Local moderate magnetically induced hyperthermia using an implant formed in situ in a mouse tumor model

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Abstract
Purpose: We investigate a new heat delivery technique for the local treatment of solid tumors. The technique involves injecting a formulation that solidifies to form an implant in situ. This implant entraps superparamagnetic iron oxide nanoparticles (SPIONs) embedded in silica microbeads for magnetically induced moderate hyperthermia. Particle entrapment prevents phagocytosis and distant migration of SPIONs. The implant can be repeatedly heated by magnetic induction. Methods: We evaluated heating and treatment efficacies by means of thermometry and survival studies in nude mice carrying subcutaneous human colorectal carcinomas. At day 1, we injected the formulation into the tumor. At day 2, a single 20-min hyperthermia treatment was delivered by 141-kHz magnetic induction using field strengths of 9 to 12 mT under thermometry. Results: SPIONs embedded in silica microbeads were effectively confined within the implant at the injection site. Heat-induced necro-apoptosis was assessed by histology on day 3. On average, 12 mT resulted in tumor temperature of 47.8 degrees C, and over 70% tumor necrosis that correlated to the heat dose [...]

Reference

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Local Moderate Hyperthermia Treatment Induced Magnetically through an Implant Formed In Situ in a Mouse Tumor Model

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**Running head**

Local hyperthermia by magnetic implant in mouse

**Keywords**

magnetic induced hyperthermia; superparamagnetic nanoparticles; implant; precipitating polymers; subcutaneous xenograft; necrotizing colocolcarcinoma; survival; thermometry.
Abstract

Purpose: We investigate a new technique of heat delivery through an injectable formulation forming an implant in situ, aimed at the local treatment of solid tumors by magnetically-induced moderate hyperthermia. Superparamagnetic iron oxide nanoparticles (SPIONs) embedded in silica microbeads, are entrapped into a solid ethylene-vinyl alcohol copolymer matrix formed at the injection site. Phagocytosis and distant migration of SPIONs been thus prevented, this implant can be repeatedly heated by alternative magnetic induction.

Methods: We evaluated heating and treatment efficacies by thermometry and survival studies led in nude mice bearing subcutaneously a human colon carcinoma tumor. At D1, we injected the formulation into the tumor. At D2, a single 20-min hyperthermia treatment was delivered by 141-kHz alternative magnetic induction for field strengths of 9 to 12 mT, under thermometry monitoring.

Results: SPIONs-silica microbeads were durably confined within the implant at injection site. Heat-induced necrosis was assessed by histology at D3. On average, 12 mT resulted in an intra-tumoral temperature of 47.8°C, with over 70 % tumor necrosis correlated to the dose (i.e. AUC = 282 °C·min), whereas 9 mT induced a intra-tumoral temperature of 40.0 °C (i.e. AUC = 131 °C·min) without morphologically identifiable necrosis. Survival after treatment was significantly improved with 10.5 or 12 mT compared to non-implanted and implanted controls, leading to respective median survival times of 27 and 37 days vs. 12 and 21 days.

Conclusion: Resulting in 45 % of animals one year survival without tumor recurrence, 12-mT magnetically-induced moderate hyperthermia through injectable implants revealed high efficiency.
Hyperthermia, the therapeutic application of heat, has revealed large benefits in oncology. Moderate tissue temperatures (40 to 46 °C) associate with cytotoxic protein destabilization and denaturation. Cellular defense consists in tolerance reactions against heat stress, closely connected with the induction of heat shock proteins. This tolerance is inhibited above the temperature threshold near to 42°C and results in a pronounced increase in cell death rate[1]. Moderate hyperthermia turns the vascular deficiency of a tumor to therapeutic gain through relative enhanced sensitivity to heat damages. A variety of reference therapies in oncology: radiotherapy, brachytherapy, chemotherapy or a combination of them, has been synergistically combined with moderate hyperthermia[2,3].

The outcome of a hyperthermia treatment correlates to the heat dose administered[4], which is dependent upon anatomical situation and heating modalities. Local hyperthermia aims at confining heat delivery to the lesion site. Despite constant improvements in heating localization of external or interstitial modalities based on standard heat application means (radiofrequencies, microwaves and ultrasounds), physical limitations still hinder the treatment of deep-seated lesions[5]. Magnetic fields are able to cross the diamagnetic body of a patient without losses. Superparamagnetic iron oxide nanoparticles (SPIONs) exposed to alternative magnetic field convert magnetization energy in heat dissipation[6,7]. Their administration allows for magnetically-induced modality of hyperthermia[8]. Magnetic fluid hyperthermia[9], the alternative magnetic induction of dextran coated SPIONs interstitially injected, has achieved promising clinical success[10-12]. As a major drawback of current techniques of administration, SPIONs fade ineluctably out from the injection site into lymphatic and blood circulation or are sequestrated in macrophage, exposing patients to potentially toxic hazards[13].

This report describes an original approach of magnetically induced local moderate hyperthermia through an in situ-formed implant entrapping SPIONs embedded in silica microbeads. After injection in aqueous environment and precipitation from their organic solvent formulation, water-insoluble polymer chains entangle and form a matrix holding the dispersed superparamagnetic microbeads. Avoiding their phagocytosis and distant migration, SPIONs are protected and durably confined at the injection site. The opportunity of repeating the implant heating in the long term could bring therapeutic benefits. Thermometry and survival studies were performed in a model of human colorectal carcinoma tumor subcutaneously.
engrafted in nude mice to assess heating efficiency and treating potential of local moderate magnetically-induced hyperthermia delivered through the implant formed in situ.

**Material and Methods**

*Magnetics beads*

They consisted of micron-sized particles containing 32% w/w of nanometric iron oxide particles of 10-nm mean diameter. They were synthesized as described by Chastellain et al.[14]. Briefly, tetramethoxysilane (45 ml) was added to a 2 M solution of Fe(NO$_3$)$_3$·9H$_2$O in ethanol (44.4 g iron salt in 55 ml ethanol). The mixture was stirred vigorously for 10 min, transferred to a sealed glass container and allowed to gel at 50 °C. The obtained brown gel was ground and classified by sieving. Particles smaller than 100 micrometers were thermally treated at 500 °C for 24 hrs, followed by high-energy attrition milling for 1 hr. Measurements by Superconducting QUantum Interference Device (SQUID) ascertained superparamagnetism. The Specific Absorption Ratio (SAR), which reflects the heating capacities, is conventionally defined as the slope of the initial temperature rise multiplied by specific heat capacity. In our case, this was in the order of 18 W/g iron oxide for a 12-mT magnetic field.

*Injectable formulation*

The ethylene vinyl alcohol copolymer EVAL™ 105-B (EVAL Europe, Zwijndrecht, Belgium) was dissolved at 8 % (w/v) in pharmaceutical grade dimethyl sulfoxide (DMSO) (Gaylord Chemical Corp., Los Angeles, USA). By ultrasound and vigorous mechanical stirring, the beads were suspended at 40 % (w/v) in the resulting polymer solution. The preparation was finally sterilized by 15 min steam autoclaving at 121°C. As flocculated sedimentation occurred during storage, energetic shaking before use restored suspension homogeneity.

*Alternating magnetic field generator*
The field generator used (TIG 2.5, Hüttinger Elektronik GmbH, Freiburg, Germany) consisted of an alternating current generator feeding the coil inductor (internal, external diameters and length of the horizontal coil were 54, 64 and 46 mm respectively). A conical tube mouse restrainer was introduced within the coil so that the implanted tumor was positioned at the center. With a small pick-up coil calibrated using a teslameter, we found a linear relation between the magnetic field amplitude at 141 kHz and the generator peak voltage in volts. Current intensity was adjusted to impose the voltage corresponding to the chosen field strength.

*Tumor model*

Tumors generated by subcutaneous injection of the human Co112 colon carcinoma cells were maintained by serial subcutaneous transplantation in Swiss nude mice[15]. About 15 mm\(^3\) of excised and minced tumor was subcutaneously engrafted into the right flank of 5-week old Swiss nude mice and the nodule was allowed to grow for 4 to 6 weeks. Care was taken to implant the tumor above hind leg musculature in order to avoid detrimental heat exposure of intestinal tissues. The obtained tumor showed peripheral angiogenesis, necrosis in the tumor center core and a pseudocapsule composed of connective tissue. Central necrosis is a common feature in Co112 tumors, observed also in *in vitro*-grown multicellular spheroids and liver metastases[16,17].

*Mice*

One-month old female Swiss nude mice were supplied by Charles Rivers (Iffa Credo, Saint Germain sur l’Arbresle, France). Animal experiments were performed according to the ethical principles of laboratory animal care and Swiss legislation. Experiments were specifically approved by the official committee of animal research surveillance of the local authority. Animals were maintained in SPF animal house under a 12 hrs light and 12 hrs darkness cycle with normal diet, *ad libitum*, respecting a maximum of five animals per cage. Animals were euthanized by asphyxia under CO\(_2\) saturated atmosphere.

*Implantation*
Since small animals are notably sensitive to organic solvent, we set the injection volume to 0.25 ml. The DMSO dose injected was below the mouse intraperitoneal LD$_{50}$ (13 g/kg[18]). The formulation was slowly (over 1 to 2 min) injected into the tumor through a 22G needle. Systemic and local toxicities were limited. Thanks to the brownish color and stiffness of the implant, we could manage and verify correct implant distribution through tissue darkening and induration. The implant first entered into the necrotic core and then extended towards the surrounding pseudocapsule to reach the peripheral border of tumor. To avoid distant leakage, we paid special attention to needle positioning to distribute the implant uniformly without accumulation in a part of the nodule. The procedure gave rise only to transient perinodular edema, which was spontaneously resorbed by the time we proceeded to alternating magnetic field stimulation.

**Thermometry**

We monitored temperature with a fluoroptic thermometer (Luxtron, Santa Clara CA, USA) with three optic fiber probes of 200 μm diameter, reporting every second temperatures with 0.1 °C accuracy. A one-point calibration at 20.0 °C was performed before each experiment. Data were acquired using Physitemp software (Luxtron, Santa Clara CA, USA).

**In vivo investigations protocols**

1) **Thermometry studies:** 0.25 ml of the EVAL solution in DMSO 8 % (w/v) containing 40 % (w/v) microbeads was injected (Day 1). After 24 hrs, the animal was exposed to a 20-min alternating magnetic field (141 kHz), under general anesthesia by halothane (Day 2). We investigated five magnetic field strengths: 9, 10, 10.5, 11 and 12 mT with respectively n = 5, 4, 5, 3 and 5 animals per group. Temperatures were monitored in the tumor, on the skin over the tumor, and in the hollow of the brachial plexus. The animals were sacrificed 24 hrs later (Day 3) for standard histology. Tumor size in the animals used for thermometry studies was in the range of 0.1 to 0.3 cm$^3$.

2) **Survival studies:** For the survival investigation protocol, 0.25 ml 8% (w/v) EVAL solution in DMSO containing 40 % (w/v) beads was injected (Day 1). After 24 hrs, animals were exposed to an alternating magnetic field (141 kHz) for 20 min (Day 2). For temperature monitoring two thermometry probes were affixed to the skin over the tumor, and one fixed over the brachial plexus. Animals were sacrificed when the tumor volume reached ten times
the initial volume. We investigated four groups: control with neither implant nor magnetic field (n = 6), implanted control (no magnetic field) (n = 7), 10.5 mT treated (n = 7), 12 mT treated (n = 11). Animals were assigned to different treatment and control groups in order to obtain similar mean tumor sizes: respective mean initial tumor sizes and standard deviations were 59 (± 54) mm$^3$ for the control group, 52 (± 44) mm$^3$ for the implanted control group, 53 (± 33) mm$^3$ for the 10.5-mT treated group and 64 (± 42) mm$^3$ for the 12-mT treated group. In a multigroup comparison, tumor size in the different groups was indeed very similar (Friedman test: p > 0.8).

Histology

Tumor and part of the surrounding tissues (overlying skin and adjacent muscle fascia or peritoneum) were fixed in buffered neutral formalin (1:10). Slices of 3 mm in thickness were embedded in paraffin (through alcohol dehydration and xylol clearing). Sections of 5 μm in thickness were stained with hematoxylin and eosin. The ratio of necrotic tumor to whole tumor volume was semi-quantitatively scored from 0 to 100 %. Microphotography was performed with an Olympus BX40 microscope (Olympus Corp., Center Valley, USA).

Imaging

For magnetic resonance imaging, we used a MRI scanner (Achieva 1.5T, Philips, Eindhoven, The Netherlands). For micro-computerized tomography, we used a Micro-CT scanner (Skyscan 1076, Kontich, Belgium)

Statistics

StatView version 5.0 (SAS Institute Inc.©) software was used for statistical analysis. Statistical significance was considered at p < 0.05. For the thermometry study, the Kruskall-Wallis test, the Mann-Whitney U test and the Spearman correlation test were used. For the survival studies, the Friedman test and Kaplan Meyer analysis were used.
Intra-tumoral precipitation of injectable implant fills necrotic core and peripheral extensions

Injection of the formulation was accompanied by mild acute toxicity[19,20]. The injected 0.25-ml volume in a 25 g mouse would translate to 700 ml in a 70 kg patient. Systemic manifestations, most probably due to DMSO, consisted of the observation of transient fatigue and ocular irritation with eyelid ptosis. Locally, we observed edema which was rapidly reversed along with solvent clearance.

As previously observed in other experiments, Co122 tumors showed extensive central necrosis (up to 50%)[21]. Similar necrotic centers have been observed in Co112 multicellular spheroids grown in vitro[16] or in liver metastases[17]. At electron microscopy level, the Co112 tumor spheroids developed junctional complexes and desmosomes, while oxygen measurements had shown severe central hypoxia[22].Histology confirmed that the implant was invariably present in the necrotic tumor core and extended towards the viable peripheral rim of cells. Occasionally, an implant extension had leaked into peritumoral loose connective tissue, especially in the case of small and mostly vital tumors. However, distant leakage was rarely observed because of the existence of a peritumoral stromal pseudocapsule confining the implant to the site of the neoplasm.

The implant formed in situ can heat the tumor by magnetic induction at 141 kHz on the whole temperature range of moderate hyperthermia as function of field strength

Thermograms had a similar shape (Fig. 1 A). After a steep increase over the first 5 min, temperatures reached a plateau corresponding to equilibrium between implant heat production and dissipation through diffusion and convection. The observed plateau temperature was not due to a diminishing response capacity of the superparamagnetic beads, since stepwise field increase in a separate experiment produced stepwise increasing temperatures throughout the 25-min exposure (Fig. 1 B). In order to have a parametric value of this equilibrium, we defined the Equilibrium Temperature (E.T.) as the averaged temperature over the last 15 min of magnetic field application. Table 1 shows that the mean tumor site E.T. increased with magnetic field strength (Spearman $\rho = 0.724; p < 0.001$). We observed the lowest mean tumor site E.T. (40.0 °C) for the group treated with the lowest magnetic field strength, i.e. 9 mT (Table I). Treatments with intermediate magnetic field strengths of 10 to 11 mT led to
intermediate mean values of tumor site E.T. between 42.7 to 43.5 °C that were not statistically different. For the group treated with 12-mT magnetic field strength, the mean tumor site E.T. of 47.8°C observed was significantly larger than for all other magnetic field strengths.

The area under curve (AUC) of temperature as a function of time is a parameter related to the heat dose delivered during a given treatment[23,24]. For the group treated with the highest magnetic field strength (12 mT), we obtained a more than two-fold increase in mean AUC (282.5 °C·min) as compared to the group treated with 9 mT (131.1 °C·min). Mean AUC for intermediate magnetic field strengths of 10 to 11 mT were again intermediate (Table 1). When considering the whole range of investigated magnetic field strengths, AUC was positively correlated with the 5 different magnetic field strengths applied (Spearman ρ = 0.718; p = 0.001).

Above threshold temperature, increasing the heat dose delivered magnetically increases the extent of induced necrosis to whole tumor for higher magnetic field strengths

The necrosis to tissue ratio, as observed by histology, was not significantly different between injected and non-injected controls, suggesting that the implant does not induce necrosis. We then assessed the heating efficiency microscopically in terms of coagulation necrosis of vital tumor and adjacent tissues. In the case of low heat delivery, it appeared that the extent of spontaneous and heat-induced necrosis overlapped. However, between moderately and highly heated tumors the extent of the necrosis was quite different. Fig. 2 shows heat-induced damage patterns in two tumors treated at magnetic field strengths of 10 and 12 mT, respectively. For treatments reaching more than 44 °C, the observed extensive tumor necrosis was associated with coagulation necrosis of immediately adjacent connective and muscle tissues attributable to heat, since this was absent in mice injected with implant alone or at lower heat induction. Likewise, the skin, especially over the implanted tumor, showed heat-induced necrosis. In some animals, muscle tissue at the peritoneal side of the tumor showed signs of thermal damage. For treatments reaching less than 42 °C, no significant necrosis attributable to heat was observed. Heat-induced necrosis was notably not observed in implanted mice exposed to the magnetic field strength of 9 mT and was only occasionally found in mice exposed to 10 or 10.5 mT, clearly depending on developed heat. It was found that AUC was well correlated with heating efficiency at tissue level, quantified as the percentage of tumor necrosis (Spearman ρ = 0.445; p < 0.01).
Heat delivery through magnetically-induced heating of implant is highly efficient in treating solid tumor for a magnetic field strength of 12 mT

In groups of animals matched for tumor size, magnetically induced heating of the implant prolonged survival time as defined by reaching 10 times initial tumor size. After a single 20-min treatment, a median survival time of 27 days was observed for the group treated with a 10.5-mT magnetic field strength (Fig 3). Median survival time increased further to 37 days for mice treated with 12 mT, as compared with 12 days for non-implanted controls and 21 days for implanted controls. Kaplan-Meyer analysis revealed significant differences between the 10.5-mT treated group and the non-implanted control group (p < 0.05), while the 12-mT treated group was significantly different when compared to the implanted and non-implanted controls (p<0.05 and p<0.01, respectively). Finally, while at 10.5 mT one complete response was observed, this was the case for 5 of 11 animals (45 %) treated with 12 mT and complete responses persisted at one year when mice were sacrificed.

Computerized Tomography allows for precise implant imaging

We studied implant precipitation pattern in vivo through Magnetic Resonance Imaging (MRI) and Computerized Tomography (CT) of two implanted and non-treated mice. In MRI, SPIONS led to a susceptibility artifact, masking partly the implanted tumor (fig. 4A). We confirmed entrapment of the beads at the injection site through absence of distant artifacts. In contrast, micro-computerized tomography allowed detailed imaging of the implant, confirming intra-tumor precipitation pattern of the implant (fig. 4B). Due to the high density of the implant, this first CT imaging experiment is shown in the bone density window, impeding soft tissue visualization.

Discussion

Our results show the feasibility of an injected formulation of superparamagnetic beads that solidifies upon contact with interstitial fluid. The resulting solid implant provides a device responding to alternating magnetic field exposure with biologically active heat delivery.

A gradual injection gave enough time for the formulation to spread into the tumor before subsequent precipitation and solid implant formation in situ. Most tumors in our model based
on the Co112 cell line had a necrotic center that was easily filled up in a first step. Then, precipitation extended towards peripheral tumor spaces. This last step was critical with regard to eventual occurrence of leakage. The central implant formed was particularly appropriate for magnetically induced heating of the tumor.

The magnetic field used did not induce any direct measurable effect in mice without implant and would be entirely compatible for human application according to current knowledge. The investigated 9- to 12-mT field strengths induced a tissue temperature rise from mild hyperthermia in the range of 39 – 42 °C up to cytotoxic moderate hyperthermia in the range of 42 – 48°C. Treatments at higher temperatures were associated with extensive tumor necrosis and collateral damage mainly to adjacent skin. It is worthwhile mentioning that the tissue origin is of importance: mouse tissues are more sensitive to heat than the human tissues used here in form of the human tumor transplant.

Under alternating magnetic induction, the measured skin temperatures were generally inferior to intra-tumor temperatures, as long as heating remained at non-toxic levels. However, on reaching toxic heat levels, skin temperature rose to intra-tumoral levels (see Table 1). These observations underline the importance of cooling by tissue perfusion[25,26]. When skin blood perfusion breaks down as a consequence of heat damage to vasculature, the cooling effect of blood perfusion stops and skin temperature raises to a value close to the tumor temperature.

Around larger extensions of confluent implant aggregates, necrosis was wider than around thin implant extensions. It is likely that the latter were delivering lesser heat to surrounding tissues[27]. Certainly, this could result from a combination of different factors: first, a threshold mass for dissipating significant heat[28] from implant center to distant tissue areas, and, secondly, a differential effectiveness of vascular cooling[29]. Indeed, since cooling efficiency is directly related to the contact area between implant and tissue, whereas heating power is proportional to implant mass, small implants that present a higher surface/mass ratio will be more efficiently cooled. Measurement of necrosis rim width from biological relevant implants and in vitro or in vivo thermal mapping studies indicated a necrosis extent in the order of 2-3 mm, compatible with previously published observations[30].

The survival study revealed an important therapeutic potential for a single 20-min treatment based on the sole cytotoxicity of hyperthermia. We observed a minor growth delay in the group implanted but not submitted to magnetic field induction heating. We could not exclude local toxicity of DMSO but this growth delay is more likely due to implant precipitation in capillaries and secondary hemostasis, leading to antineoplastic effects through hypoxia.
Magnetically induced hyperthermia treatment, however, did increase significantly the median survival time. Some definitive complete responses were observed with a rate that depended on the magnetic field amplitude. It is well known that temperature distribution in tumor is essential in treatment response analysis[31]. Its generation is directly related to magnetic field strength and implant localization, and its control depends on thermometry. The suboptimal heating of peripheral tumor cells allows for tumor relapse when tissue escapes the cytotoxic area of the temperature gradient. In comparison to the 10.5-mT single 20-min treatment, these considerations corroborate the better outcome by increasing the magnetic field strength to 12 mT.

Our approach might ultimately benefit from imaging in order to be able to control implant injection directly and, if necessary, the exact localization of a thermometry probe. Clinically, imaging could allow a control of the correct implant size and localization to further improve heat delivery. While MRI appeared most sensitive to the presence of the superparamagnetic beads, leading locally to some artifacts, CT appeared most appropriate for implant imaging without any obvious artifacts in its vicinity.

In conclusion, these results demonstrate the efficiency of superparamagnetic particles embedded in the in situ forming implant to deliver hyperthermia in a therapeutically relevant range[32]. In a necrotizing tumor model, sustained moderate hyperthermia could be generated at clinically relevant field strengths, revealed a high potential for hyperthermia therapy.

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References


Figure 1. Thermograms representing the tumor temperature as a function of time. **A**, during single 20-min treatments in a 141 kHz alternating magnetic field, for magnetic field strengths of 9 mT (n = 5), 10 mT (n = 4), 10.5 mT (n = 5), 11 mT (n = 3), 12 mT (n = 5). **B**, during two stepwise 25-min treatments in an 141-kHz alternating magnetic field. Magnetic field strength was 9 mT from 0 to 10 min, 10 mT from 10 to 15 min, 11 mT from 15 to 20 min, 12 mT from 20 to 25 min.
Figure 2. Histology (HE) following a 20-min hyperthermic treatment at 10mT (left: A and C) and 12 mT (right: B and C) for which were reported equilibrium temperatures of 41.5 and 46.2°C, respectively. Upper pictures at 20 X magnification (A and C) show the implanted tumor in contact with the external stromal pseudocapsule (PC) and the skin (S), illustrating the extent of tissue damage. Thanks to the brownish color of SPION our implant (I) is easily identifiable. Lower pictures (C and D) at 200 X magnification detail the cellular toxicity. Contrasting with the tumor treated at 10 mT, the tumor treated at 12 mT displays a large area of necrotic tumoral tissue (NT), easily distinguishable from viable tumor tissue (VT).
Figure 3. Survival curves. Dotted line: control group (C), n = 6; dashed line: implanted control group (IC), n = 7; normal line: 10.5-mT treated group, n = 7; bold line 12-mT treated group, n = 11. Note that in the group treated with a 12-mT alternating magnetic field, 5 of 11 mice survived 12 months after treatment without tumor relapse.
Figure 4. Implant imaging. **A**, MRI imaging of Swiss nude mouse bearing a subcutaneous tumor injected with 0.25 ml of the implant formulation, with a T1 weighted sequence. Tumor zone is enclosed in the dotted white circle, highlighting the susceptibility artefact caused by SPION entrapped in the implant. **B**, Micro-computerized tomography of Swiss nude mouse bearing a subcutaneous tumor punctured with 0.25 ml of the implant formulation. In transversal section, we can precisely localize the implant (in white) and the tumor enclosed within the dotted black circle. The SPIONs entrapped in the implant allowed for X ray absorption with an absorption density close to bone density (see for instance spinal vertebrae and iliac wing enclosed in the dotted white circle). Note that the window is adjusted for bone density without further soft tissue contrast refinements or use of contrast agent.