation dose-enhancement therapy. Their biological
507 distribution at the cellular scale will be the key factor for providing large dose-enhancements and
508 this will determine their therapeutic efficacy.

509

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518

519 **Conflict of interest:**

520 The authors have no relevant conflicts of interest to disclose.

521

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