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Magnetic core mesoporous silica nanoparticles doped with dacarbazine and labelled with 99mTc for early and differential detection of metastatic melanoma by single photon emission computed tomography

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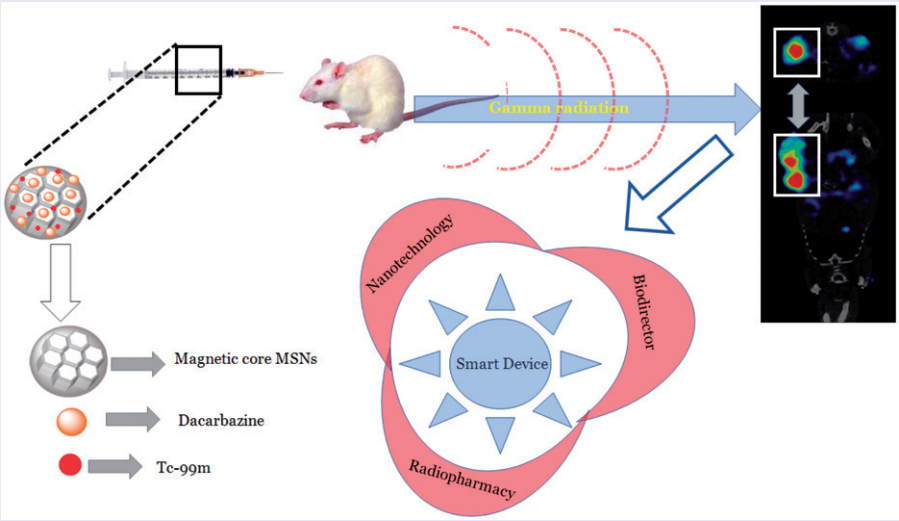
ABSTRACT

Cancer is responsible for more than 12% of all causes of death in the world, with an annual death rate of more than 7 million people. In this scenario melanoma is one of the most aggressive ones with serious limitation in early detection and therapy. In this direction we developed, characterized and tested *in vivo* a new drug delivery system based on magnetic core-mesoporous silica nanoparticle that has been doped with dacarbazine and labelled with technetium 99 m to be used as nano-imaging agent (nanoradiopharmaceutical) for early and differential diagnosis and melanoma by single photon emission computed tomography. The results demonstrated the ability of the magnetic core-mesoporous silica to be efficiently (>98%) doped with dacarbazine and also efficiently labelled with 99mTc (technetium 99 m) (>99%). The *in vivo* test, using inducted mice with melanoma, demonstrated the EPR effect of the magnetic core-mesoporous silica nanoparticles doped with dacarbazine and labelled with technetium 99 metastable when injected intratumorally and the possibility to be used as systemic injection too. In both cases, magnetic core-mesoporous silica nanoparticles doped with dacarbazine and labelled with technetium 99 metastable showed to be a reliable and efficient nano-imaging agent for melanoma.



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Introduction

Cancer is one of the most devastating diseases in the world and comes from time to time becoming a global epidemic problem. The absence of effective treatment for most of tumours, especially inoperable metastatic ones, raises the risk classification [1,2]. Cancer is a lethal disease, characterized by the development of cellular abnormalities, causing division, rapid and uncontrolled growth due to the combination of mutagenic stages [1–5]. Is responsible for more than 12% of all causes of death in the world, with an annual death rate of more than 7 million people [6,7].

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Among the types of cancer, skin cancers are the most common in countries with high ultraviolet (UV) incidence and represent an important part of the global number of diagnosed cases of cancer. The early detection of this type of cancer is still inefficient. Although with a low lethality, in some cases, due delayed diagnosis, this cancer can lead to severe ulcerations and physical deformities. Nonetheless, is most aggressive types, like metastatic melanoma (MM) this delay may lead to serious metastasis process followed by death [1,8,9]. The estimated number of new cases of cutaneous melanoma in 2016 was estimated to be in the range of 5–10% of all cases of skin cancer in the United States. The estimated number of new cases of cutaneous melanoma in 2016 was 76,380, representing 4.5% of all new cases of cancer in the world [7,10,11].

There are few therapeutic alternatives for the treatment of cutaneous melanoma and the only drugs approved by the Food and Drug Administration (FDA) are Dacarbazine (DTIC) and high doses of interleukin-2 (HIL-2), both with low response rates: between 10 and 20%. None of the agents used for the treatment of melanoma have demonstrated an increase in overall survival, increasing the necessity for new agents for both: diagnosing and therapy [4,12–14].

In order to overcome all the limitations regarding the imaging exam for melanoma, with special attention for early detection, we developed and tested mesoporous silica nanoparticle with magnetic core doped with dacarbazine and radiolabeled with 99mTc to be used as a nanoradiopharmaceutical for single photon emission tomography coupled with computed tomography.

Materials and methods

Preparation of magnetic core-mesoporous silica nanoparticles

Reagents and materials

Iron (III) chloride hexahydrate, iron (II) tetrachloride hexahydrate, oleic acid, hexadecyltrimethylammonium bromide (CTAB) and tetraethyl orthosilicate (TEOS) were purchased from Sigma (St. Louis, MO). Ammonia solution (32%), ethanol and ethyl acetate were purchased from Scharlau (Barcelona, Spain). Chloroform was obtained from Acros Organics (Geel, Belgium). Distilled water was used in all reactions.

Synthesis of oleate-coated iron oxide nanoparticles

Iron oxide nanoparticles (Fe_3O_4 magnetite nanocrystals) were obtained by a modified coprecipitation method [1]. Briefly, 12 g of iron (III) chloride hexahydrate was mixed with 4.9 g of iron (II) chloride tetrahydrate in 50 ml of water at 80 °C under a flow of argon and mechanical stirring. Ammonia solution 32% (19.53 ml) was carefully added and the mixture turned completely dark. Oleic acid (2.13 ml) was added after 30 min and the reaction was left stirring at 80 °C for another 90 min. The reaction was cooled down and centrifuged at 9500 rpm for 10 min. The resulting black precipitate was washed three times with distilled water and three times with ethanol and then dried under vacuum overnight. In order to prevent their oxidation, the oleate-coated iron oxide nanoparticles were kept in chloroform giving a dark brown ferrofluid.

Synthesis of magnetic core-mesoporous silica

In a typical procedure, 100 mg of CTAB was dissolved in 10 ml of water, followed by addition of 0.74 ml of the ferrofluid (8.88 mg/ml). The mixture was placed in a probe sonicator (Branson 450 Sonifier, Thermo Fisher Scientific Co., Waltham, MA) for 2 min, giving an oil-in-water emulsion. Then, the mixture was heated to 65 °C to evaporate the chloroform and achieve an effective phase transfer from chloroform to water. The resulting transparent aqueous suspension was added to a solution of 30 ml of water and 0.548 ml of ammonia (32%), which was then, heated up to 75 °C. Then, 0.5 ml of TEOS was added dropwise followed by addition of 3 ml of ethyl acetate. The reaction was stirred at 350 rpm and 75 °C during 3 h. Then, the reaction was placed on an ice bath and the nanoparticles were collected by centrifugation (9500 rpm, 10 min). Afterward, the sample was washed with ethanol twice and dried under vacuum overnight. The final magnetic core-mesoporous silica was calcined in air at 550 °C for 5 h.

Characterization of magnetic core-mesoporous silica

Powder X-ray diffraction

The synthesized materials were characterized by powder X-ray diffraction (PXRD), transmission electron microscopy (TEM) and N_2 adsorption-desorption analysis. PXRD measurements were obtained using a Bruker AXS D8 advance diffractometer (Bruker, Billerica, MA) equipped with CuKα radiation and working at 40 kV/40 mA. PXRD measurements were performed at high angle ($2\theta = 15°–68°$) and low angle range ($2\theta = 1.3°–8.3°$).

Transmission electron microscopy

TEM images were taken on a 100 kV JEOL JEM-1010 microscope operated with AMT image capture engine software. TEM samples were prepared by adding 10 µl of nanoparticles suspended in distilled water onto carbon-coated copper grids. The statistical analysis of the data obtained from TEM images was performed using Origin Pro software (OriginLab, Northampton, MA).

N_2 adsorption-desorption

N_2 adsorption-desorption measurements were conducted in a TriStar II Plus surface area and porosity analyser from Micromeritics (Norcross, GA). The specific surface area of the material was determined from the adsorption-desorption isotherm by applying the BET model. The pore volume and average pore size was estimated by using the BJH model.

Magnetic properties from the magnetic core-mesoporous silica

Magnetization measurements were performed on a superconducting quantum interference device (SQUID) magnetometer

at 298 K. All the measurements were performed at room temperature

Doping magnetic core-mesoporous silica nanoparticles with dacarbazine

Magnetic core mesoporous silica nanoparticles (magnetic core-mesoporous silica) were loaded with dacarbazine. In order to get the loaded magnetic core-mesoporous silica nanoparticles, 300 μg (300 μl of dacarbazine solution) of Fauldacar® (Libbs, Sao Paulo, Brazil) (dacarbazine) was stirred (24 h) at room temperature with 100 μg of magnetic mesoporous silica. After this period the magnetic mesoporous silica soaked with dacarbazine solution was dried under low pressure at 28 °C temperature for 12 h, until completely dry. The dried powder containing solely magnetic mesoporous silica doped with dacarbazine were individualized and set aside for later labelling with 99mTc.

The calculation to establish the amount of dacarbazine necessary to complete dope the magnetic mesoporous silica was done considering the size of the magnetic mesoporous silica (100 nm) applied to the circumference volume (Cv) equation:

$$Cv = \pi r^3$$

Where Cv: circumference volume; π: constant pi; r: radius of the circumference.

In this case, we have that the weight of one single magnetic core-mesoporous silica nanoparticles is about: 1×10^{-15} g. Thus, in 100 μg of magnetic core-mesoporous silica nanoparticles, we have approximately 10^{12-13} nanoparticles with a surface area of approximately 872 m^2/g. This means that using an excess of dacarbazine (300 μg) that has a molar mass of 182.18 g mol^{-1} we kept a ratio of 3:1dacarbazine/magnetic core-mesoporous silica nanoparticles.

Labelling magnetic core-mesoporous silica doped with dacarbazine with 99mTc

The labelling process was done by the direct radiolabeling process [15–18]. In this methodology, we used 150 μg of magnetic mesoporous silica doped with dacarbazine. Briefly, 100 μCi (approximately 300 μl) of 99mTc has incubated with a stannous chloride ($SnCl_2$) solutions (80 μg/ml) (Sigma-Aldrich, St. Louis, MO) for 20 min at room temperature. Then this solution was incubated with 150 μg magnetic mesoporous silica doped with dacarbazine for another 10 min in order to label their structures.

Quality control of the labelling process with 99mTc

In order to confirm the efficacy of the magnetic mesoporous silica doped with dacarbazine labelling process, paper chromatography was done using Whatman paper n° 1 using 2 μl of the labelled-nanoparticle and acetone (Sigma-Aldrich, St. Louis, MO) as mobile phase. The radioactivities of the strips were verified in a γ-counter (Perkin Elmer Wizard® 2470, Shelton, CT).

Entrapment efficacy – 99mTc EE%

Due limitations to perform the entrapment efficacy of the dacarbazine into the magnetic core-mesoporous silica nanoparticle we performed the 99mTc entrapment efficacy. We decided to use this indirect method for the calculation of EE, based on the quality result from the labelling process with the dacarbazine. In this direction, we previously labelled the dacarbazine with 99mTc and then, doped the magnetic core-mesoporous silica nanoparticles as described in the Section Doping magnetic core-mesoporous silica nanoparticles with dacarbazine.

Doping stability of the dacarbazine into magnetic core-mesoporous silica

In order to evaluate the stability of the doping process using our methodology, we dispersed about 50 μg of the magnetic mesoporous silica doped with previously labelled dacarbazine (99mTc labelled dacarbazine) into 1 ml of albumin solution (20% solution of human albumin Grifols®, Barcelona, Spain). After 10 min of contact with the magnetic mesoporous silica doped with 99mTc dacarbazine, the human albumin solution (20%) was centrifuged (10,000 rpm for 10 min) and the supernatant and the precipitate was collected and the radioactive counted on a gamma counter (Perkin Elmer Wizard® 2470, Shelton, CT).

Cytotoxicity assay

MV3 human melanoma cells, obtained from Dr. C. Marcienkewicz (Temple University Center for Neurovirology and Cancer Biology, Philadelphia, PA), were cultured in Dulbecco's Modified Eagle's Medium (DMEM), enriched with 10% FBS (foetal bovine serum), 3.7 g/l sodium bicarbonate, 5.2 g/l HEPES (4–(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 0.5 U/ml penicillin and 0.5 mg/ml streptomycin at 37 °C/ 5% CO_2. After reaching confluence, cells were detached by a brief treatment with 0.1/0.01% trypsin/ ethylenediaminetetraacetic acid (EDTA), collected by centrifugation, resuspended in fresh 10% FBS medium DMEM and cultured (104 cells/well) on 96-well flat plates, overnight. After that, cells were treated with magnetic core-mesoporous silica doped with dacarbazine (0.1–10 μg/ml) in fresh 1% FBS medium DMEM, at 37 °C in humidified 5% CO_2. After 72 h, MTT (3–(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed as previously described by Rosa et al. [19]. Briefly, cells were incubated with MTT (1 mg/ml) in 1% FBS DMEM, in the dark at 37 °C, for 2 h, allowing MTT be reduced to formazan crystals by viable cells. The formazan crystals formed were dissolved in isopropanol for 30 min and the optical densitometry obtained using a microplate reader (BIO-RAD, Hercules, CA) with 570 nm filter. For calculation, a standard curve was built using increasing concentrations of adhered MV3 cells (103–5 × 104 cells/well) cultured overnight at 37 °C in 5% CO_2 atmosphere, to perform. The MTT assay as described. Results are shown as percentage of control, of two independent experiments performed in triplicate.

In vivo *analysis*

Animals

Animals were treated in accordance with protocols approved by the Institutional Animal Care and Use Internal Review Board of the Institute of Energy and Nuclear Research under the number 181/2017. Balb/c mice with free access to water and food were included in this study, after reaching maturity at 8 weeks of age.

Tumour xenograft models

SK-MEL-37 cells (Memorial Sloan-Kettering Cancer Center, New York, NY) were cultured in RPMI (Gibco, Life Technologies, Gaithersburg, MD) supplemented with 10% of foetal bovine serum (Gibco, Life Technologies, Gaithersburg, MD) and 50 μg/ml of gentamicin (Gibco, Life Technologies, Gaithersburg, MD). Mycoplasma contamination in cultured cells was excluded using Lonza Mycoplasma Detection Kit (Lonza, Basel, Switzerland).

Tumours were established by subcutaneous (sc) injection of 1×10^5 SK-MEL-37 cells at the right flank of eight-week-old female Balb/c nude mice. Tumour size was monitored for 3 weeks and measured by a calliper. Mice were observed three times per week for evidence of distress, ascites, paralysis or excessive weight loss.

Micro single photon emission computed tomography imaging

Approximately 4 weeks after inoculation, tumours had reached a volume of 0.3–0.6 cm^3. On the day of the experiment, 37 MBq of magnetic core-mesoporous silica doped with dacarbazine-99mTc was injected by two via: a) in the lateral tail vein and b) intratumorally. After 2 h post-injection imaging experiments were conducted on an Albira micro-PET/SPECT/CT imaging system (Bruker Biospin Corporation, Woodbridge, CT). MicroSPECT/CT images were acquired under general anaesthesia (isoflurane/O_2) and heating at 37 °C. The single photon emission computed tomography (SPECT) data for each mouse was recorded via static scans, (a 40 min SPECT scan (FOV 80 mm), followed by a 10 min CT scan (FOV 80 mm, 35 kV, 400 μA)). The microSPECT/CT scans were reconstructed with Albira software (Bruker Biospin Corporation, Woodbridge, CT) with ordered subsets expectation maximization (OSEM) and filtered back projection (FBP) algorithms, for SPECT and CT, respectively, and, images were processed with the PMOD software (PMOD Technologies, Zürich, Switzerland).

Statistical analysis

Statistical analyses were performed using Origin Pro version 8 (OriginLab, Northampton, MA) software. Results are shown as means ± standard deviation (SD). *p* Values less than .05 were considered significant.

Results and discussion

Characterization of magnetic core-mesoporous silica

The structure periodicity of the mesoporous material was confirmed by PXRD, which showed a sharp peak at the low-angle region for both as-made (S0–1) and calcined (S0–2) magnetic core-mesoporous silica (Figure 1). The slight shift of the peak to higher angles indicates shrinkage of the silica matrix due to the condensation of silanol groups during the calcination process. PXRD analysis at high angles confirmed

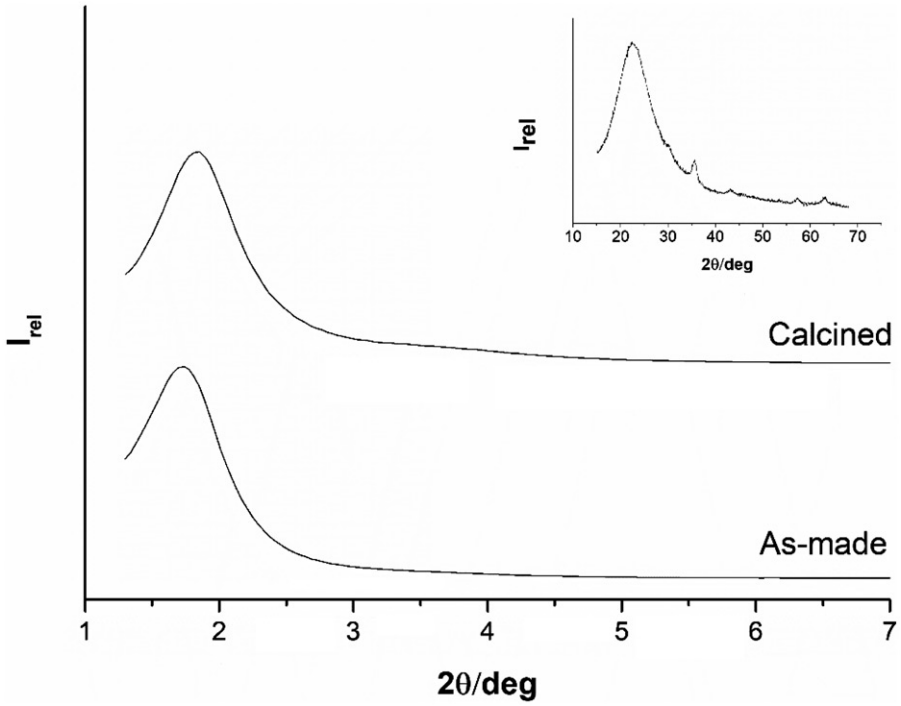

Figure 1. Powder X-ray diffraction patterns of (bottom) as-made magnetic core-mesoporous silica (S0–1) and calcined magnetic core-mesoporous silica (S0–2). Inset shows the peaks corresponding to magnetite nanocrystals and the characteristic broad peak of amorphous silica.

the presence of magnetic cores within the structure (see inset in Figure 1).

The mesoporous structure of S0–2 magnetic core-mesoporous silica was also analysed by TEM and the size of the primary nanoparticles was determined by image analysis (58.9 ± 8.1 nm, $n = 100$). The data was represented in a histogram, which shows the particle size distribution of the S0–2 nanoparticles (Figure 2).

The N_2 adsorption-desorption isotherms of the magnetic core-mesoporous silica presented a typical type IV behaviour (Figure 3), characteristic of mesoporous materials. From the isotherm curve, a specific surface area of 872 m^2/g was estimated along with a pore volume of 0.85 cm^3/g and an average pore diameter of 3.15 nm.

The magnetization properties of the developed nanoparticles were determined using an SQUID magnetometer. The magnetic curves of the material (Figure 4) show the superparamagnetic behaviour produced by the Fe_3O_4 nanocrystals in the core of the nanoparticles. The obtained saturation magnetization value for the final S0–2 nanoparticles (1.6 emu/g) is consistent with those reported for similar magnetic core MSNs.

Labelling magnetic core-mesoporous silica doped with dacarbazine with 99mTc

The magnetic core-mesoporous silica nanoparticles doped with dacarbazine were successfully labelled (>98%) with 99mTc generating a clear suspension with visible precipitate, that passed through 200 nm filter.

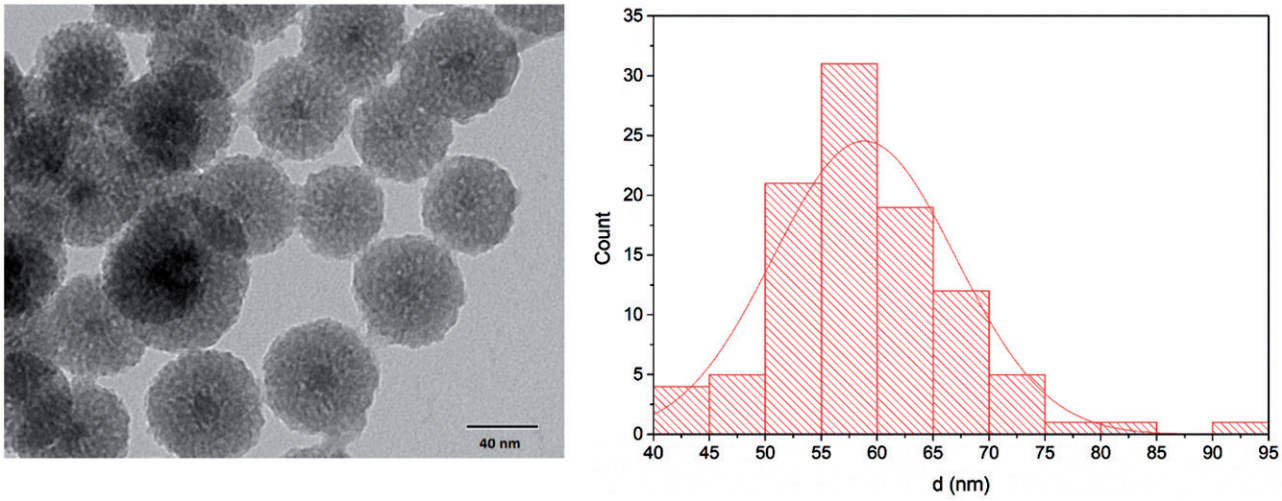

Figure 2. Left: TEM images of calcined S0–2 nanoparticles. Right: Size histogram and normal size distribution of calcined S0–2 nanoparticles.

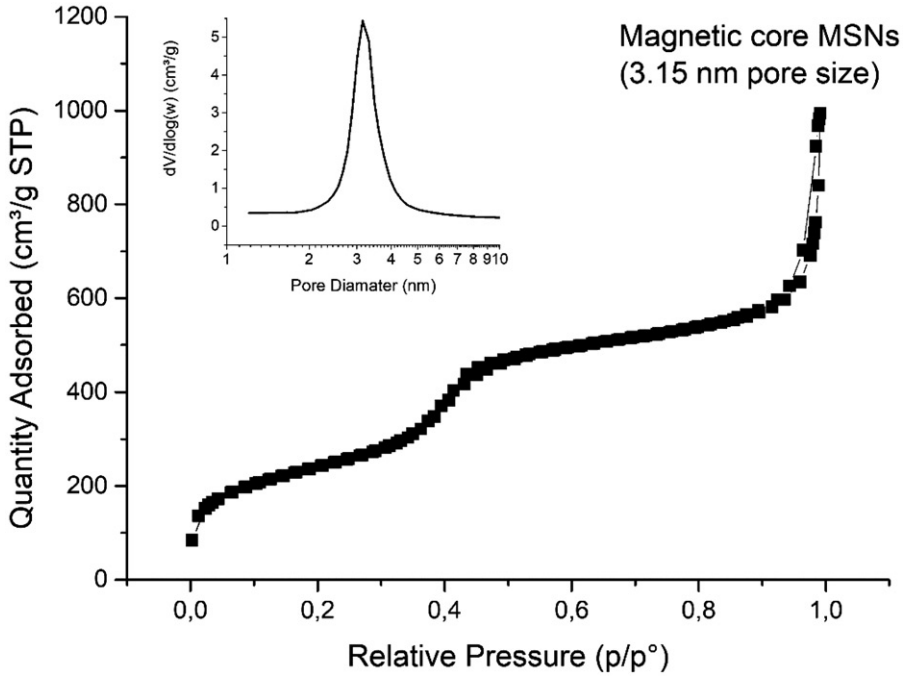

Figure 3. N_2 adsorption-desorption isotherm of calcined S0–2 nanoparticles. Inset shows the pore size distribution of the material.

Quality control of the labelling process with 99mTc

The effectiveness of the labelling process was confirmed by paper chromatography which indicated almost no significant dissociation of technetium-99 m from the magnetic core-mesoporous silica nanoparticles doped with dacarbazine for a period as long as 8 h, as shown in Table 1.

Entrapment efficacy – 99mTc EE%

In order to calculate the amount of 99mTc and, in an indirect way, determine the amount of dacarbazine that has been absorbed/doped into the magnetic mesoporous silica we calculated the entrapment efficacy (EE%) of 99mTc-dacarbazine into the magnetic mesoporous silica, using the formula:

$$99\text{mTc EE\%} = \frac{\text{Total amount of 99mTc used} - \text{Total amount of 99mTc in the supernatant}}{\text{Total amount of 99mTc used}} \times 100$$

The result found for the 99mTc EE% was 98.3% ± 0.7. This result confirmed that almost all the 99mTc used (labelled with dacarbazine) was trapped in the surface of the mesoporous silica.

The quality control of the solely dacarbazine labelling process with 99mTc showed that over 98% of the 99mTc remains connected with dacarbazine for a period as long as 8 h (as shown in Table 2), corroborating our decision to use this indirect way to calculate the amount of dacarbazine.

Doping stability of the dacarbazine into magnetic core-mesoporous silica nanoparticles

The values found in the supernatant, containing exclusively the human albumin solution 20% was 1.7% of the dose of the 99mTc used to label the Dacarbazine. This confirms that 98.3% of the dose remained in the precipitated; corroborating that dacarbazine doping process is stable and viable, under mimetic biological conditions.

Cytotoxicity assay

The MTT test (Figure 5) demonstrated the inability of the magnetic core-mesoporous silica doped with dacarbazine to kill melanoma cells. This result is desired since the amount of dacarbazine used to dope the magnetic core-mesoporous silica was 10% of the total amount necessary to develop a therapeutic effect. Thus, this result confirmed the safety aspect of the drug delivery system. Otherwise, the main goal

Table 1. Percentage of labelled magnetic core-mesoporous silica nanoparticles doped with dacarbazine observed over time, after ascending chromatograms of 99mTc compared with free pertechnetate ($Na99mTcO_4^-$).

Time (h)	Labelling (%) magnetic core-mesoporous silica doped with dacarbazine
0	99.8 ± 1.2%
1	99.1 ± 0.6%
2	99.3 ± 0.9%
4	98.5 ± 0.4%
8	98.1 ± 0.8%

Table 2. Percentage of labelled dacarbazine observed over time, after ascending chromatograms of 99mTc compared with free pertechnetate ($Na99mTcO_4^-$).

Time (h)	Labelling (%) dacarbazine
0	99.7 ± 0.7%
1	99.5 ± 0.9%
2	98.3 ± 0.9%
4	98.4 ± 1.3%
8	98.0 ± 0.7%

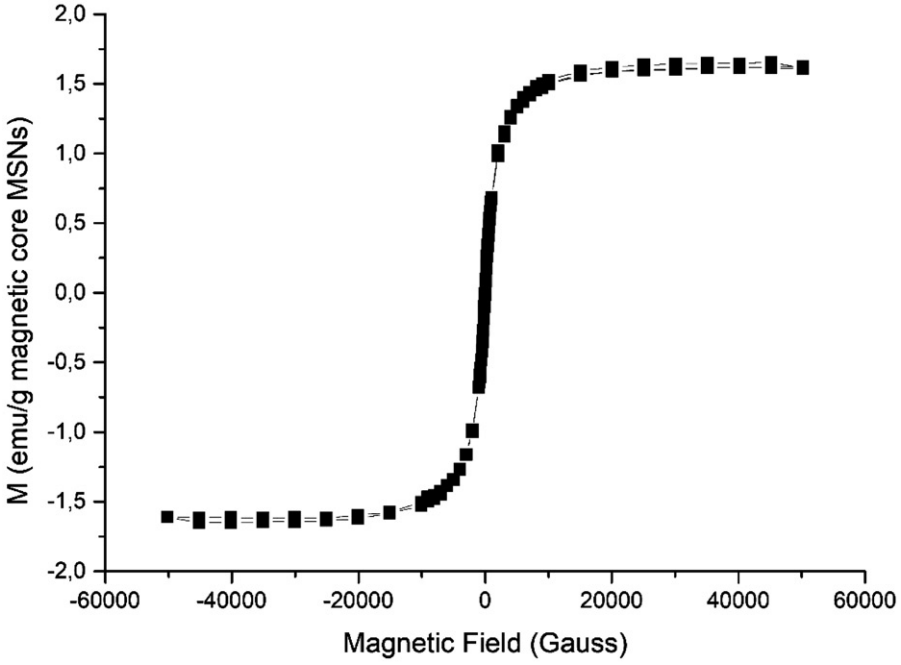

Figure 4. Magnetization curves of calcined S0–2 nanoparticles measured at 298 K.

of this smart device is to be used as imaging agent (nanoradiopharmaceutical). So, no therapeutic action is required.

Studying the Figure 6(A) is possible to observe that even after a 2 h post-injection period is possible to observe the highly accumulation of magnetic core-mesoporous silica doped with dacarbazine and labelled with 99mTc, confirming that in this kind of application the magnetic core-mesoporous silica mesoporous silica doped with dacarbazine and labelled with 99mTc has a long EPR (enhanced permeability and retention) effect, corroborating the possible use of this drug delivery system as an imaging agent for melanoma. The Figure 6(B), showed that even after a 2 h post-injection period the amount of magnetic core-mesoporous silica doped with dacarbazine and labelled with 99mTc was not high enough. This happened due to the high uptake in liver and spleen. We believe that for a systemic injection along post-injection (4 h) period is necessary, at least 4 h of post-injection will be necessary to form an image similar to image 5 A. This extra time is the necessary for the magnetic core-mesoporous silica doped with dacarbazine and labelled with 99mTc overcome the mononuclear phagocyte system.

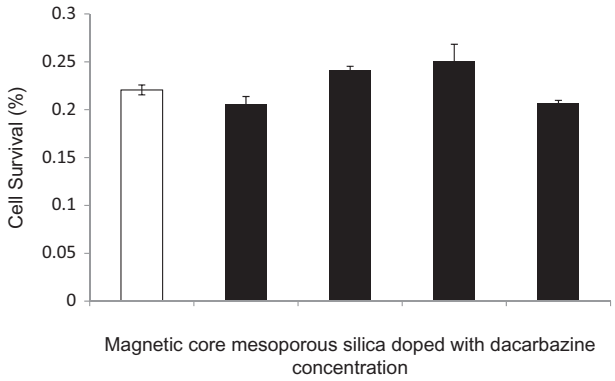

Figure 5. MCF-7 cell survival after treatment for 24 h with different dilutions of magnetic core-mesoporous silica doped with dacarbazine. The first column (white) is the control (just saline). The second column has used a concentration of 10 μg/ml of magnetic core-mesoporous silica doped with dacarbazine, the third column has used the concentration of 5 μg/ml, the fourth column has used the concentration of 2.5 μg/ml and the fifth column was used the concentration of 0.1 μg/ml.

Conclusions

In this study, we developed, characterized and tested *in vivo* magnetic core-mesoporous silica nanoparticles doped with dacarbazine and labelled with 99mTc as imaging agent for melanoma to be used in SPECT. The results suggested that this drug delivery system was able to reach the tumour by both via: systemic and intratumorally. Is important to notice

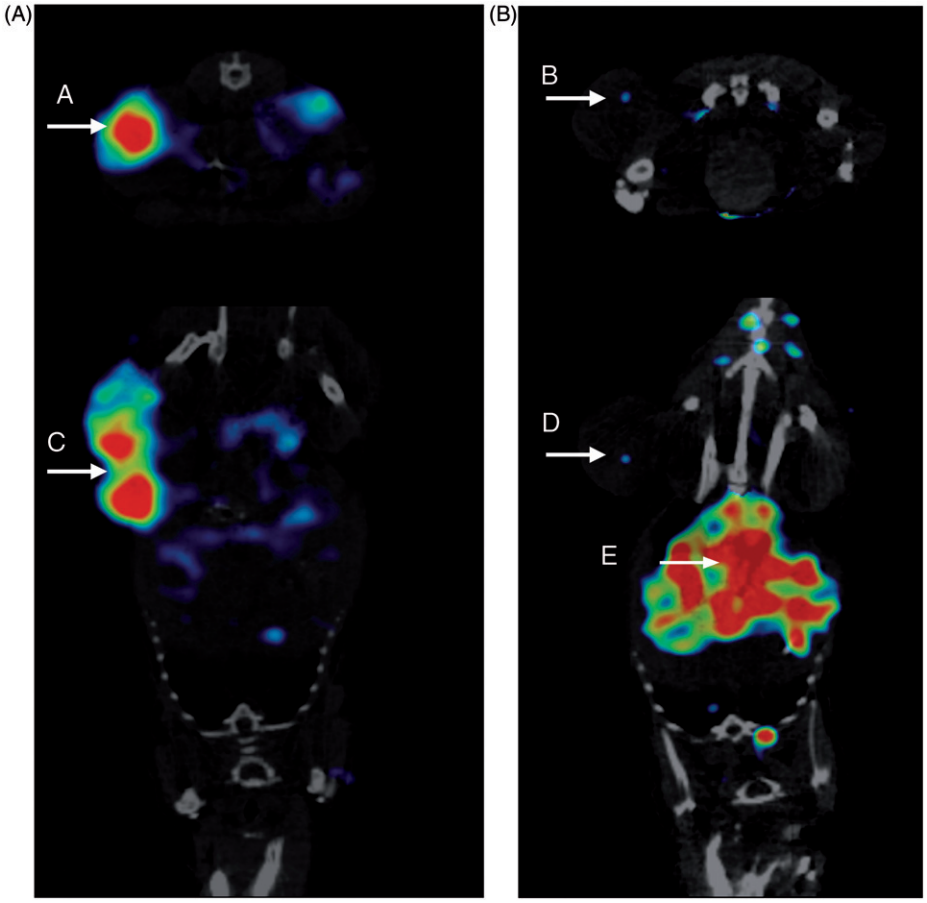

Figure 6. Micro-SPECT/CT imaging of inducted mice with melanoma. In figure (6A) is the intratumoural 2 h post-injection magnetic core MSN doped with dacarbazine and labelled with 99mTc. In the arrow, A and C are possible to observe the high uptake the confirmation of the EPR effect. And (6B) is the tail vein 2 h post-injection magnetic core MSN doped with dacarbazine and labelled with 99mTc. Is possible to observe in arrow B and D the begging of the uptake by the tumour of magnetic core-mesoporous silica doped with dacarbazine and labelled with 99mTc. In arrow E is possible to observe the high accumulation of magnetic core-mesoporous silica doped with dacarbazine and labelled with 99mTc by liver and spleen.

that the intratumoural via corroborated the enhanced permeability retention (EPR) effect of the magnetic core-mesoporous silica doped with dacarbazine and labelled with 99mTc. The systemic via, however, showed that a longer period post injection is desired to reach the tumour.

In both cases, the magnetic core-mesoporous silica doped with dacarbazine and labelled with 99mTc may be used as a SPECT imaging agent for differential and early detection of melanoma. Nonetheless, the presence of a magnetic core in this silica may improve the use of this nanoparticle for MRI (imaging) and hyperthermia (therapy), besides the use proposed in this article. Thus, developing a perfect theranostic agent.

Disclosure statement

No potential conflict of interest was reported by the authors.

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