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1 Biocompatible and stable magnetosome minerals coated
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4 hyperthermia treatment of tumors.

5 *Chalani Mandawala^{1,2}, Imène Chebbi¹, Mickael Durand-Dubief¹, Raphael Le Fevre^{1,3}, Yasmina*
6 *Hamdous^{1,4}, François Guyot², Edouard Alphandéry^{1,2,*}*

7
8 ¹Nanobacterie SARL, 36 boulevard Flandrin, 75016, Paris, France

9 ²Institut de minéralogie et de physique des Matériaux et de Cosmochimie, UMR 7590 CNRS, Sorbonne
10 Universités, Muséum National d'Histoire Naturelle, Université Pierre et Marie Curie, IRD, 61 rue
11 Buffon, 75005, Paris, France.

12 ³Equipe de Paléomagnétisme, Institut de Physique du Globe de Paris, Paris Cité, Université Paris
13 Diderot, UMR 7154 CNRS, 1 rue Jussieu, Paris, France.

14 ⁴Laboratoire d'Imagerie et Modélisation en Neurobiologie et Cancérologie, Campus Universitaire, Bât.
15 440, 15 rue Georges Clemenceau, 91406 Orsay Cedex, France.

16
17 *CORRESPONDING AUTHOR EMAIL ADDRESS:

18 edouardalphandery@hotmail.com

19 **ABSTRACT**

20 Magnetic hyperthermia, in which magnetic nanoparticles are introduced into tumors and exposed to an
21 alternating magnetic field (AMF), appears promising since it can lead to increased patients life
22 expectancy. Its efficacy can be further improved by using biocompatible iron oxide magnetosome
23 minerals with better crystallinity and magnetic properties compared with chemically synthesized
24 nanoparticles (IONP – Iron Oxide Nanoparticles). To fabricate such minerals, magnetosomes are first
25 isolated from MSR-1 magnetotactic bacteria, purified to remove potentially toxic organic bacterial
26 residues and stabilized with poly-L-lysine (N-PLL), citric acid (N-CA), oleic acid (N-OA), or carboxy-
27 methyl-dextran (N-CMD). The different coated nanoparticles appear to be composed of a cubo-
28 octahedral mineral core surrounded by a coating of various thickness, composition, and charge, and to
29 be organized in chains of various lengths. *In vitro* anti-tumor and heating efficacy of these
30 nanoparticles were examined by bringing them into contact with GL-261 glioblastoma cells and by
31 applying an AMF. This led to a specific absorption rate of 89-196 W/gFe, measured using an AMF of
32 198 kHz and 34-47 mT, and to a percentage of tumor cell destruction due to nanoparticles exposed to
33 AMF of 10±3 % to 43±3 % depending on the coating agent. It indicated the potential of these
34 nanoparticles for the magnetic hyperthermia tumor treatment.

35 **KEYWORDS**

36 Magnetosomes, magnetotactic bacteria, magnetosome minerals, minerals, magnetic hyperthermia,
37 alternating magnetic field.

38

39

40

42 **INTRODUCTION**

43 For more than a decade, magnetic nanoparticles are widely used for several biomedical
44 applications, (1,2), such as gene, drug and radionuclide delivery, magnetic bio-separation, (3), magnetic
45 resonance imaging, MRI, and for the magnetic hyperthermia treatment of tumors carried out both *in*
46 *vitro*, (4), (5), and *in vivo* on animals, (6), (7), and humans, (8). This treatment has proven its efficacy
47 for glioblastoma treatment, (9), leading to an average survival time of 13 months following diagnosis,
48 compared with 4 to 6 months with conventional treatments, (Maier-Hoff2007 et Maier-Hoff 2010).

49 Magnetic hyperthermia is considered as a nontoxic approach to cancer therapy, in which
50 biological tissues are exposed to moderate temperatures of 43°C to 46°C allowing selective destruction
51 of tumor cells, (11). In magnetic hyperthermia, magnetic nanoparticles are usually administered to
52 tumors and heated several times by applying an alternating magnetic field of strength 5-20 mT and
53 frequency 50-200 kHz, (12). Most of the tested nanoparticles are chemically synthesized
54 superparamagnetic iron oxide nanoparticles, SPION, (13). In this study, we introduce another type of
55 iron oxide nanoparticles, which is synthesized by a strain of magnetotactic bacteria, MTB, called MSR-
56 1 *Magnetospirillum gryphiswaldense*. MTB were originally discovered by Salvatore Bellini in 1963,
57 (14), and reintroduced by Richard Blakmore in 1975, (15). MTB synthesize intracellular magnetic
58 nanoparticles, called magnetosomes, whose magnetic moments align parallel to the earth magnetic field,
59 (16). Magnetosomes act as a compass to guide magnetotactic bacteria in the direction of the earth
60 magnetic field, (17). They are made of a crystallized mineral core composed of magnetite (Fe_3O_4),
61 which is surrounded by an organic layer, (18), (19). They are usually organized in chains, preventing
62 their aggregation, a property which is not usually found among chemical nanoparticles. Moreover, due
63 to their sizes of 30 to 120 nm, high level of crystallinity, ferrimagnetic properties, they heat more
64 efficiently than most chemically synthesized nanoparticles under application of high frequency

65 magnetic fields, (20). The efficacy of magnetosomes isolated from MTB has already been demonstrated
66 and the concept of evidence established on a murine model with breast cancer, (21). However, the
67 previously tested magnetosome suspensions, which did not undergo any specific treatment, contained
68 pyrogenic endotoxins, which need to be removed for further use in a medicinal preparation, (22).

69 In this article, we describe a method to produce magnetosome minerals, from which the organic
70 layer originating from MTB has been mostly removed, and which thus contains very low endotoxin
71 concentrations while organizing in stable suspensions. In this method, magnetosomes are extracted from
72 MTB and treated chemically to remove most of the organic material surrounding magnetosome
73 minerals. To prevent their aggregation, magnetosome minerals are then coated with the four following
74 biocompatible coating agents, which have already been used for the stabilization of chemically
75 synthesized nanoparticles: i), poly-L-lysine, PLL, (23), ii), citric acid, CA, (24), iii), oleic acid, OA,
76 (25), (26), or, iv), carboxy-methyl-dextran, CMD, (27). The properties of these coated magnetosome
77 minerals, such as endotoxin concentration, coating thickness, possible arrangement in chains,
78 cytotoxicity tested according to ISO 10993-5, *in vitro* heating, internalization and antitumor efficacy
79 under the application of an AMF are examined and compared with those of pyrogenic magnetosome
80 chains directly extracted from magnetotactic bacteria, MC, and Iron Oxide Nanoparticles (IONP), which
81 are chemically synthesized and currently used for the magnetic hyperthermia treatment of cancer,
82 (11,28). We decided to use IONP since: i) they are ferrimagnetic iron oxide nanoparticles similar in
83 composition and magnetic properties to the magnetosomes but with lower values of coercivity and
84 Mr/Ms (valeurs à reprendre de l'article de Raphael?), (Branquinho and al. (2013), Kasten and al (2014),
85 Zadnik and al (2014)), ii) they are commonly used in the study of magnetic hyperthermia
86 (ToryanaBrown). Since coated magnetosomes are intended to be used on humans, we have followed
87 regulatory guidelines (ISO 10993 standards) for the assessment of their biocompatibility.

88

89 **EXPERIMENTAL**

90 **Materials**

91 **Iron Oxide Nanoparticles (IONP).** IONP (10-00-102), which are starch coated magnetite
92 nanoparticles, were purchased from Micromod Partikeltechnologie, GmbH, Rostock, Germany. We
93 estimated that IONP contain an endotoxin concentration of 140 EU/ml per mg of iron.

94 **Growth of MSR-1 magnetotactic bacteria.** *Magnetospirillum gryphiswaldense* strain MSR-1
95 (DSM6361) was purchased from Deutsche Sammlung von Mikro-organismen und Zellkulturen
96 (Brunswick, Germany). First, MSR-1 cells are deposited on solid activated charcoal agar medium, and
97 incubated at 29 °C under microaerobic conditions during 7 days, (29). Then, several black-brown
98 colonies are collected from the solid agar medium, containing (completer) and are cultivated and
99 amplified at 29 °C under stirring. Cells are then introduced in a 35 L fermentation medium, containing
100 in 1L of medium 118 ml of 85% lactic acid, 18 ml of 25% to 28% ammonia, 2.4 g of magnesium sulfate,
101 6 g of potassium phosphate, 0.2 ml of propylene glycol, 6 g of yeast extract and 7 ml of mineral elixir
102 (30). Fermentation is carried out at 29-30 °C under agitation at 200 rpm during 5 days. During
103 fermentation, pH is maintained at 6.9 by adding an acidic feeding medium containing an iron source.
104 Growth of magnetotactic bacteria is stimulated by bubbling oxygen in the growth medium.
105 Temperature, agitation speed, pH, feeding pump flow and oxygen concentration, are monitored and
106 adjusted using an EZ controller and a BioXpert software from Applikon Biotechnology.

107 **Magnetosomes isolated from magnetotactic bacteria, MC.** After fermentation, MSR-1 cells
108 are concentrated and washed in water using tangential flow filtration. To lyse the bacteria and obtain a
109 suspension containing pyrogenic chains of MC, concentrated MSR-1 cells are resuspended in 5M
110 NaOH, (31), and heated at 60 °C during 2 hours. Then they are sonicated four times in the presence of a
111 solution of PBS 1X at 10 W during 20 sec, to remove all lysis bacterial cells remains (32).

112 **Uncoated magnetosome minerals, N.** MC then undergo the following four treatments: (i), they
113 are re-suspended in a solution containing 1% Triton X-100 and 1% SDS and are then heated at 50 °C
114 overnight; (ii), they are mixed in phenol at pH 8 and then heated at 60 °C during 2 hours in a 25 KHz
115 sonicating bath (SB); (iii), they are re-suspended in chloroform and heated at 60 °C during 2 hours; (iv),
116 they are mixed with a 1 M NaOH solution and heated at 60 °C during 1 hour in the SB, (33), (34) to
117 remove all proteins and lipids. After bacterial lysis and each of the five treatments with detergents,
118 magnetosomes are isolated from non-magnetic organic debris using a neodymium magnet. The
119 supernatant is then removed and replaced by a detergent. Uncoated magnetosome minerals labelled N
120 containing a low percentage of residual organic materials are thus obtained. They are autoclaved and
121 stored at -80 °C.

122 **Coated magnetosome minerals, N-PLL, N-CA, N-OA, and N-CMD.** Coating procedures are
123 carried out under sterile conditions, using a sterile flow hood. To prepare the different suspensions of
124 coated magnetosome minerals, four different solutions are first prepared containing: i), 300 mg of poly-
125 L-lysine, PLL, hydrobromide powder dissolved in 6 ml of pyrogen-free water, ii), 105 mg of citric acid,
126 CA, monohydrate powder dissolved in 6 ml of pyrogen-free water, iii), 800 mg of oleic acid, OA, in 40
127 ml of pyrogen-free water, iv), 840 mg of carboxy methyl dextran, CMD, powder dissolved in 12 ml of
128 pyrogen-free water. They are filtered with a polyether sulfone filter of 0.2 µm and their pH values are
129 adjusted at 10.5, 6, 11.5 or 4.1 for the PLL, CA, OA and CMD solutions, respectively. 1.5 mL of a
130 suspension of uncoated magnetosome minerals at 20 mg of iron /ml is then positioned against a
131 neodymium magnet of remanence 1.3 T during 5 minutes. The supernatant is removed and replaced by
132 6 mL of a PLL solution at 50 mg /ml, 6 mL of a CA solution at 17.5 mg /ml, 7.5 mL of an OA solution
133 at 20 mg/ml, or 6 mL of a CMD solution at 70 mg/ml. The different mixtures are then sonicated in the
134 SB during 5 hours at 37°C for N-PLL, in the SB during 5 hours at 90°C for N-CA, using a sonicating
135 finger at 10 W during 1h30 for N-OA, or in the SB overnight at room temperature for N-CMD. The
136 protocols for obtaining stable nanoparticles with these different coating agents have been adapted from

137 previously described coating conditions used with chemically synthesized iron oxide nanoparticles:
138 Babic & al. (2008) for N-PLL (35), Kotsmar & al. (2010) for N-CA (36), Jain & al. (2005) and Yang &
139 al. (2009) for N-AO (37,38) and Liu & al. (2011) for N-CMD (39). Protocols resulting from these
140 articles have been modified in order to have a manufacturing process without harmful products. After
141 sonication, the different suspensions of coated magnetosome minerals are centrifuged at 13000 g during
142 90 minutes, the supernatant is removed and replaced by pure water. A neodymium magnet is then
143 positioned against the tube containing the different suspensions of coated magnetosome minerals, the
144 supernatant is removed and replaced by pure water.

145 **Characterization of different nanoparticles suspensions**

146 **Quantification of iron concentration.** To verify total iron concentration of each nanoparticle
147 suspension, nanoparticles are first mixed with a 12 N hydroxide chloride and hydrogen peroxide to
148 produce Fe^{3+} ions complexed with 2 moles per liters of potassium thiocyanate. Iron concentration is then
149 measured at 476 nm with a spectrophotometer (UviLine 9400 Secomam).

150 **Transmission electron microscopy (TEM).** To determine the morphology, size, dispersion of
151 the different nanoparticles, 5 μL at 100 $\mu\text{g/ml}$ of each nanoparticle suspension mixed in water are
152 deposited on top of a carbon-coated copper grid (300 mesh from Oxford instruments). They are dried at
153 room temperature and examined using a JEOL JEM-2100 apparatus using a LaB6 gun operated at
154 200kV. Nanoparticle size and size distribution are estimated by measuring nanoparticle diameters on
155 500 nanoparticles using the Image J software.

156 **Nanoparticle stability in suspension.** The colloidal stability of each nanoparticle suspension is
157 evaluated using 1 mg of a homogenized nanoparticle suspension mixed in 1 ml of water and placed in a
158 quartz cuvette. The variation of the absorption of nanoparticle suspensions with time is measured at 476
159 nm during 20 minutes using a UviLine 9400 Secoman spectrophotometer. The preparation was carried
160 out two days before the first stability measurement. For each nanoparticle suspension, the stability

161 measurements are the sum of the measurements carried out during each day within 1 month on three
162 batches of nanoparticles (triplicates). The measurements are performed during 20 minutes after manual
163 shaking. Data are averages of three different measurements.

164 **Zeta potential measurements.** Electrokinetic potential or Zeta potential, related to nanoparticle
165 surface charge, is measured at 25°C using a Zetasizer Nano ZS from Malvern Instruments for each type
166 of nanoparticle dispersed in water, at a pH, which is varied between 2 and 12 using a NaOH or HCl
167 solution. Results are averages of three different measurements.

168 **FT-IR measurements.** Fourier transform infrared (FT-IR) spectra are measured on lyophilized
169 powders containing the different nanoparticles using a Bucker Vertex 70 ATR Pike Germanium. Each
170 sample spectrum has a 1 cm^{-1} resolution and is obtained for wavenumbers varied between 4000 and 400
171 cm^{-1} .

172 **CHNS measurements.** A CHNS elemental analyzer (Flash 2000 CHNS Analyzer, Thermo
173 Scientific) is used to determine the carbon and nitrogen contents of each lyophilized nanoparticle
174 suspension, containing 3 mg of iron of the different nanoparticle suspensions. Data are averages of three
175 measurements.

176 ***Limulus amoebocyte lysate (LAL)*** assay used to estimate endotoxin concentrations in
177 nanoparticle suspensions. This assay is carried out on each nanoparticle suspension to determine
178 endotoxin concentrations, using a Pierce LAL Chromogenic Endotoxin Quantitation Kit (88282
179 ThermoScientific). 1 ml of each suspension is washed with pyrogen-free water and heated at 70°C over
180 10 minutes to denature any residual protein that could interfere with the LAL assay. 25 μl of each
181 suspension containing 10 μg in iron are introduced in a 96-well and maintained at 37 °C during the
182 experiment. 25 μl of the LAL solution are added to initiate the reaction. After 10 minutes of reaction, 50
183 μl of the chromogenic substrate are added to the well during 6 minutes and the amount of endotoxins is
184 detected. Finally, 25 μl of acetic acid are added to stop the reaction. The optical density of the obtained

185 suspension is measured at 405 nm using a microplate reader. The endotoxin concentration is then
186 estimated using the calibrating curve provided with the kit. To verify that the LAL test does not interfere
187 with the nanoparticles, a recovery rate, defined as $C_{\text{total}}/(C_1+C_2)$ is measured, where C_{total} is the
188 endotoxin concentration of the nanoparticle suspensions mixed with a known amount of endotoxin of
189 0.5 UE/mL, C_1 being the concentration of endotoxins in the different suspensions of nanoparticles and
190 $C_2 = 0.5$ UE/mL. The estimated recovery rate during the different steps is lower than 50%, indicating
191 that the nanoparticles did not interfere with LAL test. Data are averages of three measurements.

192 **Cell culture**

193 **Mouse (GL-261) and Rat (RG2) glioblastoma cells** GL-261 cells were purchased from NCI-
194 Frederick (Sample number: 0507812) and cultured in RPMI 1640 medium with L-glutamine (Hyclone)
195 supplemented with 20% of Foetal Bovine Serum (Gibco) and 1% with streptomycin-penicillin solution
196 (10 units penicillin; 10 μg /ml of streptomycin from Hyclone), at 37°C in 5% CO₂. Rat glioblastoma
197 cells (RG2) were purchased from ATCC (CRL-2433) and cultured in DMEM medium (Hyclone)
198 supplemented with 10% of Foetal Bovine Serum (Gibco), 0.11 g/L of sodium pyruvate (Hyclone),
199 penicillin G sodium (50 units /ml from Hyclone) and 50 μg /ml of streptomycin sulfate (Hyclone) at
200 37°C in 5% CO₂.

201 **Mouse fibroblast cells, BALB/c 3T3 clone 31 (3T3).** 3T3 cells were purchased from ATCC
202 (CCL-163) and cultured in DMEM medium (Hyclone) supplemented with 5% of Newborn Calf Serum
203 (Hyclone), 4 mM of L-glutamine, 0.5 mL of streptomycin-penicillin solution (10 units penicillin; 10 μg
204 /ml of streptomycin from Hyclone), and 20 mM of 1M HEPES (Hyclone), at 37°C in 5% CO₂. For all
205 experiments, confluent cell monolayers are trypsinized with 0.25% Trypsine-EDTA (Gibco).

206 ***In vitro* cytotoxicity assay of the different nanoparticles.**

207 **Neutral red uptake (NRU) assay according to ISO10993-5.** Cytotoxicity of different
208 nanoparticles is determined using the NRU assay on healthy BALB/c 3T3 cell lines according to the
209 protocol described in the standard ISO 10993-5. This assay is based on the accumulation of the neutral
210 red dye in the lysosomes of viable cells. 1.10^4 cells per well are seeded in a 96-well plate and incubated
211 overnight at 37°C in 5% CO₂. The following day, the culture medium is removed and replaced by 100
212 µl of complete medium with different nanoparticle and iron concentrations of 15.6, 31.2, 62.5, 125, 500,
213 or 1000 µg/ml; cells are incubated at 37°C in 5% CO₂ during 24 hours. Then, cells are washed once
214 with a solution containing 150 µl of PBS with calcium and magnesium chloride. 100 µl of a Neutral
215 Red solution at 50 µg/ml is added to the cells and incubated during 3 hours at 37°C in 5% CO₂.
216 Following exposure to 3T3 cells, cells are washed again with 150 µl of PBS and 150 µl of Neutral Red
217 desorbing fixative (glacial acetic acid solution: ethanol: water ; 1%: 50%: 49%) is added followed
218 gentle shaking for 10 min to complete dissolution. Absorbance at 540 nm is measured using a Multiskan
219 FC microplate reader. The percentage of cells inhibition (% Inhibition), is calculated using the formula:
220
$$\% \text{ Inhibition} = \left(1 - \left(\frac{DO_{\text{sample}}}{DO_{\text{control}}} \right) \right) \times 100$$
, where DO_{sample} is the absorbance of cells with nanoparticles
221 and DO_{control} is the absorbance of cells only. These experiments are carried out in triplicate.

222 **MTT assay.** Cytotoxicity of different nanoparticles on GL-261 and RG2 cell lines is determined
223 using the MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay. 5.10^3 cells per
224 well are seeded in a 96-well plate and incubated overnight at 37°C in 5% CO₂. The following day, the
225 culture medium is removed and replaced by 100 µl of complete medium with different nanoparticle and
226 iron concentrations of 15.6, 31.2, 62.5, 125, 500, or 1000 µg/ml; cells are incubated at 37°C in 5% CO₂
227 during 72 hours. Then, cells are washed once with a solution containing PBS with calcium and
228 magnesium chloride. 100 µl of a MTT solution at 1 mg/ml is added to the cells and incubated during 2
229 hours at 37°C in 5% CO₂. The MTT solution is carefully removed and replaced by 100 µl of an
230 isopropanol solution. The plates are mixed thoroughly to dissolve purple formazan crystals and
231 incubated at 37 °C during 4 hours to ensure that all crystals are dissolved. Then the optical density

232 representing the viable cell number resulting from the solubilized purple formazan is estimated at 540
233 nm using a Multiskan FC microplate reader. And the percentage of cells inhibition (% Inhibition), is
234 calculated from the formula: $\% \text{ Inhibition} = \left(1 - \left(\frac{\text{DO}_{\text{sample}}}{\text{DO}_{\text{control}}} \right) \right) \times 100$, where $\text{DO}_{\text{sample}}$ is the absorbance
235 of cells with nanoparticles and $\text{DO}_{\text{control}}$ is the absorbance of cells only. These experiments are carried
236 out in triplicate. To get rid of the interference between the MTT assay and the nanoparticles, we
237 subtracted the value of the optical density of the assembly containing cells and nanoparticles to the
238 value of the optical density obtained after adding MTT to the assembly. The same protocol was
239 followed with Neutral Red.

240 ***In vitro* nanoparticle antitumor and heating efficacies as well as nanoparticle cellular**
241 **internalization in the presence of the AMF.**

242 **Magnetic hyperthermia set-up.** In an attempt to get close to *in vivo* treatment conditions, the magnetic
243 hyperthermia experimental set-up was not adiabatic. We used an induction system and a coil of 7 cm to
244 expose the mixture of cells and nanoparticles to an alternating magnetic field (AMF) of 34-47 mT and
245 198 kHz.

246 **Hyperthermia treatment with AMF.** *In vitro* studies are carried out using 1 mg in iron of the different
247 nanoparticle suspensions brought into contact with GL-261 cells and exposed during 30 minutes to an
248 alternating magnetic field of 198 kHz and strength of 34 to 47 mT. 2.5×10^5 GL-261 cells are seeded in a
249 35 mm Petri dish and incubated for 24 hours at 37°C in 5% CO₂. After 24 hours, the culture medium is
250 removed and replaced by 2 ml of complete medium with or without 1 mg (concentration = 0.5 mg/mL et
251 pas 1 mg/mL ?) of the different nanoparticles. Samples are then, or not for the control, exposed during
252 30 minutes to either one of the following two magnetic treatments. In the first magnetic treatment,
253 temperature is maintained at 43°C - 46°C by applying an alternating magnetic field of frequency 198
254 kHz and strength adjusted manually between 34 and 47 mT. In the second one, an alternating magnetic

255 field of 198 kHz and strength 34 mT is applied. The temperature is measured by the infrared camera
256 (ThermoPro™ EasIR-2 Thermal Imager) and infrared images are analyzed by the provided analyzer
257 software.

258 The second magnetic treatment is used to measure the specific absorption rate (SAR), measured
259 in Watt per gram of iron, of the different nanoparticles mixed with GL-261 cells. SAR is estimated
260 using the formula: $SAR = C_{\text{water}} \cdot \left(\frac{\delta T}{\delta t}\right) \cdot \left(\frac{1}{C_{\text{iron}}}\right)$, where $C_{\text{water}} = 4.2 \text{ J/(g.K)}$ is the specific heat capacity of
261 water, $\Delta T/\delta t$, measured in °C/s, is the initial slope of the temperature variation with time and C_{iron} ,
262 measured in g of iron per g of water, is the iron concentration in the different nanoparticle suspensions.
263 The choice of these parameters seems accurate since heat capacities of culture medium and water are
264 similar, (ref), and nanoparticle concentrations do not change significantly when nanoparticles are mixed
265 in culture medium (vérifier). Data are averages over three measurements.

266 **Flow cytometer measurements.** Following these two magnetic treatments, cells are washed
267 once with a solution containing PBS with calcium and magnesium, and incubated during 24h with 2 ml
268 of complete medium at 37°C in 5% CO₂. In order to harvest cells, the culture medium is removed and
269 replaced by 500 µl of Trypsin-EDTA. 2 ml of complete medium are added to neutralize Trypsin-EDTA
270 and to obtain a cell suspension whose cell viability is measured with a flow cytometer. Before and after
271 one magnetic treatment, the percentage of living cells is estimated. For that, 5 µL of 20 mg /ml of
272 propidium iodide (PI) is introduced in 500 µl of a GL-261 tumor cell suspension mixed with the
273 different nanoparticles. A Flow cytometer (Beckton Dickinson FACS Calibur 3C, BD Biosciences) is
274 used to excite PI with an argon laser at 488 nm and to detect PI emission with a FL3-H detector. PI,
275 which only penetrates in inactivated cells, is used to estimate the percentage of inactivated cells. Twenty
276 thousand cells per sample were measured to determine the percentage of living cells.

277 **Internalization of nanoparticles.** Before and after one magnetic treatment, the quantity of iron
278 of nanoparticles internalized in GL-261 cells is also measured. GL-261 tumor cells are first washed

279 twice with PBS to remove nanoparticles from cell surface and it is verified by optical microscope
280 observation that nanoparticle aggregates do not remain at the cell surface and that the quantity of iron
281 actually mainly corresponds to the quantity of iron internalized in cells, whether it corresponds to
282 crystallized or dissolved iron. 500 μ l of GL-261 tumor cells are collected for cell counting and 1 ml of
283 each cell suspension is used to measure the quantity of iron contained per cell. For that, cell suspensions
284 are first centrifuged at 13000g during 10 minutes. The supernatant is removed and the cell pellet
285 containing cells and nanoparticles is re-suspended with 250 μ l of HCl:HNO₃ (3:1) solution and
286 incubated overnight. This HCl:HNO₃ (3:1) solution dissolves crystallized iron oxide into Fe²⁺ and Fe³⁺
287 ions and denatures cell membranes. 50 μ l of each sample are mixed with 50 μ l of HCl (6N), 50 μ l of
288 H₂O₂ (20%) and 50 μ l of potassium thiocyanate (2M). This mixture induces the formation of a complex
289 between iron (III) and thiocyanate ions. Measurement of iron internalized in cells is estimated by
290 measuring the concentration of this complex by absorption at 476 nm. Hyperthermia treatments and
291 internalization studies were carried out without washing cells following nanoparticle incubation in the
292 first case and with washing cells following nanoparticle incubation in the second case. In this way, we
293 could study nanoparticle toxicity towards cells as well as nanoparticle cellular internalization in the
294 presence of AMF.

295

296 **RESULTS AND DISCUSSION:**

297 To produce a magnetosome suspension, which can be used as a medicinal product, four steps are
298 followed: i), MSR-1 magnetotactic bacteria are first cultivated, ii), pyrogenic magnetosomes chains are
299 then extracted from these bacteria, called MC, iii), MC are purified to yield a suspension of
300 magnetosome minerals, called N, which only contains a small quantity of organic material coming from
301 these bacteria, iv), magnetosome minerals are then coated with PLL, CA, OA, and CMD to produce
302 four different coated magnetosome minerals, called N-PLL, N-CA, N-OA, and N-CMD, respectively.

303 **Characterization of samples containing uncoated magnetosome minerals extracted from whole** 304 **MSR-1 magnetotactic bacteria and purified**

305 MSR-1 magnetotactic bacteria, which are used in this study to produce magnetosomes, are
306 characterized using transmission electron microscopy (TEM). When 5 μ l of a suspension containing
307 these bacteria are deposited on top of a carbon-thin-film-covered grid, dried and observed by TEM,
308 Fig. Fig. 1(a) shows that each bacterium typically contains a long chain of 30 magnetosomes,
309 (Faivre2008).). Despite of better heating properties compared to their chemical counterparts, (20), (40),
310 magnetosomes from MTB are not currently used as a medicinal product, most probably due to the
311 presence of endotoxins, including lipopolysaccharides, which could contaminate magnetosome
312 medicinal preparations. Since magnetosome formation results from the invagination of the bacterial
313 membrane, (40), endotoxin concentration at magnetosome surface might be higher than the tolerated
314 concentration.

315 In this study, MC are therefore extracted from magnetotactic bacteria with NaOH and then
316 treated with a series of different detergents (SDS, Triton X100, phenol and chloroform) at a temperature
317 of 60°C in the presence of sonication to remove most of the organic material, including endotoxins,
318 coming from MSR-1 magnetotactic bacteria. Uncoated magnetosome minerals are thus obtained and
319 characterized.

320 When 2 μ l of a suspension of uncoated magnetosome minerals are deposited on top of a carbon grid and
321 observed by TEM, no remains of organic material remain visible (Fig.1(c)). These uncoated
322 magnetosome minerals aggregate strongly and have a mean size of 43 nm (Fig. 2(a)), which is larger
323 than that of 21 nm, observed for IONP (Fig. 1(b)). Treatments involved in magnetosome extraction and
324 purification did not significantly modify magnetosome morphology and size, with reference to those
325 observed in whole magnetotactic bacteria. The FT-IR spectrum of a lyophilized suspension of uncoated
326 magnetosome minerals, presented in Figure 3(a), shows two dominant peaks attributed to iron oxide at
327 609 cm^{-1} and 673 cm^{-1} , (42). When they are contained inside magnetotactic bacteria, magnetosome iron
328 oxide composition consist of magnetite,(43). After extraction and purification, saturating isothermal
329 remanent magnetization (SIRM) measurements, carried out on uncoated magnetosome minerals, reveal
330 a maghemite composition, (44). Additional peaks observed in FT-IR spectra at 1041 cm^{-1} , 2926 cm^{-1} ,
331 and 3267 cm^{-1} , (Fig.3(a)), are attributed to PO, C-H, NH_2 and OH vibrational modes, respectively.
332 These signals are due to residual organic material remaining at the magnetosome mineral surface after
333 purification. The quantity of this organic material is further estimated by CHNS measurements carried
334 out on a homogenized lyophilisate of uncoated magnetosome minerals. These measurements reveal the
335 presence of $2.39 \pm 0.04\%$ of carbon residue coming from whole magnetotactic bacteria in 1 mg of
336 uncoated magnetosome minerals (Fig. 2(b)). Concerning the endotoxin concentration in the suspension
337 of uncoated magnetosome minerals, it lies between 10 and 160 EU /ml per mg of iron as estimated by a
338 LAL test. This indicates that uncoated magnetosome minerals are much less pyrogenic than suspensions
339 of whole magnetotactic bacteria, characterized by endotoxin concentrations larger than 1.10^5 EU /ml per
340 mg of iron, and then suspensions containing MC extracted from magnetotactic bacteria by a unique
341 NaOH treatment, which possess endotoxin concentrations lying between 2000 and 12000 EU /ml per
342 mg of iron. However, uncoated magnetosome minerals tend to aggregate as revealed by TEM image
343 shown in Figure 1(c), and by absorption measurements at 480 nm of a suspension containing 1 mg /ml
344 in iron of uncoated magnetosome minerals. Their absorption signal decreases rapidly by 80% in 20
345 minutes (Fig. 2(c)). Moreover, the variation of the surface charges of these uncoated magnetosome

346 minerals as a function of pH is shown in Fig. 2(e). It shows a zeta potential increase from -15 mV to 0
347 mV between pH 5 and 6 followed by a zeta potential decrease from 0 to -20 mV between pH 6 and 7.
348 Such a large variation in zeta potential, observed within a relatively narrow range of pH, could be
349 explained by magnetosome aggregation, which is believed to be dependent on surface charge, (21). Zeta
350 potential with measurements of the surface charge make it possible to establish the degree of interaction
351 between nanoparticles, in our example a significant variation in the surface charge would indicate
352 nanoparticles more or less aggregated devoid largely of the layer of original biological material. For
353 medical applications, it is essential to use suspensions that are stable since aggregation can lead to
354 embolism *in vivo*, and can also prevent a thorough magnetosome administration and a uniform
355 magnetosome heat production.

356 **Characterization of suspensions containing coated magnetosome minerals**

357 Administration to an individual of a magnetosome suspension requires the use of a stable
358 suspension. To achieve this aim, magnetosome minerals are coated with PLL, CA, OA, or CMD, chosen
359 for their good solubility in water, biocompatibility and low toxicity, (35–39)
360 . TEM measurements carried out on suspensions containing the four different coated magnetosome
361 minerals, N-PLL, N-CA, N-OA, N-CMD, respectively deposited on carbon grids for TEM observations
362 (Figs. 1(d) to 1(k)) which reveal the presence of a coating material surrounding magnetosome mineral
363 cores with average thicknesses between 4 and 6 nm for N-PLL, 2 and 5 nm for N-CA, 3 and 5 nm for
364 N-CMD and lower than 2 nm for N-OA. TEM images of Figures 1(d) to 1(k) show that N-PLL, N-CA,
365 N-OA, and NCM-D are arranged in chains with preferential crystallographic common orientations, as
366 presented elsewhere, (ref), and demonstrated for magnetosomes directly extracted from AMB-1
367 magnetotactic bacteria, (44). Chemically synthesized iron oxide nanoparticles are rarely reported to
368 organize in chains. When an organization in chains of such nanoparticles is described, (45), their
369 behavior contrasts with that observed with coated magnetosome minerals. Indeed, chemically
370 synthesized nanoparticles do not appear to have preferential alignments and are usually

371 superparamagnetic. FT-IR spectra of lyophilized suspensions of N-PLL, N-CA, N-OA and N-CMD,
372 provide further support for the presence of the various coating agents at the magnetosome mineral
373 surfaces. For N-PLL, peaks at 1546 cm^{-1} , 1651 cm^{-1} and 3266 cm^{-1} are attributed to the NH, C=O and
374 NH_2 bonds of poly-L-lysine respectively (Fig. 3(c)). Concerning N-CA, the peaks at 1631 cm^{-1} and
375 3250 cm^{-1} are due to C=O and OH bonds of citric acid (Fig. 3(d)). Regarding N-OA, the peaks at 1427
376 cm^{-1} , 1546 cm^{-1} and 3250 cm^{-1} are attributed to C-O, C=O and OH bonds of oleic acid (Fig. 3(e)).
377 Similarly, for N-CMD, the peaks at 1034 cm^{-1} and 3250 cm^{-1} arise from the C-O and OH bonds of
378 carboxy-methyl-dextran. CHNS measurements, carried out on lyophilized suspensions of N-PLL and N-
379 OA show a percentage of carbon of $4.91 \pm 0.09\%$ and $8.16 \pm 0.02\%$ respectively, which is higher than
380 that of $2.39 \pm 0.04\%$, which is estimated for uncoated magnetosome minerals (Fig. 2 (b)). This result
381 suggests that in N-PLL and N-OA coating material is added to mostly uncoated magnetosome minerals.
382 By contrast, for N-CA and N-CMD, CHNS measurements reveal a percentage of carbon of $2.41 \pm$
383 0.16% and $2.37 \pm 0.02\%$, which is similar to that estimated for uncoated magnetosome minerals. In N-
384 CA and N-CMD, it is therefore possible that residual organic material at the surface of uncoated
385 magnetosome minerals has been replaced by the coating material. The presence of coating in
386 nanoparticles mixed in suspension can also be observed from zeta potential measurements, which
387 indicate that N-CA, (35), N-OA, (46), and N-CMD, (38), are negatively charged at pH 7, a behavior
388 which could be due to the presence of carboxylic and hydroxyl functional groups at the surfaces of N-
389 CA, N-OA, or N-CMD (Figs. 2(e) and 2(f)), while N-PLL appear positively charged at pH 7, a property
390 that could come from the presence of a tertiary amine function (pK_a of PLL = 10.5) at N-PLL surface
391 (Fig. 2(e)). By contrast to uncoated magnetosome minerals, all four coated magnetosome minerals
392 appear to be stable in suspension. Indeed, the absorption of homogenized suspensions containing 1
393 mg/ml of N-PLL, N-CA, N-OA and N-CMD, measured at 480 nm, decreases by less than 40% in 20
394 minutes (Figs. 2(c) and 2(d)). The magnitude of this absorption decrease is comparable to that observed
395 for stable chemically synthesized nanoparticles IONP. Coating therefore leads to well dispersed N-PLL,

396 N-CA, N-OA and N-CMD, as shown in the TEM images presented in Figures. 1(e, g, i, k) and confers
397 stability to the four different coated magnetosome minerals suspensions in water. Therefore,
398 administration of these suspensions to human, which usually requires less than 20 minutes, seems
399 feasible. The biocompatibility of these nanoparticles is first demonstrated using an LAL assay, which
400 shows that the endotoxin concentration of N-PLL, N-OA, N-CA, and N-CMD suspensions is 21-160
401 EU/ml per mg of iron for N-PLL, 20-130 EU/ml per mg of iron for N-CA, 10-105 EU/ml per mg of iron
402 for N-OA, 23-140 EU/ ml per mg of iron for N-CMD. These concentrations are lower than 160 EU/ml
403 per mg of iron, an endotoxin concentration comparable to that of 140 EU /ml per mg of iron, measured
404 for chemically synthesized nanoparticles, IONPs.

405 **Cytotoxicity of the different nanoparticles towards healthy 3T3 cells in the absence of magnetic** 406 **treatment**

407 Their biocompatibility is further determined following ISO 10993 standards. Such standards are
408 followed since uncoated and coated magnetosome mineral are both considered as medical devices given
409 that their dominant mode of action does not involve any immunological, pharmacological or metabolic
410 effect but only heat. Cytotoxicity of suspensions containing various concentrations of IONP, uncoated
411 and coated magnetosome minerals, *i.e.* between 16 $\mu\text{g}/\text{mL}$ and 1 mg/mL , is estimated on healthy 3T3
412 cells using a NRU assay according to ISO 10993-5 standard. ISO 10993-12 recommends using a
413 concentration of 6 cm^2/ml for medical devices with a high surface to volume ratio such as nanoparticles,
414 corresponding to 22 $\mu\text{g} /\text{ml}$ for magnetosomes, (47) .Therefore the tested concentration range includes
415 concentrations that are above the concentration of 6 cm^2/ml recommended by ISO 10993-12. 3T3 cell
416 viability is measured after cellular incubation in the presence of the different nanoparticles during 24
417 hours. Figure 4(a) shows the percentage of 3T3 cell inhibition as a function of nanoparticle
418 concentration, measured in mg of iron per ml. When 3T3 cells are brought into contact with IONP, N,
419 N-PLL, N-CA, N-OA and N-CMD, Figure 4(a) shows that the average percentage of cell inhibition
420 remains below $\sim 30\%$, suggesting that the different nanoparticles are not cytotoxic below 1 mg per ml

421 according to the criteria of ISO 10993-5 standard. These experiments also indicate that inhibitory
422 concentrations leading to 50% cell inhibition, IC_{50} , of the different nanoparticles are high and larger
423 than 1 mg per mL, indicating that these different nanoparticles are not cytotoxic towards healthy 3T3
424 cells at these tested nanoparticle concentrations.

425 **Cytotoxicity of the different nanoparticles towards glioblastoma GL-261 and RG-2 cells in the** 426 **absence of magnetic treatment**

427 Cytotoxicity of suspensions containing IONP, uncoated and coated magnetosome minerals, is
428 further evaluated on glioblastoma GL-261 and RG2 cells using a MTT assay. Percentage of cell
429 inhibition is estimated as a function of the different nanoparticle concentrations, varied between 15.6 μg
430 per mL and 1 mg per mL, after nanoparticle incubation during 24 hours with GL-261 (Figure 4(b)) or
431 RG2 (Figure 4(d)) cells. Uncoated iron oxide particles display low cytotoxicity towards 3T3, GL-261
432 and RG2 cells, with a percentage of cell inhibition remaining below 20% for all tested concentrations in
433 Figures 4(a), 4(b) and 4(d). As observed with IONP, N-PLL and N-CA reach a larger than 30%
434 percentage of cell inhibition at 1 mg/mL and appear as observed with IONP to be more cytotoxic
435 towards GL-261 and RG2 cells than towards 3T3 cells (Figures 4(a), 4(b) and 4(d)). By contrast,
436 Figures 4(a), 4(b), and 4(d), show that N-OA are less cytotoxic towards GL-261 and RG2 cells than
437 towards 3T3 cells. N-CMD display a rather unusual behavior with significant cytotoxicity towards 3T3
438 and RG2 cells (Figure 4(a) and 4(d)) and low cytotoxicity towards GL-261 cells (Figure 4(b)). IC_{50}
439 values on GL-261 and RG2 cells, respectively of 269 and 355 $\mu\text{g}/\text{ml}$ for N-PLL, 606 and 733 $\mu\text{g}/\text{ml}$
440 for N-CA, larger than 1 mg /ml and 919 $\mu\text{g}/\text{ml}$ for N-CMD, and larger than 1 mg /ml for uncoated
441 magnetosomes, N-OA and IONP. In the absence of AMF application, optimal coating materials, which
442 may correspond to those leading to the largest cytotoxicity towards tumor cells and to the lowest
443 cytotoxicity towards healthy cells, may therefore be poly-L-lysine and citric acid.

444 After 72 hours of incubation of the different nanoparticles, the percentage of cell inhibition is

445 measured as a function of nanoparticle concentration, varied between 15.6 $\mu\text{g}/\text{ml}$ and 1 mg/ml , on GL-
446 261 (Figure 4(c)) and RG2 (Figure 4(e)) cells. Compared with 24 hours, the cytotoxicity is enhanced at
447 72 hours, leading to IC_{50} values, on GL-261 and RG2 cells, respectively larger than 1 mg/ml for N,
448 653 and 672 $\mu\text{g}/\text{ml}$ for N-CMD, 271 and 433 $\mu\text{g}/\text{ml}$ for N-CA, 224 $\mu\text{g}/\text{ml}$, and more than 1 mg/ml for
449 IONP, 271 and 303 $\mu\text{g}/\text{ml}$ for N-OA, and 6 and 197 $\mu\text{g}/\text{ml}$ for N-PLL, respectively. IC_{50} values are
450 lower towards GL-261 cells than towards RG2 cells. Given that cytotoxicity towards tumor cells is
451 increased with incubation time, magnetic hyperthermia treatment efficacy may not decrease when
452 nanoparticles stay in the tumor.

453 Cytotoxicity of the various coated magnetosome minerals on GL-261 cells without AMF is due
454 to the coating since uncoated magnetosome minerals are characterized by an absence of cytotoxicity. It
455 could be explained on the one hand by cytotoxic properties of the coating agents surrounding the
456 magnetosome minerals (Suppl. Fig. 1 (a)) and on the other hand by variations in dispersion properties of
457 the magnetosome minerals, as a function of their coatings, (23). Compared with commonly used
458 cytotoxic cancer drugs characterized by IC_{50} values of 16.3 ng/ml for doxorubicin, (48), 4.1 $\mu\text{g}/\text{ml}$ for
459 tamoxifen, (48), 22 to 56 ng/ml for cisplatin, (49), and 96 to 120 ng/ml for carboplatin, (49), N-OA, N-
460 CA, N-CMD, and N-PLL, possess much higher IC_{50} values. By contrast to conventional cytotoxic
461 cancer drugs, the main mode of action involved in tumor cell destruction using magnetic hyperthermia
462 with N-OA, N-CA, N-CMD, and N-PLL, thus does not come from their cytotoxicity, which would
463 require much lower IC_{50} values. Instead it comes from heat generated by AMF application.

464 **Cell destruction, internalization, heating properties of the different nanoparticles in the presence**
465 **of glioma GL-261 cells under alternative magnetic field application.**

466 To measure the specific absorption rate (SAR) of IONP, uncoated and coated magnetosome
467 minerals, 1 mg/mL in iron of these different nanoparticles is brought into contact with GL-261 cells
468 during 24 hours and then exposed during 30 minutes to an alternating magnetic field of frequency 198

469 kHz and average field strength of 34 mT. The variation with time of the average spatial temperature
470 distribution over the whole Petri dish containing the cells mixed with the various nanoparticles is
471 presented in Figure 5(a). From the initial slopes of the plots of Figure 5(a), $0.018\text{ }^{\circ}\text{C}/\text{sec.} < \Delta T/\delta t <$
472 $0.047\text{ }^{\circ}\text{C}/\text{sec.}$, average SAR are estimated as $\sim 96\text{ W/gFe}$, $\sim 73\text{ W/gFe}$, $\sim 89\text{ W/gFe}$, $\sim 141\text{ W/gFe}$, \sim
473 100 W/gFe , $\sim 196\text{ W/gFe}$ for N, IONP, N-PLL, N-CA, N-OA, and N-CMD, respectively (Table 1).
474 After 30 minutes of application of the alternating magnetic field, the maximum temperatures reached
475 are measured as $39.4\text{ }^{\circ}\text{C}$, $35.0\text{ }^{\circ}\text{C}$, $33.7\text{ }^{\circ}\text{C}$, $41.8\text{ }^{\circ}\text{C}$, $42.3\text{ }^{\circ}\text{C}$ and $50.8\text{ }^{\circ}\text{C}$ for N, N-PLL, N-CA, N-OA,
476 and N-CMD, respectively. In petri dishes, N-CMD, N-CA and N-OA lead to higher SAR values and
477 maximum temperatures as well as to a more homogenous temperature distribution, where the latter may
478 be defined as the temperature distribution that yields the largest percentage of heated area at $43\text{-}46\text{ }^{\circ}\text{C}$
479 (table 2), a range of temperature that is reported to produce antitumor efficacy in hyperthermia
480 treatment, (ref montrant que l'hyperthermie a lieu pour des temperatures de chauffage superieures à 43-
481 $46\text{ }^{\circ}\text{C}$), compared with uncoated magnetosomes. The opposite behavior is observed for N-PLL having
482 smaller SAR values and yielding smaller maximum temperatures and less homogenous temperature
483 distribution than uncoated magnetosomes (Fig. 5(b)). This difference in behavior may be explained by
484 different thicknesses and properties of the coatings. Indeed, as observed in the TEM image of Fig. 1(e),
485 the largest coating thickness of 6.4 nm and possible changes in magnetosome morphology and chain
486 length, observed in N-PLL, leads to the lowest heating rates. Assuming that Brown relaxation is
487 occurring within these large nanoparticles as previously reported, (ref à trouver), the presence of such
488 thick coating could decrease N-PLL rotation motions or friction with the viscous surrounding, hence
489 minimizing the amount of heat produced. By contrast, magnetosome minerals with a thin coating seem
490 to heat more, possibly due to better thermal conductivity. Optimal coating thickness, leading to
491 enhanced heat production, appear to lie between $2\text{ and }4.5\text{ nm}$ as is the case for N-CA, N-OA and N-
492 CMD and is close to the coating thickness of $\sim 6\text{ nm}$ of magnetosomes before purification, (ref à
493 rajouter). As a whole, N-PLL, N-CA, N-OA, and N-CMD, all lead to higher SAR values and equivalent

494 or better heat distribution than IONP, suggesting that they all possess promising heating properties to
495 carry out magnetic hyperthermia.

496 Next, we examine how efficiently N, IONP, N-CA, N-PLL, N-CMD, and N-OA can reach *in*
497 *vitro* temperatures of 43- 46°C, which are typical temperatures desired for magnetic hyperthermia, (10:
498 faux). For that, 1 mg of the different nanoparticles is brought into contact with GL-261 cells during 24
499 hours and then exposed, or not for the control, to a heat treatment at 43- 46°C during 30 minutes. Heat is
500 maintained at these temperatures by applying an alternating magnetic field of frequency 198 kHz and
501 average strength of 34-47 mT. While for N-CA, N-OA, and N-CMD, a magnetic field strength of 33 to
502 40 mT is needed to reach an average temperature in the Petri dish of 45°C after 30 minutes of treatment,
503 leading to a more homogenous temperature distribution (Table 2) than for N and IONP (Figure 6(b)), a
504 different behavior is observed for N-PLL that require the application of a higher magnetic field of 47
505 mT to reach an average temperature of 42 °C after 30 minutes of treatment and yield a less homogenous
506 temperature distribution (Table 2) than for uncoated magnetosomes and IONP (Fig. 6(b)).

507 We now turn to a comparison between *in vitro* antitumor efficacy against GL-261 tumors of N-
508 CA, N-PLL, N-CMD, and N-OA, with that of uncoated magnetosomes and IONP. As shown in Figure
509 6(a), for all nanoparticles studied, the percentage of GL-261 living cells decreases in the presence of
510 heat treatment at 43-46 °C. While for N-OA and N-CMD, GL-261 cell destruction appears to be the
511 most efficient, leading to a decrease in the percentage of living cells of 30-40 ±2% following heat
512 treatment, close to that of 53% ± 2.2% observed with IONP, such decrease is only 10-16 ± 2% for N-
513 PLL, N-CA and uncoated magnetosomes, lower than for IONP. For magnetic hyperthermia, it is
514 desirable to use nanoparticles that can induce cell destruction at low magnetic field strength to prevent
515 eddy currents. Therefore, N-OA and N-CMD seem to be the most efficient nanoparticles since their
516 relatively high percentage of cell destruction of 30-40 ±2% is correlated with relatively high
517 temperatures of 52-53 °C reached during 30 minutes of application of a magnetic field of relatively low
518 strength of 33-40 mT (Fig. 6(a)). Although IONP yield a relatively high percentage of cell destruction of

519 53% \pm 2.2%, they seem to be less promising since the relatively high temperature of 48 °C that they
520 reach requires the application of an alternating magnetic field of high strength of 47 mT during 30
521 minutes (Fig. 6(b), which may produce Eddy currents resulting in global warming of the whole
522 organism, (Ref. : « Effects of size distribution on hysteresis losses of magnetic nanoparticles for
523 hyperthermia », Rudolf Hergt, Silvio Dutz and Michael Roder (2008)). N-PLL and uncoated
524 magnetosomes appear to be the less promising nanoparticles since they induce the smallest percentages
525 of cell destruction of 16% \pm 2.3%, obtained at relatively low temperatures of 42-48 °C by applying
526 magnetic fields of high strength of 47 mT (Fig. 6(b)).

527 To examine whether *in vitro* antitumor efficacy is due to cellular internalization of the different
528 nanoparticles, N-PLL, N-OA, N-CA, N-CMD, uncoated magnetosomes, and IONP, are exposed to the
529 same heat treatment as above at 43-46 °C. The different nanoparticles are removed from the cell surface
530 by washing and it is verified by optical microscopy that nanoparticle aggregates do not remain at the
531 cell surface, so that the quantity of internalized nanoparticles, whether composed of crystallized or
532 dissolved iron, can be measured. As shown in Figure 7, after heat treatment, the amount of internalized
533 iron either increases from 1 to 4 pg per cell for N-PLL, from 2 to 18 pg per cell for N-CA, or remains
534 relatively unchanged at 0.5 to 4 pg per cell for uncoated magnetosome minerals, N-OA, N-CMD and
535 IONP. High cellular internalization of N-CA in the presence of the heat treatment at 54 °C (Figure 6(b))
536 may possibly be explained by N-CA high affinity for cellular membrane, as it is the case for
537 superparamagnetic nanoparticles coated with citric acid,(50). In the literature, anionic maghemite
538 nanoparticles have indeed been shown to have a high affinity for cellular membrane mainly due to
539 electrostatic interactions, (51). These behaviors may also take place with N-CA and promote their
540 cellular internalization.

541 On the one hand, N-CA that are prone to the highest level of internalization, produce a small
542 decrease in the percentage of GL-261 living cells of only 10% \pm 2.8% following heat treatment at 54 °C,

543 which may be due to the relatively limited cytotoxicity of citric acid ($IC_{50} \sim 606 \mu\text{g/ml}$), (50). This
544 hypothesis is further supported by analyzing the behavior of MC, which internalize and lead to
545 enhanced cytotoxicity following magnetic field application (Suppl. Figs. 1(a) and 1(b)). In this case,
546 cytotoxicity may arise from bacterial residues that enter inside cells following magnetic field
547 application. On the other hand, nanoparticles that appear to yield most efficient cell destruction, *i.e.* N-
548 OA and N-CMD with percentages of cell destruction of $43\% \pm 2.9\%$ and $30\% \pm 2.0\%$ respectively, do
549 not internalize much in cells, suggesting that internalization may not be the main factor responsible for
550 nanoparticle cytotoxicity. Instead, *in vitro* antitumor efficacy following alternating magnetic field
551 application may be due to aggregation of nanoparticles at the cell surface, to homogenous heating,
552 mechanic chocks between nanoparticles and cell membranes, or to extracellular hyperthermia, (52),
553 which could result in cell lysis. Chemical nanoparticles coated with OA have already been used to
554 induce toxicity *in vitro* under the application of an alternating magnetic field, (53), reinforcing the idea
555 that N-OA are suitable for the magnetic hyperthermia treatment of tumors.

556 Coated magnetosome minerals also appear promising for magnetic hyperthermia, since N-PLL have
557 been shown to efficiently destroy both subcutaneous GL-261 and intracranial U-87 glioblastoma tumors
558 under AMF applications, as presented in details elsewhere, (43), (54).

559 CONCLUSIONS

560 In this study, we describe a process for purifying iron oxide nanoparticles extracted from
561 magnetotactic bacteria and removing most of the organic material, including endotoxins. The
562 nanoparticles are then stabilized with four different biodegradable and biocompatible coating agents.
563 These coated magnetosome minerals are characterized by a mineral crystallized core composed of
564 maghemite, which is surrounded by a layer of coating agent and are arranged in chains of coated
565 particles. Sedimentation and electro kinetic potential measurements reveal that they have good colloidal
566 stability at physiological pH 7.4, which is a good criterion for injecting nanoparticles into tumor.

567 Moreover, their endotoxin concentrations are below 160 EU/ml per mg and comparable to that of
568 chemically synthesized nanoparticles IONP. Cytotoxicity assays reveal that the percentage of healthy
569 3T3 cell inhibition by N-PLL, N-CA, N-OA, and N-CMD at concentrations varied between 16 $\mu\text{g/ml}$
570 and 1 mg/ml is lower than 30% indicating that, according to ISO 10993-5 standard, these nanoparticles
571 are not toxic. The SAR, measured when these nanoparticles are brought into contact with GL-261 cells
572 and exposed during 30 minutes to an alternating magnetic field of 198 kHz and strength 34 mT, lie
573 between 89 and 196 W/gFe, larger than the SAR of 73 W/gFe, measured for chemically synthesized
574 nanoparticles IONP, currently used to carry out magnetic hyperthermia treatment of tumors. *In vitro*
575 anti-tumor efficacy of N-PLL, N-CA, N-OA, and N-CMD is also examined by bringing them into
576 contact with GL-261 cells and by heating them to 43-46°C under application of an alternating magnetic
577 field of 198 kHz and 34-47 mT. Decrease in the percentage of living GL-261 cells following magnetic
578 heat treatment is the largest for N-CMD and N-OA and the lowest for N-CA and N-PLL. Interestingly,
579 N-CA internalize efficiently in GL-261 cells following magnetic heat treatment, while the opposite
580 behavior is observed for N-CMD and N-OA. Therefore, efficient GL-261 tumor cell destruction does
581 not seem to be correlated with a high level of nanoparticle internalization, but instead with high SAR
582 values of ~ 100 -196 W/gFe and with homogeneous heating at the scale of a Petri dish, measured for N-
583 CMD and N-OA. Although IONP yield a significant percentage of cell inhibition in the presence of the
584 AMF, this is achieved by using an AMF of high strength (47 mT), which should be avoided in humans,
585 since it can lead to Eddy currents and global warming of the organism. These results indicate that coated
586 magnetosome minerals are good candidates to carry out the magnetic hyperthermia treatment of tumors.
587 SAR values and *in vivo* biodistribution should both be optimized to produce the most efficient magnetic
588 hyperthermia.

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719 and reference materials [Internet]. [cited 2016 Oct 26]. Available from:
720 <https://www.iso.org/obp/ui/#iso:std:53468:en>. To estimate that 22 μ g/mL corresponds to 6 cm² per
721 ml, we use the formula: $22 \mu\text{g/mL} = 6 \div (2.2 \times 10^{15} \times 12,15 \times 10^{-11})$, where 2.2×10^{15} per gram is the
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744

745 **FIGURES and TABLE:**

746 **Figure 1: TEM images of whole magnetotactic bacteria, IONP, uncoated and coated magnetosome**

747 **minerals.** (a), Transmission electron microscopy images of a magnetotactic bacterium *Magnetospirillum*

748 *gryphiswaldense* used in this study and containing a chain of magnetosomes; (b), chemical nanoparticles

749 IONP; (c), magnetosome minerals without coating, N; (d,e), magnetosome minerals coated with either

750 poly-L-lysine, N-PLL; (f,g), citric acid, N-CA; (h,i), oleic acid, N-OA; (j,k), carboxy-methyl-dextran,

751 N-CMD.

752 **Figure 2: Physicochemical properties of uncoated and coated magnetosome minerals.**

753 (a), Size distribution of uncoated magnetosome minerals, measured over 500 magnetosomes. (b),

754 Weight percentage of carbon and nitrogen in the different nanoparticles, measured by CHNS. (c) and

755 (d), Variation with time of the absorbance, measured at 480 nm, of suspensions containing of 1mg/mL

756 in iron of uncoated, coated magnetosome minerals and IONP. (e) and (f), Variation of Zeta potential of

757 uncoated, coated magnetosome minerals and IONP as a function of pH. These results were obtained

758 from triplicates. The error bars represent standard deviations (SD°).

759 **Figure 3: FTIR spectra of IONP, uncoated and coated magnetosome minerals.**

760 Fourier transform infrared, FT-IR spectra of, (a), lyophilized uncoated magnetosome minerals, N; (b),

761 lyophilized IONP; (c), lyophilized magnetosome minerals coated with poly-L-lysine, N-PLL; (d), with

762 citric acid, N-CA; (e), oleic acid, N-OA; (f), carboxy-methyl-dextran, N-CMD.

763 **Figure 4: Percentages of 3T3, RG2, and GL-261 cell inhibition in the presence of IONP, uncoated and**
764 **coated magnetosome minerals.**

765 (a), Percentage of 3T3 cell inhibition after 24 hours of 3T3 cell incubation with various concentrations

766 of uncoated, coated magnetosome minerals and IONP; (b), Percentage of GL-261 cells inhibition after

767 24 hours of 3T3 cell incubation with various concentrations of uncoated, coated magnetosome minerals

768 and IONP; (c), Percentage of GL-261 cell inhibition after 72 hours of 3T3 cell incubation with various
769 concentrations of uncoated, coated magnetosome minerals and IONP.

770 **Figure 5: Heating properties of IONP, uncoated and coated magnetosome minerals, in the presence**
771 **of GL-261 cells and AMF application.**

772 (a), Variation of temperature of GL-261 cells brought into contact with 1mg/mL of uncoated and coated
773 magnetosome minerals and exposed (or not) to an alternating magnetic field of frequency 198 kHz and
774 strength $H = 34$ mT. (b), Spatial temperature distribution of concentration 1 mg /ml of N, N-PLL, N-
775 CA, N-OA, N-CMD, and IONP mixed with GL-261 cells and exposed to an alternating magnetic field of
776 frequency 198 kHz and average field strength of 34 mT during 30 min.

777 **Figure 6: Percentage of cell inhibition and quantity of heat produced by the various nanoparticles**
778 **under AMF application.**

779 (a), Flow cytometry results showing the percentage of living GL-261 cells treated with or without AMF
780 with uncoated, coated magnetosome minerals and IONP, (b), Spatial temperature distribution within the
781 Petri dish of N, N-PLL, N-CA, N-OA, N-CMD, and IONP mixed with GL-261 cells and exposed to an
782 alternating magnetic field of frequency 198 kHz and average field strength adjusted between 34 and 47
783 mT to maintain the temperature of cells mixed with the nanoparticles at 45 °C during 30 min.

784 **Figure 7: Quantity of iron coming from the various nanoparticles internalized in cells or localized at**
785 **cell surface after and before AMF application.** Quantity of iron per cell (pg) for cells treated with or
786 without AMF.

787 **Table 1:** $\Delta T/\delta t$ estimated in °C/s; specific absorption rate, estimated in Watt per gram of nanoparticle in
788 iron, temperature variation, and percentage of heated area at 43-46°C for uncoated, coated magnetosome
789 minerals and IONP brought into contact with GL-261 cells and exposed to an alternating magnetic field
790 of 198 kHz and strength 32 mT applied during 30 minutes.

791 **Table 2:** Percentage of heated area at 43-46°C for uncoated, coated magnetosome minerals and IONP
792 mixed with GL-261 cells and exposed to an alternating magnetic field of 198 kHz and strength of 32 mT
793 applied during 30 minutes.

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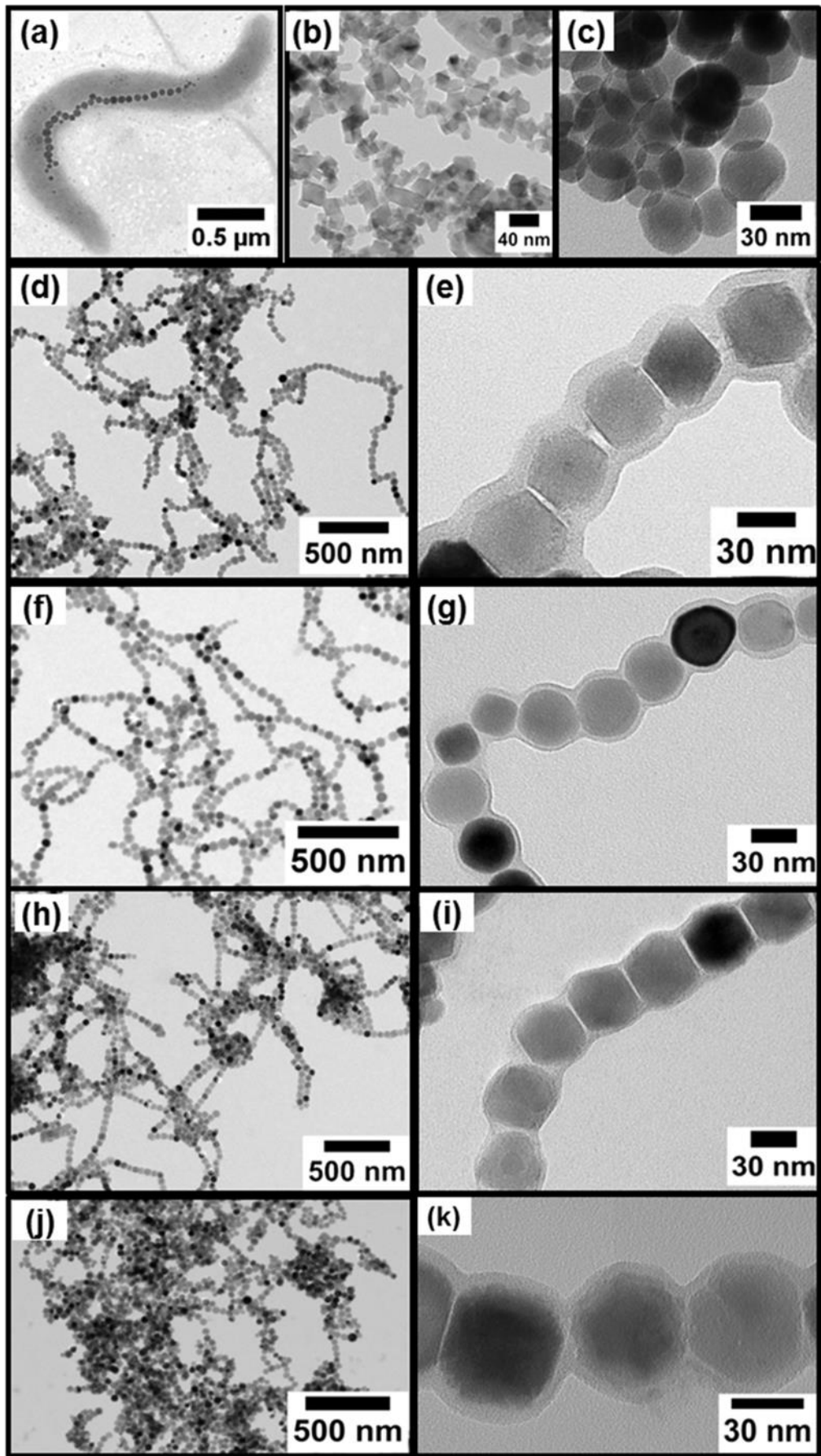


Figure 1

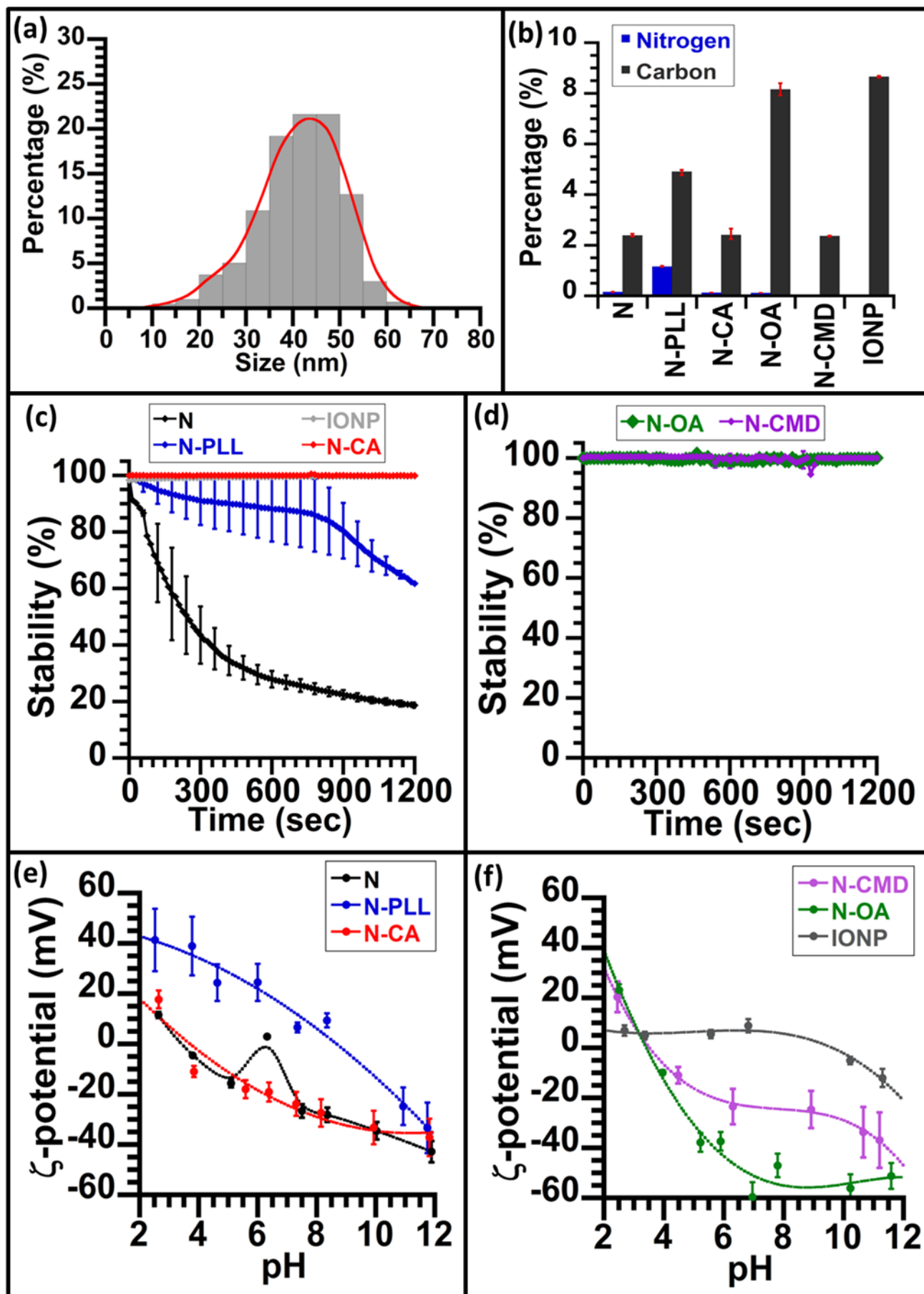


Figure 2

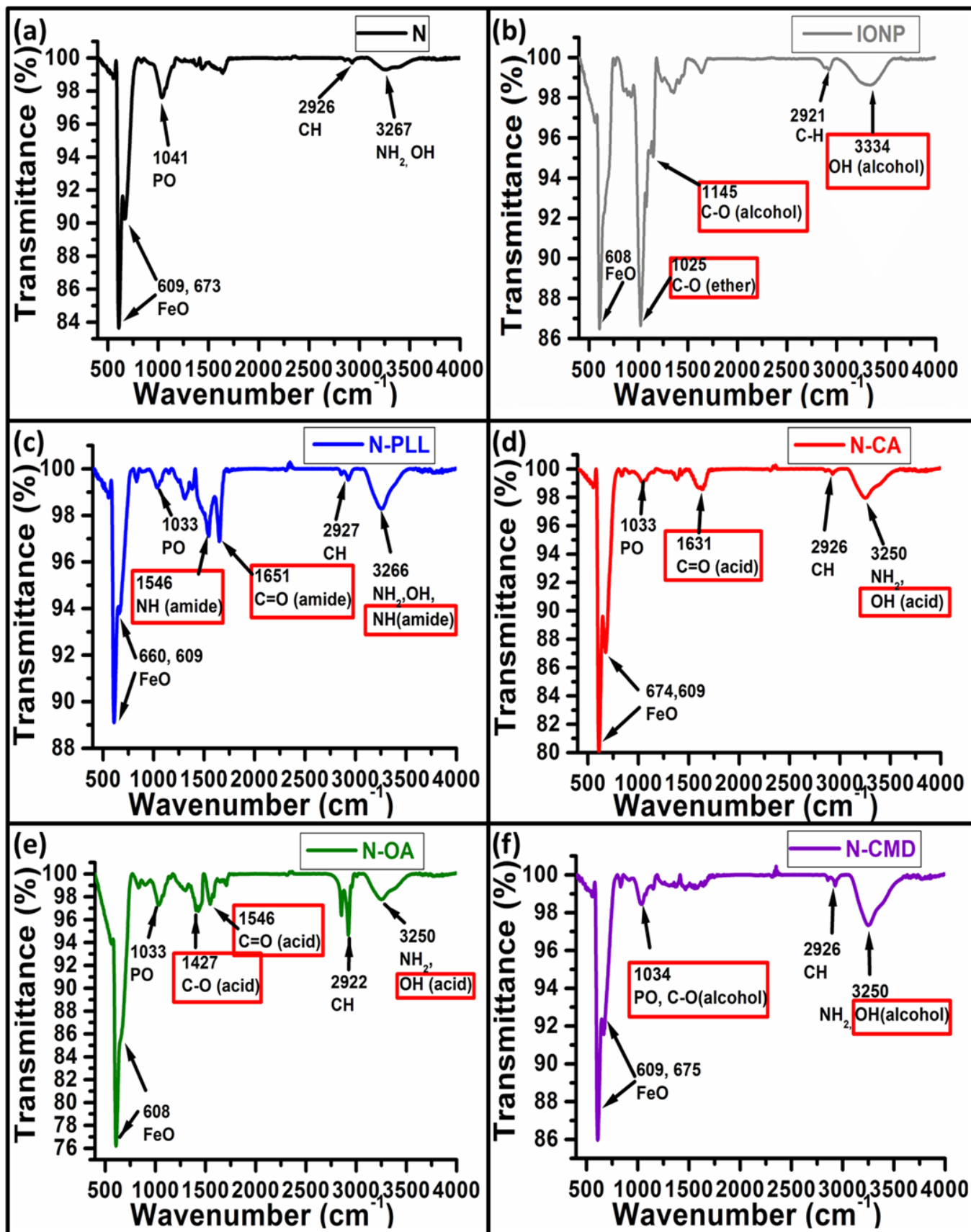


Figure 3

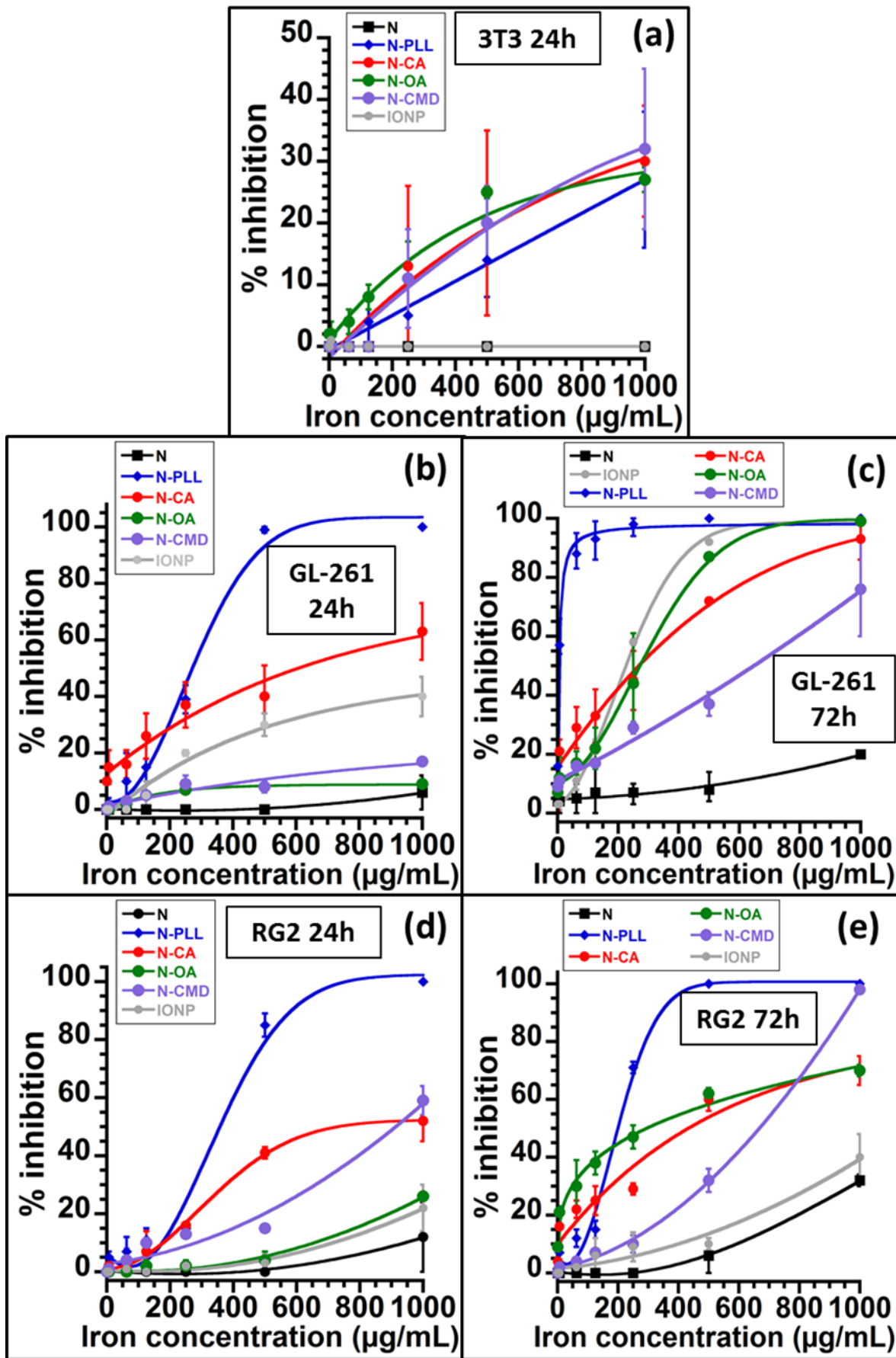


Figure 4

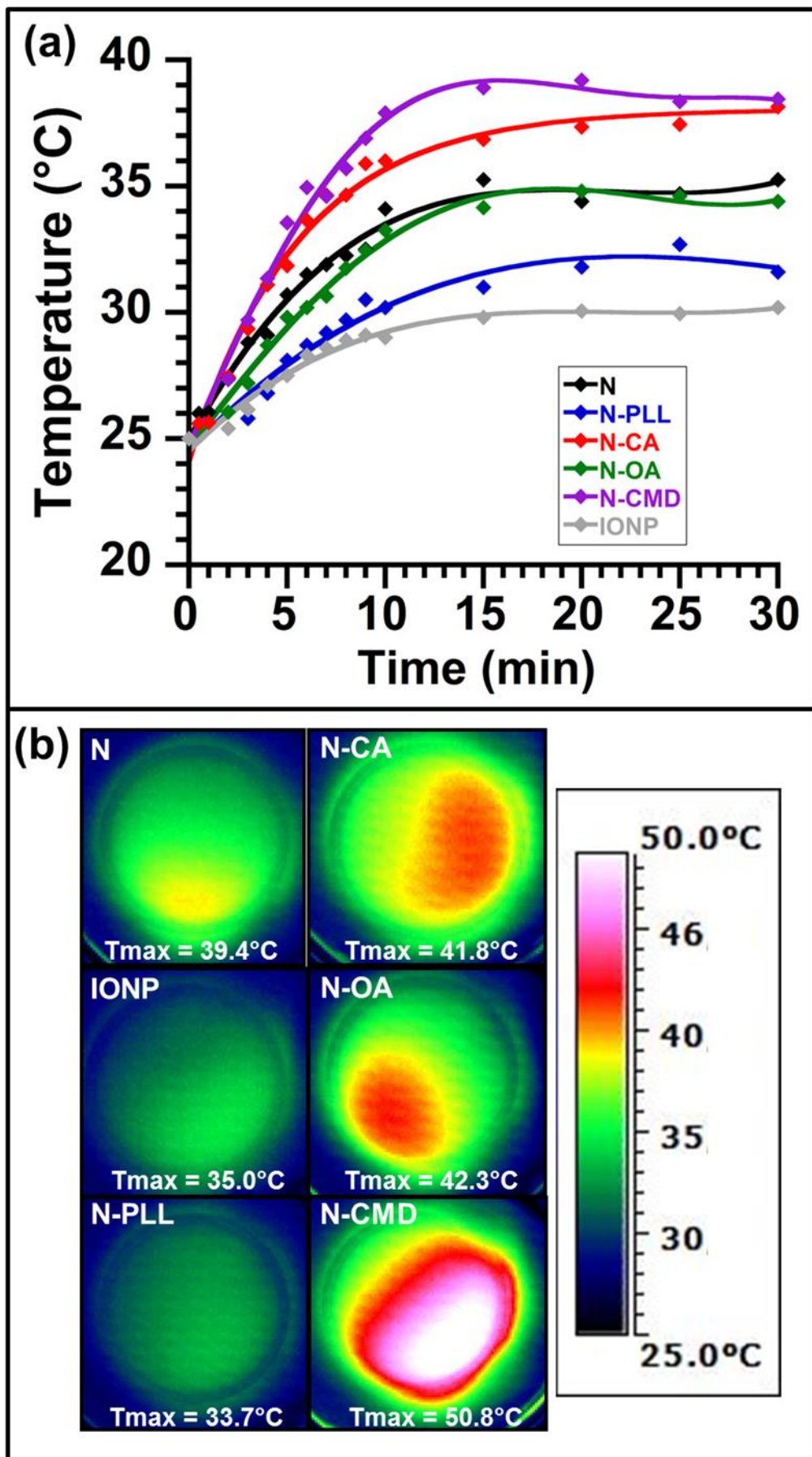


Figure 5

	$\Delta T/\delta t$ (°C/s)	SAR (W/g of iron)	Percentage of heated area at 43- 46 °C (%)
N	2.30E-02	96	0.0
IONP	1.80E-02	73	0.0
N-PLL	2.10E-02	89	0.0
N-CA	3.40E-02	141	12.7
N-OA	2.40E-02	100	12.4
N-CMD	4.70E-02	196	90.1

Table 1

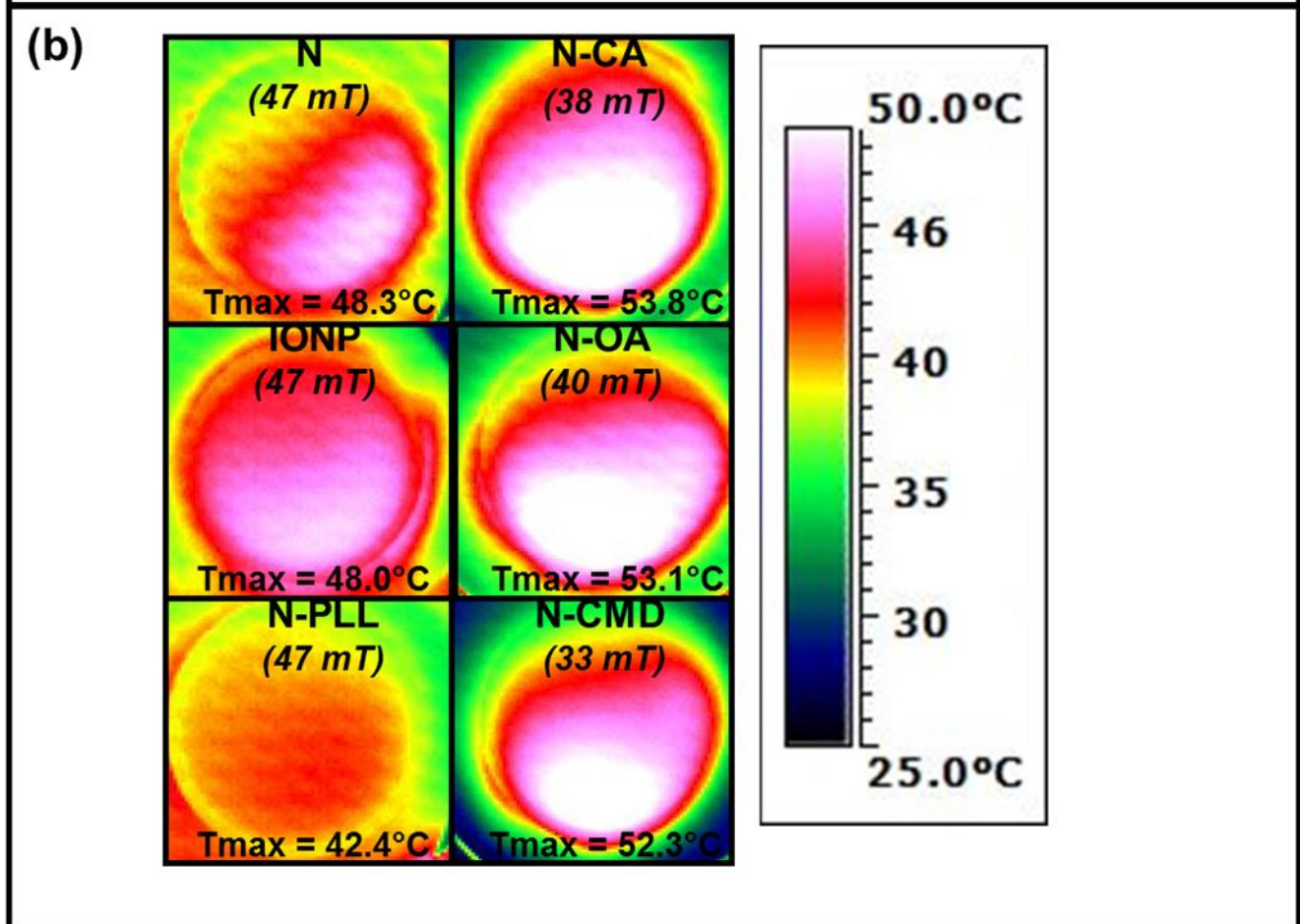
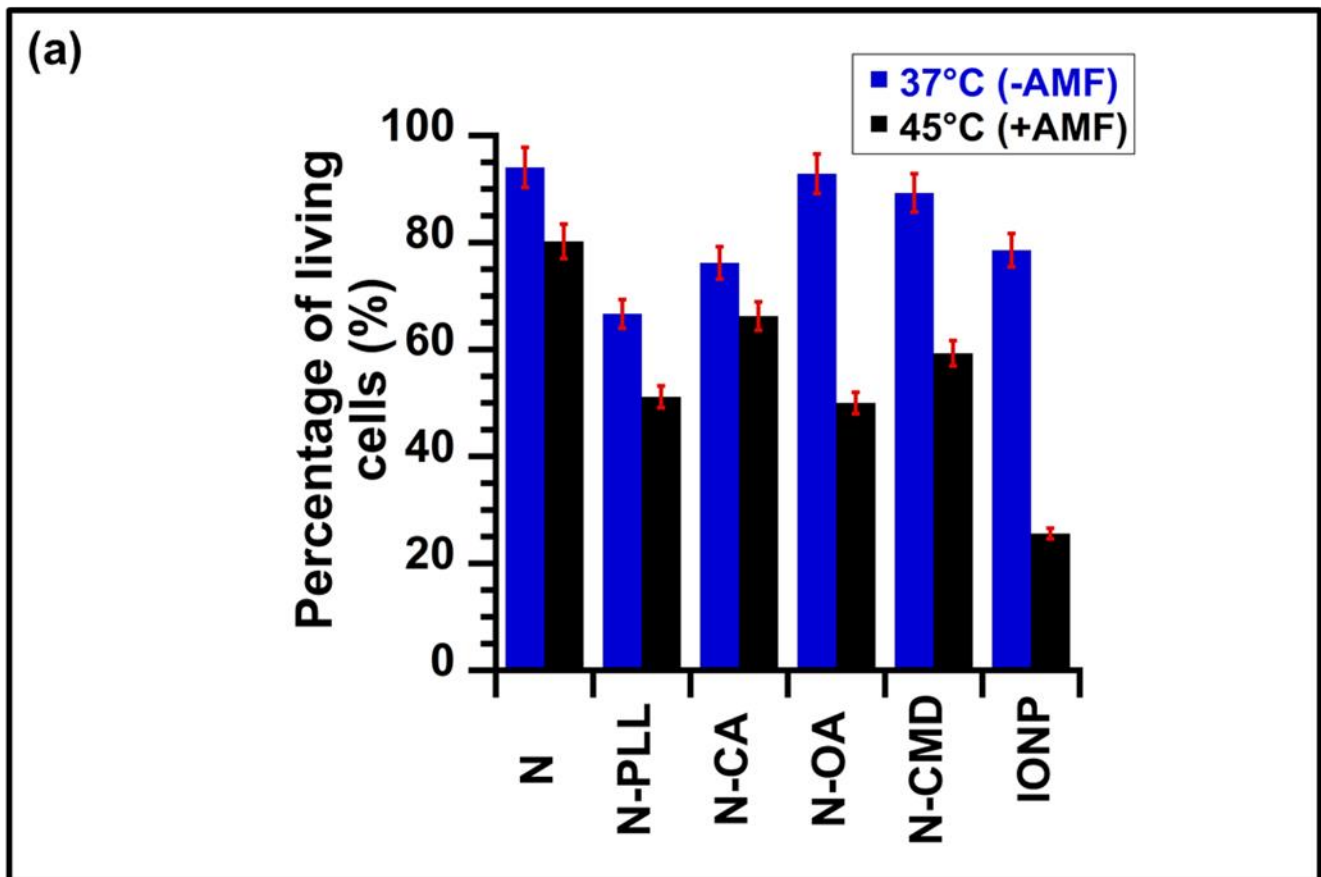


Figure 6

	Percentage of heated area at 43- 46 °C (%)
N	89.4
IONP	100.0
N-PLL	32.0
N-CA	100.0
N-OA	100.0
N-CMD	100.0

Table 2

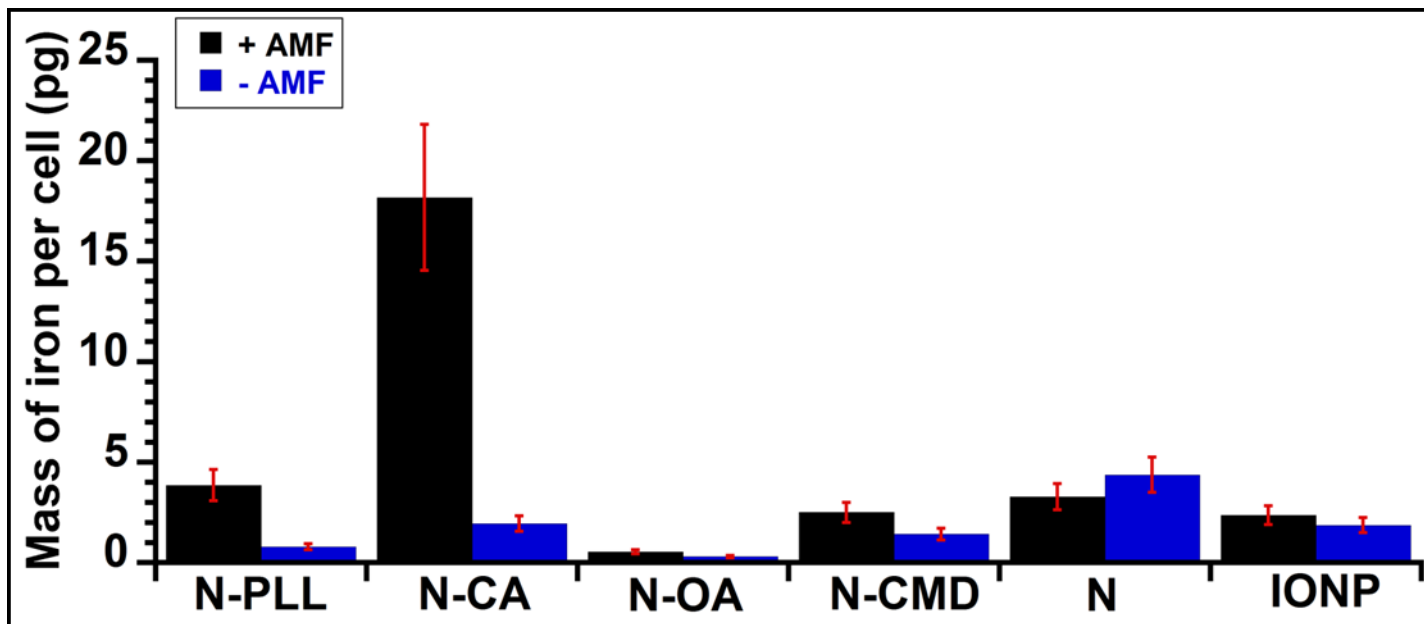


Figure 7

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