

# Chains of Magnetosomes Extracted from AMB-1 Magnetotactic Bacteria for Application in Alternative Magnetic Field Cancer Therapy

Edouard Alphanféry, Imène Chebbi, Stéphanie Faure, François Guyot, Olivier  
Seksek

► **To cite this version:**

Edouard Alphanféry, Imène Chebbi, Stéphanie Faure, François Guyot, Olivier Seksek. Chains of Magnetosomes Extracted from AMB-1 Magnetotactic Bacteria for Application in Alternative Magnetic Field Cancer Therapy. ACS Nano, American Chemical Society, 2011, 5 (8), pp.6279-6296. <10.1021/nn201290k>. <hal-01547088>

**HAL Id: hal-01547088**

**<http://hal.upmc.fr/hal-01547088>**

Submitted on 30 Jun 2017

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

# Chains of magnetosomes extracted from AMB-1 magnetotactic bacteria for application in alternative magnetic field cancer therapy.

E. Alphandéry<sup>+</sup>, I. Chebbi<sup>‡</sup>, S. Faure<sup>‡</sup>, F. Guyot, O. Seksek<sup>\*</sup>

<sup>+</sup>Institut de minéralogie et de physique des milieux condensés, Université Pierre et Marie Curie, 4 place Jussieu, 75005, Paris.

<sup>‡</sup>Nanobacterie SARL, 36 boulevard Flandrin, 75016, Paris.

<sup>\*</sup>Laboratoire ANbioPhy-Fre 3207, Université Pierre et Marie Curie, 4 place Jussieu, 75005, Paris.

## **Abstract:**

Chains of magnetosomes extracted from magnetotactic bacteria are shown to be highly efficient for alternative magnetic field cancer therapy. The viability of MDA-MB-231 breast cancer cells is relatively unaffected by the presence of less than ~ 1 mg of chains of magnetosomes. When these cells are exposed to an oscillating magnetic field of frequency 183 kHz and field strengths of 20 to 60 mT, up to 100 % of them are destroyed. We show that it is possible to fully eradicate a tumor xenografted on a mouse by administering a suspension containing ~ 1 mg of chains of magnetosomes within the tumor and by exposing the mouse to three heat cycles of 20 minutes, during which the tumor temperature is raised to ~ 43 °C. We demonstrate the higher efficiency of the chains of magnetosomes compared with various other materials, i. e. whole inactive magnetotactic bacteria, individual magnetosomes not organized in chains and two different types of chemically synthesized nanoparticles currently tested for alternative magnetic field cancer therapy. The efficiency of the chains of magnetosomes is attributed to three factors, (i), a high magnetosome specific absorption rate (SAR), (ii), a homogenous distribution of the chains of magnetosomes within the tumor yielding uniform heating and (iii), the faculty of the chains of magnetosomes to penetrate within the cancer cells following the application of the alternative magnetic field, which enables intra-cellular heating. Biodistribution studies reveal that chains of magnetosomes administered directly within xenografted breast tumors progressively leave the tumors during the 14 days following their administration and are then eliminated in the feces.

## 1. Introduction:

A concept has recently been proposed, which should favor the emergence of a new generation of drugs. It lies on the idea that the movement, the activation and the detection of a drug could be controlled externally by a medical device. This new approach is of special interest in the field of cancer research since an efficient treatment with limited side effects would require the use of a drug, which could be sent specifically within the cancer cells, which could be monitored in the organism, for example to verify that the drug is located within the tumor before the treatment is started, and which could be activated on demand by a medical device. A good candidate for such a drug would be magnetic iron oxide nanoparticles. These nanoparticles may indeed potentially be guided within the cancer cells using a magnetic field, detected using MRI (magnetic resonance imaging) and heated by being exposed to an alternative magnetic field. In this case, the destruction or elimination of tumors would occur by increasing the tumor temperature typically within the range of 37-45 °C for hyperthermia (1, 2, 5) or above 45 °C for thermoablation, (3). In previous work, the heat has been induced using chemically synthesized nanoparticles, mainly in the form superparamagnetic iron oxide nanoparticles (SPION), which were either mixed in solution or mixed with cells or administered to a living organism (1-8). The anti-tumoral activity of these heated nanoparticles has been evaluated both on animal models and clinically on humans (1-8). The efficiency of this type of thermotherapy has been demonstrated on several cancers including brain cancer, (6), prostate cancer, (7), breast cancer, (8), and skin cancer, (4). Research in this area has also led to industrial developments. In addition to Nanobacterie, there are at least three companies, (9), that develop cancer therapy using the heat generated by magnetic nanoparticles when the latter are exposed to an alternative magnetic field. The patterns that have been published by these companies describe various ways of using the heat generated by chemically synthesized magnetic nanoparticles to carry out alternative magnetic field (AMF) cancer therapy, (10).

Although significant progress has been made in the area of nanoparticle cancer therapy, concerns have been raised regarding the toxicity induced by the presence of the chemically synthesized nanoparticles in the body, (4). In order to minimize the potential side effects arising during the clinical treatments, the quantity of nanoparticles administered needs to be as small as possible while still retaining the desired effect. For that, magnetic nanoparticles have to generate a sufficiently large amount of heat, i. e. significant specific absorption rates (SAR). Therefore, there is a need for magnetic nanoparticles having a higher heating capacity than that usually obtained with chemically synthesized nanoparticles. This will be useful to reduce the amount of magnetic material needed to heat a biological tissue or cell. This can be achieved by using nanoparticles with either large volumes or with high magnetocrystalline anisotropy, (5). In part due to their large volume, the magnetosomes synthesized by magnetotactic bacteria produce a larger amount of heat than the chemically synthesized nanoparticles when they are exposed to an oscillating magnetic field. This has been shown for bacterial magnetosomes mixed in solution, which were either contained within the magnetotactic bacteria or extracted from these bacteria and for magnetosomes arranged in chains or forming individual nanoparticles, (6, 12). In addition of producing a large amount of heat, it has been reported that the magnetosomes are not particularly toxic when they are injected in rats, (11). This suggests that there is no major hurdle for the development of a therapy, which uses bacterial magnetosomes as heating sources.

In this article, the heating efficiency and anti-tumoral activity of various suspensions containing either inactive magnetotactic bacteria, chains of magnetosomes extracted from magnetotactic bacteria or individual magnetosomes detached from the chains by heat and SDS treatments are studied. We examine which type of bacterial magnetosomes is the best candidate for the thermotherapy. The impact of the chain length on the efficiency of the therapy is also studied by comparing the efficiency of two suspensions containing chains of magnetosomes of different lengths. Moreover, the efficiency of the chains of magnetosomes is also compared with that of chemically

synthesized superparamagnetic iron oxide nanoparticles (SPION) of the same type as those used by other groups or companies to carry out alternative magnetic field cancer therapy, (13).

## 2. Materials and methods:

### 2.1 Preparation of the different types of nanoparticles used as heating sources:

We use the following six different types of nanoparticles as heating sources:

- (i) Whole inactive magnetotactic bacteria;
- (ii) Chains of magnetosomes extracted from magnetotactic bacteria, which have been cultivated in the standard conditions of the ATCC, designated as Ch-Std;
- (iii) Chains of magnetosomes extracted from magnetotactic bacteria, which have been cultivated by introducing an iron chelate (EDTA) within the bacterial growth medium, designated as Ch-EDTA;
- (iv) Individual magnetosomes extracted from magnetotactic bacteria and detached from the chains by heat and SDS treatment, designated as IM;
- (v) Chemically synthesized superparamagnetic iron oxide nanoparticles coated with citrate ions, designated as SPION@Citrate;
- (vi) Commercially available chemically synthesized superparamagnetic iron oxide nanoparticles covered by PEG molecules, designated as SPION@PEG;

*Magnetospirillum magneticum* strain AMB-1 was purchased from the ATCC (ATCC 700274). Cells were grown micro-anaerobically at room temperature (~ 25°C) in liquid culture in slightly modified revised MSGM medium (ATCC Medium 1653). In one liter, this growth medium contains 0.68 g of monobasic potassium phosphate, 0.85 g of sodium succinate, 0.57 g of sodium tartrate, 0.083 g of sodium acetate, 225 µl of 0.2 % resazurin, 0.17 g of sodium nitrate, 0.04 g of L-ascorbic acid, 2 ml of a 10 mM iron quinate solution, 10 ml of Woolf's vitamins and 5 ml of Woolf's minerals. The iron quinate solution was prepared by dissolving 0.19 g of quinic acid and 0.29 g of FeCl<sub>3</sub>.6H<sub>2</sub>O in 100 milliliter of distilled water. The solution of Woolf's minerals contained in 1 liter of distilled water 0.5 g of Nitritotriacetic acid (NTA, C<sub>6</sub>H<sub>9</sub>NO<sub>6</sub>), 1.5 g of Magnesium Sulfate HEPTA (MgSO<sub>4</sub>.7H<sub>2</sub>O), 1 g of Sodium Chloride, 0.5 g of manganese sulfate (MnSO<sub>4</sub>.H<sub>2</sub>O), 100 mg of ferrous sulfate heptahydrate (FeSO<sub>4</sub>.7H<sub>2</sub>O), 100 mg of cobalt nitrate (Co(NO<sub>3</sub>)<sub>2</sub>.7H<sub>2</sub>O), 100 mg of calcium chloride (CaCl<sub>2</sub>), 100 mg of Zinc sulfate heptahydrate (ZnSO<sub>4</sub>.7H<sub>2</sub>O), 10 mg of hydrate copper sulfate (CuSO<sub>4</sub>.5H<sub>2</sub>O), 10 mg of aluminium potassium sulfate dodecahydrate (AlK(SO<sub>4</sub>).12H<sub>2</sub>O), 10 mg of boric acid (H<sub>3</sub>BO<sub>3</sub>), 10 mg of sodium molybdate (Na<sub>2</sub>MoO<sub>4</sub>.2H<sub>2</sub>O), 2 mg of sodium selenite (Na<sub>2</sub>SeO<sub>3</sub>), 10 mg of sodium tungstate dihydrate (Na<sub>2</sub>WO<sub>4</sub>.2H<sub>2</sub>O) and 20 mg of Nickel chloride (NiCl<sub>2</sub>.6H<sub>2</sub>O). The solution of Woolf's vitamins was prepared by dissolving in 1 liter of distilled water 2.2 mg of folic acid (vitamin B9), 10.2 mg of pyridoxine (vitamin B6), 5.2 mg of Riboflavin (vitamin B2), 2.2 mg of Biotin (vitamin H or B7), 5.2 mg of thiamin (vitamin B1), 5.2 mg of nicotinic acid (vitamin B3 or PP), 5.2 mg of pantothenic acid (vitamin B5), 0.4 mg of vitamin B12, 5.2 mg of amino benzoic acid, 5.2 mg of thiotic acid and 900 mg of potassium phosphate. The pH of the growth medium was adjusted to 6.85 using a 5M sodium hydroxide solution. Stationary phase occurred when the medium became completely reduced as indicated by a change in the coloration of the growth medium, from pink to colorless.

Four different types of samples were prepared from intact whole cells of *M. magneticum* containing either whole magnetotactic bacteria, chains of magnetosomes extracted from magnetotactic bacteria with different lengths or individual magnetosomes detached from the chains by heat and SDS treatments. Cells were harvested at stationary phase by centrifugation at 8,000 rpm for 15 min. The supernatant (spent growth medium) was discarded and cells were resuspended in 3 ml of deionized water. The first sample contained whole inactive magnetotactic bacteria as shown in the TEM micrograph of Figure 1(a). To extract the chains of magnetosomes, 1 ml of the cell suspension

was recentrifuged and resuspended in 10 mM Tris•HCl buffer (pH 7.4) and then sonicated for 120 min at 30 W to lyse the cells releasing the chains of magnetosomes. Sonication times of 60 and 180 min were also tested and enabled to extract the chains of magnetosomes from the bacteria. For a sonication time of less than 60 min, the magnetotactic bacteria were not all lysed while for a sonication time of more than 180 min, aggregation began to be observed due to the presence of individual aggregated magnetosomes. After sonication, the suspension of chains of magnetosomes was magnetically separated by placing a strong magnet in neodymium (0.1-1T) next to the tube where the magnetic material was collected as a pellet. The supernate containing cells debris and other organic material was removed. The magnetosome chains were washed 10 times with a 10 mM Tris•HCl buffer (pH 7.4) in this way and were finally resuspended in sterile deionized water. A typical assembly of chains of magnetosomes extracted from the whole bacteria is shown in the TEM micrograph of Figure 1(b). The surface charge of the chains of magnetosome was measured as a function of pH using dynamic light scattering measurements (NanoZetasizer, Malvern instruments Ltd). At physiological pH, Suppl. Fig. 1(a) shows that the surface charge of the chains of magnetosomes is negative at -22 mV. The infrared measurements were carried out using a Nicolet 380 FT IR Thermo Electro. The infra-red absorption spectrum of a suspension of chains of magnetosomes was also recorded and is presented in Suppl. Fig. 1(b). It showed peaks arising from the functional groups carboxylic acid, amine, amide, phosphate (P-O), revealing the presence of both proteins and phospholipids within the suspension of chains of magnetosomes. This result suggests that both the membrane surrounding the magnetosomes and the filament binding the magnetosomes together are present in this sample, (13). Two different types of chains of magnetosomes extracted from magnetotactic bacteria have been prepared, which have been obtained by cultivating the bacteria either in the conditions of the ATCC as described above or in the presence of an iron chelate (0.4  $\mu$ M EDTA), which has been added to the standard ATCC growth medium. The presence of EDTA within the bacterial growth medium led to longer chains of magnetosomes with enhanced heating properties in solution as compared to the chains of magnetosomes prepared in the standard conditions (Suppl. Fig. 2). Individual magnetosomes (i.e. magnetosomes, which are not organized in chains) were obtained by heating the suspension of magnetosome chains for five hours at 90°C in the presence of 1% sodium dodecyl sulfate (SDS) in deionized water to remove most of the biological material surrounding the magnetosomes, i. e. most of the magnetosome membrane surrounding the magnetosomes and the cytoskeleton responsible for the alignment of the magnetosomes in each chain, (13). Individual magnetosomes were washed as described for magnetosome chains and resuspended in deionized water. The TEM micrograph of Figure 1(c) shows a typical assembly of individual magnetosomes. The individual magnetosomes possess different properties from the chains of magnetosomes. They form an aggregated assembly of nanoparticles (Figure 1(c)). They possess a surface charge, which strongly depends on their level of aggregation. When the individual magnetosomes are sonicated and dispersed in water, they possess a relatively similar surface charge than that of the chains of magnetosomes at pH 7. However, when they are aggregated, the individual magnetosomes possess a positive charge (10 mV at pH 7, Suppl. Fig. 1(a)). The individual magnetosomes are surrounded by phospholipid acid (presence of P-O peak in the infra-red absorption spectrum of Suppl. Fig. 1(b)), but not by proteins (absence of amide in the infra-red absorption spectrum of Suppl. Fig. 1(b)), suggesting that the biomaterial, which surrounds the magnetosomes has not been completely removed but has been sufficiently denatured to yield individual magnetosomes not organized in chains.

The chemically synthesized nanoparticles (SPION@Citrate) were prepared following a protocol described previously, (14). To prepare non-coated  $\gamma$ Fe<sub>2</sub>O<sub>3</sub> particles, a solution of base (dimethylamine) was first added to an aqueous micellar solution of ferrous dodecyl sulfate (Fe(DS)<sub>2</sub>) and mixed. The final reactant concentrations were  $1.3 \times 10^{-2}$  mol L<sup>-1</sup> and  $8.5 \times 10^{-1}$  mol L<sup>-1</sup> for Fe(DS)<sub>2</sub> and dimethylamine, respectively. The solution was then stirred vigorously during 2 hours at 28.5 °C and the resulting precipitate of uncoated nanocrystals was isolated from the supernatant by

centrifugation. In the second step, the precipitate was washed with an acidic solution ( $\text{HNO}_3$ ,  $10^{-2}$  mol.L $^{-1}$ ) until a solution of pH=2 were reached. Sodium citrate dissolved in water ( $[\text{Na}_3\text{C}_6\text{O}_7\text{H}_5] = 1.5 \times 10^{-2}$  mol L $^{-1}$ ) was used to coat the nanoparticles. The solution was sonicated during 2 hours at 90°C and the addition of acetone induced nanocrystal precipitation. After washing with a large excess of acetone, the precipitate was dried in air. The nanocrystals coated with citrate ions were finally dispersed in water. The pH, which was initially  $\sim 2$ , was progressively increased up to 7.4 by adding of solution of sodium hydroxide NaOH ( $10^{-1}$  mol.L $^{-1}$ ). The SPION@Citrate are composed of maghemite and possess a mean size of  $\sim 10$  nm. A TEM micrograph of the SPION@Citrate is shown in Suppl. Fig. 1(c). The detailed properties of the SPION@PEG can be obtained from the company Micromod. The information sheet (product-No: 79-00-201) provided by Micromod indicates that the SPION@PEG possess a saturating magnetization of 34 emu/g, a size of  $\sim 20$  nm, a polydispersity of less than 20 % and that they are stable in aqueous buffer for pH > 4.

## 2.2 *In-vitro* heating experiments of the various types of nanoparticles incubated in the presence of MDA-MB-231 cells:

MDA-MB-231 cells were obtained from the American Type Culture Collections (ATCC). The cells lines were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplement, which contained 10% fetal calf serum (FCS), 2 mM l-glutamine, 1 mM sodium pyruvate, 50 U/ml streptomycin (all purchased from Life Technologies Inc.). All *in-vitro* experiments were carried out at 37 °C in an incubator with 5% of CO $_2$ . Cell viability was evaluated using the so-called microculture tetrazolium assay (MTT), (15). This technique measures the ability of mitochondrial enzymes to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (purchased from Sigma, St Louis, MO, USA) into purple formazan crystals. Cells were seeded at a density of  $2 \cdot 10^4$  cells per well in 96-well flat-bottom plates (Falcon, Strasbourg, France) and incubated within the culture medium during 24 hours. Then, the medium was removed and replaced by 10% FCS-medium containing the various nanoparticles (Ch-Std, IM, SPION@Citrate and SPION@PEG) with different concentrations in maghemite ( $0.125 \text{ mg/mL} < C_{\gamma\text{Fe}203} < 1 \text{ mg/mL}$ ). These suspensions were exposed (or not for the control) to an alternative magnetic field (AMF) of frequency 183 kHz and strength of 43 mT. The treatment was carried out during 20 minutes either one time or two times. After 72 hours of incubation, the cells were washed with a phosphate buffer saline (PBS from Life Technologies) and incubated with 0.1 mL of MTT (2 mg/mL) for an additional 4 hours at 37°C. The insoluble product (composed essentially of formazan) was then dissolved by adding 100  $\mu\text{l}$  of DMSO (Sigma-Aldrich). The absorbance of the solubilized formazan was measured at 570 nm using a Labsystem Multiscan MS microplate reader. It provided an estimate of the number of functional mitochondria, a number, which is proportional to the number of living cells. The percentage of inhibition was then estimated as the number of dead cells (i. e. cells in apoptosis) divided by the total number of cells.

The percentage of living cells was estimated *in-vitro* to evaluate the efficiency of the thermotherapy. For that, the cells were seeded on Petri dishes (diameter of 30 nm with 50 000 cells per Petri dish), and grew during 24 hours. After this initial period of growth, the cells were incubated in the presence (or not for the control) of the various types of nanoparticles studied during 24 hours (D1), 48 hours (D2) or 72 hours (D3). At the end of the incubation time, the cells were exposed (or not for the control) to an AMF of frequency 183 kHz and strengths 20 mT, 43 mT or 60 mT. The treatment was carried out during 20 minutes either one time or two times. Following the treatment, the cells were washed twice with PBS. In order to harvest the cells, 250  $\mu\text{l}$  of Trypsin-EDTA were added to the adherent cells. 750  $\mu\text{l}$  of the liquid medium were added to the harvested cells to homogenize the suspension. The suspension was then centrifugated at 700G during 3 minutes, the supernate was removed and the cells were resuspended in 1 mL of PBS. In order to evaluate the percentage of living cells, 5  $\mu\text{l}$  of propidium iodide (PI) (1 mg /mL mixed in ethanol, Sigma Aldrich) was added to the cell suspensions. Since PI only penetrates within dead cells, the measurement of its fluorescence

provides an estimate of the percentage of dead cells. From this estimate, we could deduce the percentage of living cells. In order to measure the fluorescence of PI, the cells were analyzed in a flow cytometer (Beckton Dickinson FACSCalibur 3C), which contains an argon laser with an emission at 488 nm and a detector FL3-H able to detect the fluorescence of PI excited by the laser. Ten thousand cells per sample were measured to determine the percentage of living cells.

The heating properties of the cell suspensions were measured *in vitro*, using essentially the same protocol as that described above for the adherent cells. The only difference in this case is that the cells have immediately been mixed with the chains of magnetosomes and treated by application of the magnetic field. The temperature was measured with the thermocouple microprobe (IT-18, Physitemp, Clifton, USA), which measures the temperature macroscopically (i. e. the temperature of the cell suspension as a whole but not the temperature within each individual cell).

In order to estimate the number of magnetic cells, essentially the same protocol as that described above for the cells in suspension was followed. 50 000 cells contained within the liquid medium described above were incubated in the presence of the various nanoparticles during 5 to 20 minutes. During the incubation, an AMF of frequency 183 kHz and field strength of 43 mT was applied. After treatment, the magnetic cells were collected by positioning a strong magnet of 0.6 mT close to the cells in suspension. The supernate containing the non-magnetic cells was removed while the cells which had been attracted by the magnet were resuspended in 1 mL of PBS. The percentage of magnetic cells was then estimated using the flow cytometer.

### 2.3 *In-vivo heating experiments:*

All animal experiments have been conducted after approval of a protocol examined by the committee of the "Centre Léon Bérard, Ecole normale supérieure, Plateau de Biologie Expérimentale de la Souris, Lyon, France".

*In-vivo* heating experiments were carried out on 42 nude mice at 6 weeks of age, which were bought in Charles Rivers Laboratories, Arbresle, France. To prepare tumor-bearing animals, the mice were first gamma-irradiated. Approximately two millions MDA MB 231 human breast cancer cells in 100  $\mu$ l of phosphate buffer saline (PBS) were then injected subcutaneously both on the left and right flanks of the mice using a syringe (26G needle). The tumor sizes were measured using calipers every 3 days. The estimates of the volumes of the tumors were then carried out using the formula  $V = A \times B^2 / 2$ , where A is the longer and B is the shorter lateral diameter of the tumor, (16). The tumors grew during a period of 21 days until they reached a volume of approximately 100 mm<sup>3</sup>.

Before starting the treatment, the mice were anesthetized with ketamin/xylazin (100/6 mg kg<sup>-1</sup>, i.p.), which resulted in a decrease of their corporal temperature from 37°C down to 30-36°C depending on the mouse. Three mice died during the first steps of the treatment most probably due to an overestimation of the dose of anesthetic. After necropsy, the organs of these mice showed no obvious systemic congestion or infarction. Under anesthesia, the needle of the syringe containing either chemically synthesized nanoparticles or the various types of bacterial magnetosomes dispersed in sterile water was inserted longitudinally into the tumors of the mice (Suppl. Fig. 3(a)). The mice were then placed inside a coil of 6.7 cm in diameter where an alternative magnetic field was applied to them. To produce the alternative magnetic field, an alternative current was generated within the coil using a 10 kW EasyHeat power supply from Ambrell, Soultz, France. The schematic diagram of Suppl. Figure 3(b) shows the experimental set-up used to carry out the experiments. The measurements of the temperature were carried out using an implantable thermocouple microprobe (IT-18, Physitemp, Clifton, USA), specifically adapted to work in the presence of an alternative magnetic field. This thermocouple was used to obtain the rectal temperature or a local estimate of the temperature within the central part of the tumor (Suppl. Fig. 3(c)). The variation of the rectal temperature was monitored to verify that the increase of the tumor temperature was local and did

not take place within the whole body of the mice. An infrared camera (Moblr2, Optophase, Lyon, France) was used to obtain a more global picture of the variation of temperature of the tumor and of the tumor environment (Suppl. Fig. 3(d)). The cross section through which the temperature was measured is indicated in Suppl. Fig. 3(d) by a line. The variations of the tumor sizes during the 30 days following the first treatment were measured both for the unheated and heated tumors.

Anti-tumoral activity was studied by following the size evolution of tumors grown subcutaneously on both flanks of each mouse. Mice were randomly selected and divided into 13 groups. The first 6 groups were exposed to an oscillating magnetic field. One hundred microliters of suspensions containing Ch-Std for group 1, Ch-EDTA for group 2, SPION@citrate for group 3, SPION@PEG for group 4, IM for group 5, inactive magnetotactic bacteria for group 6, were administered into the tumors localized on the right flank of each mouse (table 1). After injection of the different suspensions, mice were exposed to an alternative magnetic field of frequency 183 KHz and magnetic field strength of  $\sim 43$  mT (groups 1 to 5) or  $\sim 80$  mT (group 6) during 20 min. The treatment was repeated 3 times at 3 days interval. Two different protocols have been tested with the Ch-Std, either with three different injections carried out before each heating cycle or only with one injection before the first heating cycle. These two protocols yielded similar results, most probably because of the percentage of the Ch-Std contained within the tumor, which remains high during the 3 days following the injection (See section 3.3). For the mice, which received the suspension containing the Ch-Std or the Ch-EDTA, the magnetic field had to be reduced by  $\sim 5$  mT to avoid that the temperature within the tumor exceeds  $50^{\circ}\text{C}$ . For the mice, which received the whole inactive magnetotactic bacteria, the magnetic field strength had to be increased to  $\sim 80$  mT to yield a temperature increase within the tumor. The groups 7 to 13 were considered as a control groups and were not exposed to the alternative magnetic field. These groups were composed of mice, which received into the tumors localized on their right flank, 100  $\mu\text{l}$  of Ch-Std (group 7), 100  $\mu\text{l}$  of Ch-EDTA (group 8), 100  $\mu\text{l}$  of SPION@Citrate (group 9), 100  $\mu\text{l}$  of SPION@PEG (group 10), 100  $\mu\text{l}$  of IM (group 11), 100  $\mu\text{l}$  of whole inactive magnetotactic bacteria (group 12), 100  $\mu\text{l}$  of physiological water (group 13). Finally, the tumors localized on the left flank of the mice were used as internal control and only received physiological water.

The concentrations of the different suspensions (20 mg  $\text{ml}^{-1}$  for the suspension containing IM and 10 mg  $\text{ml}^{-1}$  for the other suspensions) were chosen in such a way that they yielded similar heating properties in water. These concentrations represent the amount of maghemite contained in one milliliter of water. They were estimated in three different ways, either by measuring the absorbance of the different suspensions at 480 nm, by weighing the amount of nanoparticles or magnetosomes after lyophilization or by measuring the saturating magnetization of 20  $\mu\text{l}$  of each suspension deposited on top of a substrate using SQUID magnetometer measurement, (17). These three different types of measurements yielded the same estimate of the concentration for the suspensions containing individual magnetosomes and SPION. For the suspension containing the chains of magnetosomes, the presence of biological material surrounding the bacterial magnetosomes and the arrangement of the magnetosomes in chains led to an overestimate of the maghemite concentration by absorbance and lyophilization. Therefore the concentration of this suspension was determined using SQUID measurements. For the treatment with the whole magnetotactic bacteria, the bacterial concentration injected was  $10^8$  cells in 100  $\mu\text{l}$ . The concentration of bacterial cells was chosen in such a way that it yielded the same iron oxide concentration than that of suspension containing IM.

Histological examinations were carried out in subcutaneous tumor, liver, kidneys and lungs collected 30 days after the first injection. Samples were fixed in 10% formalin solution, embedded in paraffin and sectioned into slices of thickness 4  $\mu\text{M}$ . The sections were stained with hematoxylin-eosin (HE) and with Berlin blue to detect the presence of the bacterial magnetosomes dyed in blue. Necrosis of neoplastic cells, the number of mitoses per 3 randomly selected fields at a magnification of  $\times 400$  in

non necrotic area and the amount of pigmented cells were evaluated in pathological sections of the tumors localized on the right flank of the mice.

#### 2.4 Biodistribution studies:

Induction of human breast tumor was carried out as previously reported in section 2.3. Briefly, 54 female Swiss nude mice of 6 week of age (Charles River, Arbresle, France) received by subcutaneous injection two millions of MDA-MB-231 human breast cancer cells, (18), both on the left and right flanks. The injection of the various types of particles has been carried out 14 days after tumor implantation. Various suspensions containing Ch-Std, IM, SPION@citrate or SPION@PEG have been prepared at the concentration of 10 mg of maghemite per mL. 100  $\mu$ l of these suspensions have been injected directly within the tumors localized on the right flank at the dose of 1 mg of maghemite. The amount of maghemite contained within the different organs of the mice has been measured during the day of the injection (day 0, D0), three days after the injection (day 3, D3), six days after the injection (day 6, D6) or 14 days after the injection (day 14, D14). At the different days (D0, D3, D6 or D14), the animals were euthanized by cervical dislocation and the tissues or organs of interest (blood, liver, spleen, lungs, kidneys, tumor, feces) were collected immediately, weighted and frozen at 4°C until analysis. First, the heating efficiency of the different tumors containing the various types of particles and collected at different days was tested *ex-vivo*. For that, the tumoral tissue was inserted within a tube, which was then positioned inside a coil where the alternative magnetic field of frequency 183 kHz and field strength of 43 mT was applied during 20 minutes (EasyHeat 10 kW, Ambrell, Soultz, France). The temperature within the tumor was measured using an implantable thermocouple microprobe (IT-18, Physitemp, Clifton, USA). Second, the quantity of maghemite was determined using an instrument, the MIAtek<sup>®</sup>, which has been developed by the Company Magnisense, (19). This technology enables sensitive detection and precise quantification of magnetic nanoparticles in a biological target. For the measurements with the MIAtek<sup>®</sup>, the tissues were prepared by mechanical homogenization in ultrapure water (16 % of feces wet weight, i. e 16 g of feces diluted in 100 ml of PBS, 25% of tumor wet weight, 50% of kidney, lung, spleen wet weight and 100% liver wet weight). 100  $\mu$ L of tissues prepared in this way were placed into the detection system (MIAtek<sup>®</sup>). The calibration was carried out by measuring the MIAtek<sup>®</sup> signal of suspensions containing Ch-Std, IM, SPION@Citrate and SPION@PEG mixed in water as a function of the maghemite concentration of these suspensions, which was varied between 15  $\mu$ g/mL and 125  $\mu$ g/mL. In order to verify the estimates of the maghemite concentrations with the MIAtek<sup>®</sup>, SQUID measurements have been carried out on the samples containing the highest percentage of maghemite (the tumors and the feces). For that the saturating magnetization of the different tumors and feces containing the various types of particles was estimated. From this estimate, we could deduce the quantity of maghemite present in the different samples using the saturating magnetization of bulk maghemite (80 emu/g). The estimates deduced from the MIAtek<sup>®</sup> measurements have been compared with those deduced from the SQUID measurements. Finally, the different tumors containing the various types of particles have been heated *ex vivo* under the application of an alternative magnetic field of frequency 183 kHz and field strength of 43 mT. From the heating curves, we could deduce the SAR by measuring their slopes at 25 °C and hence the quantity of maghemite contained within the different tumors.

The estimates of the quantity of maghemite contained within the different tumors have been obtained by collecting one fifth of the total tumor volume after homogenization of the particles within the tumors. Most probably because of a non uniform homogenization, the collected tumor does not contain one fifth of the amount of the various types of particles injected. This results in large error bars in the measurements and in some cases in the detection of more particles within the tumor than the amount, which has been initially injected. However, despite of these uncertainties, the main conclusions drawn in this study remain valid.

### 3. Results and discussion:

#### 3.1 *In-vitro* heating experiments:

The MDA-MB-231 cells have first been incubated with various quantities of Ch-Std and exposed to an oscillating magnetic field of frequency 183 kHz and strength 20 mT, 43 mT or 60 mT during 20 minutes. Figure 2(a) shows that when the suspension containing the cells and the Ch-Std is exposed to the magnetic field of strength 20 mT, the temperature of the suspension does not increase. By contrast, when this suspension is exposed to a magnetic field of strength 43 mT, Figure 2(b) shows that the temperature of the suspension increases and the magnitude of this increase,  $\Delta T$ , increases with the amount of Ch-Std incubated from  $\Delta T = 2\text{ }^{\circ}\text{C}$  for  $0.125\text{ mg} < C_{\gamma\text{Fe}203} < 0.25\text{ mg}$  up to  $\Delta T = 4\text{--}6\text{ }^{\circ}\text{C}$  for  $0.5\text{ mg} < C_{\gamma\text{Fe}203} < 1\text{ mg}$  (Figure 2(b)). Figure 2(c) shows that the magnetic field strength of 60 mT induces a large increase in temperature,  $\Delta T = 16\text{ }^{\circ}\text{C}$ . The latter takes place even for the cells incubated in the absence of the Ch-Std indicating that it arises from the Foucauld's currents, i. e. the heat produced by the ions contained in the suspension and not by the Ch-Std. In order for the therapy to work, the tumor temperature needs to increase by typically 6-18  $^{\circ}\text{C}$ , where this increase in temperature is due to the Ch-Std exposed to the alternative magnetic field. Our results seem to suggest that the optimum strength of the applied magnetic field, which could be used in the therapy, is 43 mT.

The optimum conditions of the therapy correspond to those, which yield a low toxicity in the presence of the alternative magnetic field or Ch-Std used individually and a high toxicity in the presence of both the alternative magnetic field and Ch-Std. In order to determine these conditions, the percentage of living MDA-MB-231 cells has been estimated as a function of the strength of the applied magnetic field, of the quantity of Ch-Std incubated, of the incubation time and of the number of heating cycles. For that, the adherent cells have been incubated in the presence of various concentrations of Ch-Std during 24 hours (Figure 2(d)) or 72 hours (Figs. 2(e) and 2(f)) and exposed either to one heating cycles (Figs. 2(d) and 2(e)) or two heating cycles (Fig. 2(f)). In the absence of application of the alternative magnetic field, the incubation of the Ch-Std with the cells produces relatively low toxicity, i. e. more than 50 % of the cells are alive in most of the conditions tested except for the largest amount of Ch-Std incubated ( $C_{\gamma\text{Fe}203} \sim 1\text{ mg}$ ) and for an incubation time of 72 hours. Moreover, when a suspension containing solely the cells (without the Ch-Std) is exposed to an oscillating magnetic field of strength 20 mT, 43 mT or 60 mT, the percentage of living cells remains high, i. e. above 90 %, as shown in Figs. 2(d) to 2(f), indicating that the applied magnetic field is not toxic. By contrast, when the cells are incubated in the presence of the Ch-Std and exposed to the oscillating, Figs. 2(d) to 2(f) show the partial or total destruction of the cells, where the percentage of cell destruction increases with increasing magnetic field strength, incubation time, quantity of Ch-Std incubated and number of heating cycles. The optimum conditions are reached for the cells incubated 72 hours in the presence of 0.25 mg of Ch-Std and heated two times during 20 minutes by applying an alternative magnetic field of 20 to 60 mT. In these conditions, all the cells are alive in the absence of application of the alternative magnetic field, while 70 to 100 % of them are destroyed in the presence of the alternative magnetic field. Our results therefore suggest that the best conditions for the therapy are reached for the lowest quantity of Ch-Std and for the highest incubation time or number of repetition of the heating cycle.

The efficiency of the treatment has also been examined by measuring the percentage of inhibition of MDA-MB-231 cells incubated in the presence of the Ch-Std and of the various other types of nanoparticles (IM, SPION@Citrate or SPION@PEG). For that, MTT tests have been carried out following a protocol described in section 2.2. The percentage of inhibition of these cells has been estimated either in the absence of a magnetic field (Figure 3(a)) or in the presence of a magnetic field of 43 mT for a treatment carried out either one time (Figure 3(b)) or two times (Figure 3(c)). The best conditions for the treatment using the Ch-Std, i. e. those, which result in a high percentage of

inhibition of the MDA-MB-231 cells in the presence of a magnetic field and in a low percentage of inhibition in the absence of a magnetic field, are obtained for the smallest quantity of Ch-Std incubated (0.125 mg) and for the treatment carried out two times (Figures 3(a) and 3(c)). These results agree with the conclusions drawn from the measurement of the percentage of living cells presented in Figs. 2(d) to 2(f). Figs. 3(a) to 3(c) show that in all conditions tested, the percentage of inhibitions of the cells is larger for the MDA-MB-231 cells incubated in the presence of the Ch-Std than for those incubated in the presence of all other types of nanoparticles (IM, SPION@Citrate and SPION@PEG). The higher efficiency of the Ch-Std compared with that of the SPION@citrate or SPION@PEG could be explained by the higher specific absorption rate (SAR) of the magnetosomes compared with that of the SPION@citrate or SPION@PEG, (J Phys Chem C, bazyliński). Since the Ch-Std and IM are both composed of magnetosomes, the different efficiency between these two types of magnetosome arrangements can not arise from a difference in SAR. Instead, we examine if this behavior is due to different faculty of penetration of the IM and Ch-Std within the cells in the presence of the alternative magnetic field. Figure 3(d) shows the percentage of MDA-MD-231 cells, which become magnetic when the cells are incubated in the presence of the various types of nanoparticles and exposed (or not) to an alternative magnetic field of 43 mT applied during 5 to 20 minutes. The percentage of magnetic cells is high for the cells incubated in the presence of the Ch-Std (50-70 %, Fig. 3(d)) while it is very low for the cells incubated in the presence of the IM (less than 10 %). This difference in behavior between the Ch-Std and IM may be due a different type of organization between the Ch-Std, which distribute homogeneously and the IM, which are prone to aggregation. These results suggest that the high efficiency of the Ch-Std is due on the one hand to the high SAR of the magnetosomes and on the other hand to their faculty of penetration within the cancer cells in the presence of the alternative magnetic field.

### *3.2 In-vivo heating experiments:*

In the first set of mice, ~ 1 mg of Ch-Std are administered within the tumors and the latter are then heated by applying an AMF as described in more details in section 2.3. When the AMF is applied, the temperature measured at the location of the Ch-Std injection, increases by 10°C from 33°C to 43°C within 20 minutes (Figure 4(a)). These temperatures lie within the range of temperatures reached during a typical hyperthermia treatment, (ref). The infra-red measurements carried out during the heat cycle shows a large spread of temperatures higher than 37°C at the skin surface of the tumor. Figure 4(b) shows that the full width half maximum of the spatial temperature distribution is 0.75 cm, a value close to the lateral size of a tumor (1 cm), suggesting a homogenous temperature distribution within the tumor. During the 30 days following the treatment the variations in sizes of the treated tumors shown in Figure 4(c) either decreases or increases much less than those of the untreated tumors shown in Figures 4(d). The progressive disappearance of the treated tumor following the treatment can be observed in one of the mice (mouse 5) by examining the set of three photographs presented in Figure 5(a), which were taken either during the day of the treatment (D0), 14 days after the treatment (D14) or 30 days after the treatment (D30). 30 days following the treatment, Figure 5(a) shows a black spot at the position where the tumor has initially grown. The anti-tumoral activity was further revealed by histological examinations, which showed that there was no remain of tumor tissues in mouse 5. Pathological examinations of the treated tumors showed that the number of observed mitosis was low (4 in average by selected field of 300  $\mu\text{m}^2$ ) indicating a decrease in the activity of tumor proliferation. In mouse 9 and in two other mice, where the suspension containing Ch-Std is injected without application of the alternative magnetic field, the tumor size increases strongly during the 30 days following the treatment as shown in Figure 4(d). This indicates that the anti-tumoral activity was due to the heat released by the Ch-Std and not solely to the presence of the Ch-Std within the tumor. To further understand the origin of the anti-tumoral activity of the Ch-Std, a micrograph of a tumor tissue collected after the treatment is presented in Figure 5(b). It shows a homogenous distribution of iron indicated by the large blue spread, suggesting a homogenous

distribution of the Ch-Std. This behavior could explain the homogenous temperature distribution observed by infra-red measurements. The enlargements of Figures 5(b), which are shown in Figures 5(c) and 5(d), show a black region surrounding the cell nucleus, suggesting that the chains of magnetosomes have penetrated within the cells after application of the alternative magnetic field, a behavior, which agrees with the results presented in Fig. 3(d) and suggests a mechanism of intra-cellular heating. The impact of the length of the magnetosome chains on the efficiency of the thermotherapy is also examined by administering within the tumors Ch-EDTA, which are longer chains of magnetosomes than the Ch-Std. After administration of  $\sim 1$  mg of Ch-EDTA within the tumor and application of the AMF, the tumor temperature increases more rapidly than with the Ch-Std (Figures 6(a) and 4(a)). As with the Ch-Std, it was possible to completely eliminate the tumor with the Ch-EDTA in one of the mice treated as shown in Figs. 7(a) and 7(b). However, as a whole, the anti-tumoral activity is less pronounced with the Ch-EDTA than with the Ch-Std. While the size evolutions of the treated and untreated tumors averaged over the different mice studied are significantly different with the Ch-Std (Figs. 4(c)), they are relatively similar with the Ch-EDTA (Figure 6(b)). We deduce from these results that the efficiency of the thermotherapy is not only determined by the amount of heat produced by the chains of magnetosomes but also by the length of the magnetosome chains.

In order to examine if the efficiency of the chains of magnetosomes is due to the high SAR of the magnetosomes, we treat a series of mice with SPION of lower SAR than that of the magnetosomes, (J. Phys. Chem. C). We study the SPION, which penetrate within the cells and produce intra-cellular heating (SPION@Citrate) and those, which remain outside of the cells and yield extra-cellular heating (SPION@PEG). Compared with the Ch-Std or Ch-EDTA, when 1 mg of SPION@Citrate is administered within the tumor and the mice are exposed to the AMF, Figure 6(c) shows that the tumor temperature increases by a low amount of  $4^{\circ}\text{C}$  in 2 minutes. Most probably due to this small temperature increase, the thermotherapy is inefficient and the volume of the treated tumor increased at the same rate as that of the untreated tumor during the 30 days following the treatment as shown in Figure 6(d). Moreover, none of the mice shows a complete disappearance of the tumor during the 30 days following the treatment. For a typical mouse treated with the SPION@Citrate, the tumor is still there 30 days following the treatment (Figures 7(c) and 7(d)), (ref-expliquer l'efficacité avec 2 mg). After administration of  $\sim 1$  mg of the SPION@PEG within the tumor and application of the AMF, the tumor temperature of the mice does not increase at all as shown in Figure 7(e) and none of the tumors decreases in size during the 30 days following the treatment (Figures 6(f), 7(e) and 7(f)). We conclude that the higher efficiency of the Ch-Std and Ch-EDTA compared with that of the SPION@Citrate and SPION@PEG is due (at least partly) to the higher SAR of the magnetosomes.

Next, we turn to the function played by another parameter than the SAR on the efficiency of the therapy. This parameter is the organization of the magnetosomes in chains. For that, we treat a group of mice (group 6) with individual magnetosomes (IM), which possess the same SAR and composition in iron oxide than the magnetosomes organized in chains but form individual nanoparticles instead of chains. After application of the AMF, the tumor temperature of the mice treated with the IM increases by  $4^{\circ}\text{C}$  from  $31^{\circ}\text{C}$  to  $35^{\circ}\text{C}$  (Figure 8(a)), which is a much smaller temperature increase than that observed with the Ch-Std or Ch-EDTA. After 10 minutes of heating, infra-red measurements show that the temperature spread within the tumor is  $\sim 0.5$  cm, where this distance is estimated by measuring the full width half maximum of the temperature distribution shown in Figure 8(b). The temperature spread is less than that obtained with the Ch-Std, suggesting a less homogenous distribution of magnetosomes in this case. Figures 8(c) and 8(d) show that the sizes of the heated and unheated tumors both increase during the 30 days following the treatment indicating the absence of anti-tumoral activity. The increase of the treated tumor size can also be

observed in mouse 3 by examining the set of three photographs taken during the day of the treatment (D0), 14 days after the treatment (D14) and 30 days after the treatment (D30) (Figure 9(a)). Together these results indicate that neither the presence of the individual magnetosomes nor the heat that they generate in the presence of a magnetic field produce anti-tumoral activity. Pathological examinations of the tumor localized on the right flank of mouse 2 provided some explanation for this behavior. They show an important mass of necrotic cells in tumors collected 30 days after the first treatment **but** mitoses are numerous and indicated an important tumor proliferative activity with an average of 12 mitoses per selected field of 300  $\mu\text{m}^2$  in size. The Berlin blue staining of a pathological section obtained from the right tumor showed the presence of diffused dark spots (Figure 9(b)). These spots are presumed to arise from magnetosome aggregates as shown in enlarged views of Figure 9(b) presented in Figures 9(c) and 9(d). The presence of these aggregates prevents the magnetosomes from penetrating within the cells both in the absence and in the presence of the alternative magnetic field (Fig. 3(d)). With the individual magnetosomes, the absence of anti-tumoral activity could be explained in part by their relatively low in-vivo heating efficiency and in part by their tendency to aggregate, which prevents them from penetrating within the cells and yield extra-cellular heating, which is presumed to be less efficient than intra-cellular heating. In conclusion, the organization in chains of the magnetosomes is necessary to reach a high efficiency of the thermotherapy.

Finally, we also examine if it is necessary to extract the chains of magnetosomes from the whole inactive magnetotactic bacteria to yield anti-tumoral activity. For that,  $10^8$  inactive magnetotactic bacteria contained in 100  $\mu\text{l}$  of PBS have been injected within the tumors. After application of the AMF, the temperature increases by only 4  $^\circ\text{C}$  from 33 $^\circ\text{C}$  to 37 $^\circ\text{C}$  in 20 min. In this case, the absence of anti-tumoral activity is revealed by the increase in size of the treated tumors during the 30 days following the treatment and by histological examination, which reveal a pigmented area in the treated tumor with a high mitotic activity (15 mitosis in average by selected field of 300  $\mu\text{m}^2$ ).

### *3.3 Biodistribution:*

Biodistribution studies are carried out to study how long the various types of nanoparticles remain within the tumor after an intra-tumoral administration and to examine if they are well eliminated by the organism. Figure 10(a) shows the biodistribution of the Ch-Std within the tumors (estimated as the percentage of injected dose per gram of tissue) just after the injection (D0), 3 days after the injection (D3), 6 days after the injection (D6) and 14 days after the injection (D14). The three types of measurements (MIAtek<sup>®</sup>, SAR and SQUID) show essentially the same trend: The rapid decrease of the percentage of Ch-Std contained within the tumors during the 14 days following their injection (Figure 10(a)). Indeed, more than 90% of the Ch-Std has been eliminated 14 days following the injection. The Ch-Std are found essentially in the feces with 10-15% in the feces at the first day post-injection (D1) and 15 to 20 % at day 3 (D3), day 6 (D6) and day 14 (D14) post-injection (Figure 10(b)). With the Ch-Std, the route of elimination appears to be essentially fecal. Only few traces of Ch-Std (< 0.1 % ID/g of tissue) were found in the lung, kidney, liver and spleen the third day (D3) and the sixth day (D6) following the injection. No Ch-Std are found in the blood. These results suggest that the Ch-Std were rapidly excreted. Compared with the Ch-Std, Figure 10(c) shows that the percentage IM contained within the tumor decreases less significantly during the 14 days following the treatment. A lower percentage of 5 to 10% of IM is found in the feces at D3, D6 and D14 (Figure 10(d)). Together these results suggest a less rapid elimination of the IM compared with the Ch-Std. The SPION@Citrate and SPION@PEG also seem to be eliminated less easily than the Ch-Std. Figures 10(e) and 10(g) show that a large percentage of them remained within the tumors during the 14 days following their injection. In addition, almost no SPION@Citrate and SPION@PEG were found in the

feces (Figs. 10(f) and 10 (h)). This could be explained by the metabolization of these nanoparticles in free iron, which would rather be a drawback of these chemically synthesized nanoparticles compared with the magnetosomes since free iron can cause oxidative stress, (20).

**Conclusion:**

We can conclude that the anti-tumoral activity of the chains of magnetosomes is essentially due to three factors, the amount of heat that they produce when they are exposed to an alternative magnetic field, which is essentially due to their large sizes and ferromagnetic behavior, their faculty to penetrate within the cells enabling efficient intracellular destruction and their arrangement in chains, which favor a homogenous nanoparticles and hence also temperature distribution within the tissue. In the conditions that we tested (1 mg of the various types of nanoparticles administered within the tumors, the application of a magnetic field of 43 mT and 3 different heat cycles of 20 minutes) we could observe the complete disappearance of a tumor xeno-greffed on a mouse by administering chains of magnetosomes within the tumor. By contrast, no or much less efficient anti-tumoral activity was observed with superparamagnetic iron oxide nanoparticles coated by citrate ions or PEG molecules or with other types of bacterial magnetosomes, such as those contained within inactive magnetotactic bacteria or individual magnetosomes detached from the chains by heat and SDS treatments. This study shows the potential of chains of magnetosomes extracted from whole magnetotactic bacteria for application in alternative magnetic field cancer therapy.

**Acknowledgment:**

We thank Laurence Motte and Yoann Lalatonne for synthesizing the SPION@Citrate, which were used to evaluate the efficiency of the Ch-Std.

<b>Group</b>	<b>Mouse</b>	<b>Oscillating Magnetic field</b>	<b>Suspension administered containing:</b>	<b>Quantity of iron oxide or number of cells administered</b>
1	5, 6, 7, 8	Yes	Standard chains of magnetosomes (Ch-Std)	1 mg
2	9, 10, 11, 12	Yes	Chains of magnetosomes EDTA (Ch-EDTA)	1 mg
3	15, 16, 17, 18	Yes	SPION@citrate	1 mg
4	19, 20, 21, 22	Yes	SPION@PEG	1 mg
5	1, 2, 3, 4	Yes	Individual magnetosomes (IM)	2 mg
6	13, 14	Yes	Inactive magnetotactic bacteria	10 <sup>8</sup> cells
7	26, 27, 28	No	CM-Std	1 mg
8	29, 30, 31	No	CM-EDTA	1 mg
9	34, 35, 36	No	SPION@citrate	1 mg
10	37, 38, 39	No	SPION@PEG	1 mg
11	23, 24, 25	No	IM	2 mg
12	32, 33	No	Inactive magnetotactic bacteria	10 <sup>8</sup> cells
13	40, 41, 42	No	Physiological water	

**Table 1: Description of the different groups of mice used to carry out the experiments.**

## Figures:

**Figure 1:** (a) Transmission electron microscopy (TEM) micrograph of one cell of *Magnetospirillum magneticum* strain AMB-1, where the arrows indicate the localization of the chains of magnetosomes; (b) TEM micrograph of the chains of magnetosomes extracted from the bacteria; (c) TEM micrograph of individual magnetosomes detached from the chains by heat and SDS treatments.

**Figure 2:** Properties of suspended MDA-MB-231 cells incubated in the absence or in the presence of extracted chains of magnetosomes of various concentrations ( $0.125 \text{ mg/mL} < C_{\gamma\text{Fe}_{203}} < 1 \text{ mg/mL}$ , where  $C_{\gamma\text{Fe}_{203}}$  represents the concentration in maghemite of the suspensions) and exposed to an alternative magnetic field of frequency 183 kHz and various strengths ( $0 \text{ mT} < B < 60 \text{ mT}$ , where B represents the strength of the applied magnetic field). (a)-(c): Variations of temperature of suspensions containing MDA-MB-231 cells incubated in the absence or in the presence of chains of magnetosomes of various concentrations ( $0.125 \text{ mg/mL} < C_{\gamma\text{Fe}_{203}} < 1 \text{ mg/mL}$ ) when an alternative magnetic field of  $B = 20 \text{ mT}$ , (a),  $B = 43 \text{ mT}$ , (b), or  $B = 60 \text{ mT}$ , (c), is applied to these suspensions. (d)-(f): Percentage of living adherent MDA-MB-231 cells as a function of the strength of the magnetic field ( $0 \text{ mT} < B < 60 \text{ mT}$ ), which is applied once or twice during 20 minutes. The cells are either incubated during 24 hours, D1, (d) or 72 hours, D3, (e), (f) either in the absence or in the presence of the extracted chains of magnetosomes of various concentrations ( $0.125 \text{ mg/mL} < C_{\gamma\text{Fe}_{203}} < 1 \text{ mg/mL}$ ). The different symbols represent the heat produced by the suspension of cells in the absence of the chains of magnetosomes (- -) or by the suspension of the cells in the presence of the chains of magnetosomes with a concentration in maghemite of  $C_{\gamma\text{Fe}_{203}} = 1 \text{ mg/ml}$  ( $\square!\square$ ),  $C_{\gamma\text{Fe}_{203}} = 0.5 \text{ mg/ml}$  ( $\square\Delta\square$ ),  $C_{\gamma\text{Fe}_{203}} = 0.25 \text{ mg/ml}$  ( $\square, \square$ ),  $C_{\gamma\text{Fe}_{203}} = 0.125 \text{ mg/ml}$  ( $\square\vee\square$ ).

**Figure 3:** (a)-(c) Percentage of inhibition of MDA-MB-231 cells incubated in the presence of four different suspensions containing chains of magnetosomes (CM), individual magnetosomes (IM), SPION covered by citrate ions (SPION@Citrate), SPION covered by PEG molecules (SPION@PEG) as a function of the concentration in maghemite of these four suspensions. The cells are either not exposed to an oscillating magnetic field, (a), exposed once to the oscillating magnetic field, (b), or exposed twice to the oscillating magnetic field, (c). Percentage of cells, which become magnetic as a function of the incubation time, when the four suspensions mentioned above ( $0.125 \text{ mg/mL} < C_{\gamma\text{Fe}_{203}} < 1 \text{ mg/mL}$ ) are incubated in the presence of MDA-MB-231 cells and the alternative magnetic field of 183 kHz and strength of 43 mT is applied.

**Figure 4:** Study of the mice treated with suspensions containing chains of magnetosomes (mice 5 to 9). (a) The variations of the tumor and rectal temperatures when the magnetic field is applied during the treatment (mice 5 to 8). The temperature is averaged over the different mice (mice 5 to 8); (b) For a mouse showing a typical behavior, temperature distribution measured across the treated tumor 10 min after the beginning of the treatment; (c) Evolution of the normalized tumor volume for the tumor in which the suspension containing the chains of magnetosomes has been injected. The volume of the treated tumor is normalized by the volume of the tumor at the time of the treatment; (d) Same as in (c) for the control tumor in which only PBS has been injected. In mouse 9, the suspension containing the chains of magnetosomes has been injected but no magnetic field has been applied to the mouse.

**Figure 5:** Study of the mice treated with suspensions of chains of magnetosomes (mice 5 to 9). (a) Photographs of the treated tumor in mouse 5 just after the treatment (D0), 14 days after the treatment (D14), 30 days after the treatment (D30). (b) Micrograph of a tumor tissue collected 30 days after the treatment in mouse 5 showing the presence of the bacterial magnetosomes (blue color or dark contrast); (c) Enlargement of (b). (d) Enlargement of (c) showing a cell with its nucleus surrounded by bacterial magnetosomes.

**Figure 6:** (a), (c), (e): Variations of the tumor and rectal temperatures when the suspensions containing the Ch-EDTA, (a), the SPION@Citrate, (c), or the SPION@PEG, (e), are administered within the tumor and the alternative magnetic field of frequency 183 kHz and strength 43 mT is applied during 20 minutes. The treatment is repeated 3 times with a 1 day resting time between the different treatments. (b), (d), (f): Variations of the normalized tumor volume (i. e. the tumor volume measured at day 2 to day 30 following the treatment divided by the tumor volume measured during the day of the treatment) during the days following the treatment for the Ch-EDTA, (b), the SPION@Citrate, (d), or the SPION@PEG, (f). In (b), (d), (f), the error bars are the standard deviations estimated by taking into account the normalized tumor volume of each mouse.

**Figure 7:** (a), (c), (e): Photographs of the mice, which showed the best anti-tumoral activity 30 days after the treatment induced by heat for the treatment carried out using the Ch-EDTA, (a), the SPION@Citrate, (c), or the SPION@PEG, (e). (b), (d), (f): The variations of the normalized tumor volume during the days following the treatment for the mice showing the best anti-tumoral activity and treated with the Ch -EDTA, (b), SPION@Citrate, (d) and SPION@PEG, (f).

**Figure 8:** Study of mice treated with suspensions of individual magnetosomes (mice 1 to 4). (a) The variations of the tumor and rectal temperatures when the magnetic field is applied during the treatment. These temperatures are averaged over the different mice treated (mice 1 to 3); (b) For a mouse showing a typical behavior, the temperature distribution measured across the treated tumor 10 min after the treatment has started; (c) Variation of the normalized tumor volume for the tumor in which the suspension of individual magnetosomes has been injected (mice 1 to 3). The volumes of the tumors are normalized by the volume of the tumor at the time of the treatment; (d) same as in (c) for the so-called control tumor in which only PBS has been injected. In mouse 4, the suspension of individual magnetosomes has been injected but no magnetic field has been applied.

**Figure 9:** Study of the mice treated with individual magnetosomes (mice 1 to 4). (a) Photographs of the treated tumor in mouse 1 just after the treatment (D0), 14 days after the treatment (D14) or 30 days after the treatment (D30). (b) Micrograph of a tumor tissue collected 30 days after the treatment in mouse 2; (c) Enlargement of a region of (b) showing the presence of bacterial magnetosomes (blue color or dark contrast); (d) Enlargement of a region of (c) showing magnetosomes aggregates.

**Figure 10:** Percentage of nanoparticles in the tumor ((a), (c), (e), (g)) and in the feces ((b), (d), (f), (h)) at the time of the injection (D0), 3 days after the injection (D3), 6 days after the injection (D6) and 14 days after the injection (D14) for an intra-tumoral administration of suspensions containing either chains of magnetosomes, (a), (b), individual magnetosomes, (c), (d), SPION@Citrate, (e), (f), and SPION@PEG, (g), (h).

**Suppl. Fig. 1:** (a) TEM micrograph of the chemically synthesized nanoparticles (SPION@Citrate); (b) A measurement of charge at the surface of the chains of magnetosomes (CM) and individual magnetosomes (IM) as a function of the pH of the suspension containing these two types of bacterial magnetosomes; (c) The infrared spectra of the chains of magnetosomes (CM) and individual magnetosomes (IM).

**Suppl. Fig. 2:** (a-c): Properties of chains of magnetosomes extracted from magnetotactic bacteria, which have been synthesized in the absence of chelating agents. Histograms showing the distribution in magnetosome sizes, (a), and magnetosome chain lengths, (b) of this type of magnetosomes. (c): The variation with time of the temperature of a suspension of this type of magnetosomes containing 406  $\mu\text{g}/\text{ml}$  in maghemite when this suspension is exposed to an alternative magnetic field of frequency 183 kHz and magnetic field strength of either 43 mT or 80 mT. (d-f): Properties of chains of magnetosomes extracted from magnetotactic bacteria, which have been synthesized in the presence of 0.4  $\mu\text{M}$  EDTA. Histograms showing the distribution in magnetosome sizes, (d), and magnetosome chain lengths, (e), of this type of magnetosomes. (f): The variation with time of the temperature of a

suspension containing this type of magnetosomes with a concentration of 406  $\mu\text{g/ml}$  of maghemite when the suspension is exposed to an alternative magnetic field of frequency 183 kHz and magnetic field strength of either 43 mT or 80 mT.

**Suppl. Fig. 3:** (a) Schematic diagram showing the protocol of treatment. On the left tumor, only a suspension containing PBS is administered whereas on the right tumor a suspension containing the various types of nanoparticles is administered. (b) Schematic diagram showing the apparatus used to apply the alternative magnetic field. (c) A photograph showing the mouse ready for treatment. (d) A typical infra-red picture showing the distribution in temperature within the tumor of the mouse.

## References:

1. Duguet et al., *Nanomed.*, 2006, 1, 157-168.
2. De Nardo et al, *Clin. Cancer Res.*, 2005, 11, 7087s-7092s; De Nardo et al., *J.Nucl. Med.*, 2007, 48, 437-444;
3. Higler et al., *Radiology*, 2001, 218, 570-575;
4. Ito et al., *Cancer. Sci.*, 2003, 94, 308-313;
5. Ito et al., *J. Biosci. Bioeng.*, 2003, 96, 364-369; Ito et al, *Cancer Lett.*, 2004, 212, 167-175; Ito et al., *Cancer Immunol. Immun.*, 2006, 55, 320-328;
6. Johannsen et al., *Int. J. Hyperthermia*, 2005, 21, 637-647; Johannsen et al., *Int. J. Hyperthermia*, 2007, 52, 1653-1662; Jordan et al., *Int. J. Hyperthermia*, 1993, 9, 51-68; Maier-Hauff et al., *J. Neuro-Oncol.*, 2007, 81, 53-60; Jordan et al.; *J. Neuro-Oncol.*, 2006, 78, 7-14.
7. Kawai et al., *Prostate*, 2005, 64, 373-381; Kawai et al., *Prostate*, 2008, 68, 784-792;
8. Kikumori et al., *Breast Cancer Res. Treat.*, 2009, 113, 435-441 ;
9. These companies are Sirtex (an Australian company), Magforce (a German company) and Aspen Medisys (an American company previously Aduro Biotech and Triton Biosystem).
10. Sirtex: US2006167313 or WO 2004/064921; Triton Biosystems now Aspen Medisys, LLC: US2003/0028071; Magforce: US2008/0268061.
11. Habib et al., *J. Appl. Phys.*, 2008, 103, 07A307-1-07A307-3.
12. Hergt et al., *J. Phys. Condens. Matter*, 2006, 18, S2919-S2934.
13. SPION stabilized by molecules, which are not anti-tumoral such as citrate, are used by the group of Jordan. The same types of SPION but encapsulated within magneto cationic liposomes (MCM) are used by the group of Ito *et al.* SPION stabilized by PEG molecules and available commercially in the company Micromod are used by the group of De Nardo.
14. Hergt et al., *J. Phys. Condens. Matter*, 2006, 18, S2919-S2934; Hergt et al., *J. Magn. Magn. Mater.*, 2005, 293, 80-86; Timko et al., *J. Mag. Mag. Mat.*, 2009, 321, 1521-1524.
15. Bazylinski et al., *Nat. Rev. Microbiol.*, 2004, 2, 217-230.
16. Arakaki et al., *J. R. Soc. Interface*, 2005, 5, 977-999).
17. Pattern number US6251365B1
18. Sun et al., *Cancer Lett*, 2007, 258, 109-117
19. Sun et al., *J. Nanosci. Nanotechnol.*, 2009, 9, 1881-1885; Sun et al, Sun et al, *Nanotoxicology*, 2010, 4, 271-283.
20. RefJ phys chem C alphanbéry mécanismes de chauffage.
21. D. Faivre et al, *Chem. Rev.*, 2008, 108, 4875-4898.
22. Lalatonne et al., *Phys. Rev. E*, 2005, 71, 011404-1, 011404-10.
23. T. Mosmann, 1983, *J. Immunol. Methods*, 65, 55-63.
24. Sun et al., *Cancer Lett.*, 2007, 258, 109-117.
25. Alphanbéry et al., *J. Phys. Chem. C*, 2008, 112, 12304-12309.

26. Cailleau et al., *J. Natl. Cancer Inst.*, 1974, 53, 661-674.
27. Nikitin et al., 2007, *J. Magn. Mater.* 311, 445.
28. Puntarulo et al., 2005, *Mol. Aspects, Med.*, 299-312.