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(54) Title: METHOD OF MONITORING THE RELEASE FROM LIPOSOMES OF A PRODUCT OF INTEREST USING SUPERPARAMAGNETIC NANOPARTICLES.

(57) Abstract: The present application relates to a method of monitoring the membrane permeabilization of liposome and the incidental release of a compound of interest.



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METHOD OF MONITORING THE RELEASE FROM LIPOSOMES OF A PRODUCT OF INTEREST USING SUPERPARAMAGNETIC NANOPARTICLES.

FIELD OF THE INVENTION

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The present invention is to be used in the health sector, in particular in human health.

The methods and compositions herein disclosed generally relate to employing magnetic resonance techniques to monitor the delivery of a compound of interest *in vivo*. More particularly, the disclosure relates to the use of superparamagnetic nanoparticles the electrostatic surface charge of which is below -20 mV or above + 20 mV when measured in an aqueous medium at physiological pH to monitor the membrane permeabilization of thermosensitive liposomes and the incidental release, to a desired site *in vivo*, of a compound of interest.

15 BACKGROUND

Medical diagnostic imaging has evolved as an important non-invasive tool for the evaluation of pathological and physiological processes. Presently, nuclear magnetic resonance imaging (MRI) is one of the most widely used imaging modalities, demonstrating many advantages over other techniques like computed tomography. The innocuousness of the applied magnetic field typically used for clinical diagnostic results in its safe use. Proton MRI is based on the principle that the concentration and relaxation characteristics of protons in tissues and organs, when submitted to a static magnetic field, can influence the intensity of a magnetic resonance image. As a result, this technique brings a high contrast resolution, which allows for morphological imaging thanks to contrast tissue differentiation due to differences in protons density of the different tissues (depending on fat or water content). A functional imaging of certain organs can also be performed, *i.e.* blood oxygenation, or cerebral areas activation under sensorial stimuli. MRI demonstrates also a high spatial resolution, as cellular imaging is envisaged with strong magnetic field, and no depth limitations. Although MRI can be performed without the administration of contrast agents, the ability of many contrast enhancement agents to enhance the visualization of internal tissues and organs has resulted in its widespread use. Contrast enhancement agents that are useful for proton MRI are responsible for a change in the relaxation characteristics of protons, which can result in image enhancement and improved soft-tissue differentiation.

The use, as a drug delivery vehicle, of thermosensitive liposomes losing their structural integrity within a given temperature range is a promising approach to target a tumor or other tissue, but so far no efficient liposomal MRI contrast agents has been proposed.

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US 2004/0101969 describes a method of monitoring the localisation and distribution of a compound of interest released from an envirosensitive liposome, using a molecular compound, MnSO_4 , as a contrast agent.

10 WO 2008/035985 describes a trackable particulate material for drug delivery comprising a matrix or membrane material, a drug, an internal T1 magnetic resonance metal chelate contrast agent and an external T1 magnetic resonance metal chelate contrast agent, wherein the internal T1 agent is shielded from bulk water and the external T1 agent is exposed to bulk water.

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EP 2067485 describes thermosensitive liposomes for drug delivery which comprise a paramagnetic metal compound. The paramagnetic metal compound may be a metallic nanoparticle. Preferably, the paramagnetic metal compound comprises a chelating structure allowing the metal to interact with water or with another suitable source of protons as
20 chemical exchange-dependent saturation transfer (CEST) contrast agent.

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These documents all refer to contrast agents which are T1 magnetic resonance paramagnetic molecular compounds and offer only a limited sensitivity when measured *in vivo*. As a result, relatively large, and possibly toxic, doses of such contrast agents are to be administered to a given subject. This and other problems are addressed by the compositions and methods herein disclosed.

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WO2008/033031 describes a trackable MRI drug delivery particle comprising two distinct chemical contrast agents, one of them (T1 agent) exhibiting a T1 signal used to monitor drug release, and the other one (T2* agent) exhibiting a T2* signal. This document does not suggest using the T2* agent to monitor drug release.

US2009/004258 describes thermosensitive liposomes encapsulating iron oxide nanoparticles and carbofluoresceine (CF), CF being used as a drug model. Nanoparticles are capable of

generating heat when activated by an alternative magnetic field, thereby allowing permeabilization of the membrane towards CF. In US2009/004258, monitoring of the CF's release is performed by fluorescence detection.

- 5 Inventors now herein provide an advantageous method offering superior sensitivity, relative to the currently available methods. This method in particular uses low doses of non toxic charged superparamagnetic nanoparticles and allows the monitoring of liposomes delivery on a target site and also, surprisingly, the efficient monitoring of the product of interest release from said liposomes.

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SUMMARY OF THE INVENTION

Inventors herein provide a method of monitoring the liposome membrane permeabilization and the incidental release of a product of interest, the liposome comprising a thermosensitive
15 lipidic membrane encapsulating superparamagnetic nanoparticles, the electrostatic surface charge of which is below -20 mV or above + 20 mV when measured in an aqueous medium at physiological pH, the method comprising, in a first embodiment, the following steps of:

- a) measuring $T2^*$,
- b) heating the liposome at T_m or above T_m ,
- 20 c) measuring $T2^*$ after step b),
- d) obtaining the r_2^* values from the $T2^*$ values obtained from step a) and step c),
- e) determining the ratio of r_2^* before the heating step at T_m or above T_m / r_2^* after the heating step at T_m or above T_m , a ratio above 1.5 being indicative of the liposome membrane permeabilization and of the incidental release of the product of interest,
25 thereby monitoring the liposome membrane permeabilization.

In a particular embodiment, the method further comprises measuring $T2^*$, or $T2$ and $T2^*$, during the heating step b).

30 In a second embodiment, the method comprises the following steps of:

- a) measuring $T2^*$ and $T1$,
- b) heating the liposome at T_m or above T_m ,
- c) measuring $T2^*$ and $T1$ after step b),
- d) obtaining r_2^* and r_1 values from, the $T2^*$ and $T1$ values obtained from steps a) and c),

e) determining the ratio of r_{2^*}/r_1 before and after the heating step at T_m or above T_m , a ratio of r_{2^*}/r_1 before the heating step b) and of r_{2^*}/r_1 after the heating step b) above 2 being indicative of the liposome membrane permeabilization and of the incidental release of the product of interest, thereby monitoring the liposome membrane permeabilization.

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In a particular embodiment, the method further comprises measuring $T2^*$ and $T1$, and optionally $T2$, during the heating step b).

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Inventors herein surprisingly demonstrate that the thermosensitive liposome membrane permeabilization, upon heating, and the incidental release of a product of interest, results in a variation of the magnetic resonance imaging (MRI) signal, allowed by the superparamagnetic nanoparticles encapsulated in the liposome, which is of high value and easily measurable.

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Inventors herein provide a thermosensitive liposome comprising a thermosensitive lipidic membrane encapsulating superparamagnetic nanoparticles, the electrostatic surface charge of nanoparticles being below -20 mV or above + 20 mV when measured in an aqueous medium at physiological pH, and the nanoparticles being preferably usable as a diagnostic or monitoring agent. Typically, such a liposome is for use in a method, according to the present invention and as herein described, of monitoring the liposome membrane permeabilization and the incidental release of a product of interest.

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In a particular embodiment, the thermosensitive liposome is a liposome comprising a thermosensitive lipidic membrane encapsulating nanoparticles, the nanoparticles being usable as a therapeutic or as a diagnostic agent, each nanoparticle i) comprising an inorganic core the largest dimension of which is less than about 100 nm, and ii) being fully coated with an agent responsible for the presence of an electrostatic charge below -20 mV or above + 20 mV at the surface of the nanoparticle, the electrostatic charge being determined by zeta potential measurements in an aqueous medium between pH 6 and 8, for a concentration of nanoparticles in suspension varying between 0.2 and 8 g/L.

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In another aspect, the present disclosure provides kits comprising any one or more of the herein-described products, *i.e.*, thermosensitive liposomes and compositions, together with a labeling notice providing instructions for using the product(s) in the context of the herein described methods.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: TEM observations of 5 nm-sized iron oxide nanoparticles (scale bar = 200 nm)

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Figure 2: Typical elution profile obtained for iron oxide-containing liposomes

Figure 2 shows the elution profile for iron oxide-containing liposomes determined by quantification of magnetic nanoparticles by UV-visible spectroscopy (Cary 100 Varian spectrometer) via a colorimetric reaction between ferrous ions and phenanthroline. The concentration of iron oxide in liposomes ranges from 1 to 2.5 g/L.

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Figure 3: Release profile of doxorubicin

Figure 3 shows the release kinetic profile of doxorubicin (DOX)-loaded iron oxide-containing thermosensitive liposomes (TSL), determined by fluorescence measurements. Samples were aged in a medium comprising Phosphate Buffer Saline 1X (PBS 1X) and Fetal Bovine Serum (FBS) (volume ratio 1:1), at 37°C, 41°C, 43°C, and 45°C. Heating is performed via a water bath.

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Figure 4: Evolution upon heating of Magnetic Resonance (MR) relaxivities at 1.5 Tesla of iron oxide nanoparticles encapsulated in thermosensitive (TSL) and non-thermosensitive liposomes (NTSL), and free nanoparticles (free NP)

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Figure 4A shows longitudinal (r_1) relaxivities of thermosensitive liposomes (TSL), non-thermosensitive liposomes (NTSL), both loaded with 5nm-sized iron oxide nanoparticles, and free nanoparticles (free NP) at 1.5 Tesla, before (black) and after (grey) heating at 45°C.

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Heating is performed via a water bath.

Figure 4B shows transverse (r_{2*}) relaxivities of thermosensitive liposomes (TSL), non-thermosensitive liposomes (NTSL), both loaded with 5nm-sized iron oxide nanoparticles, and free nanoparticles (free NP) at 1.5 Tesla, before (black) and after (grey) heating at 45°C.

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Heating is performed via a water bath.

Figure 5: Evolution upon heating of Magnetic Resonance (MR) signal at 1.5 Tesla of iron oxide encapsulated in thermosensitive (TSL) and non-thermosensitive liposomes (NTSL), and free nanoparticles (free NP).

Figure 5 shows the MR images at 1.5 Tesla, of solutions of iron oxide nanoparticles encapsulated within thermosensitive (TSL) and non-thermosensitive liposomes (NTSL), and free nanoparticles (free NP), at 37°C and 45°C for the following dilutions (40, 20, 10 and 5 µg/mL in iron oxide). Gadolinium-based chelate Dotarem, 0.5 mM was used as a control (control).

Figure 6: MR signal and T2*-mapping of iron oxide nanoparticles-encapsulated in thermosensitive liposomes (TSL) before and after High Intensity Focused Ultrasound (HIFU)-induced heating.

Figure 6 shows MR images and T2*-mapping of iron oxide nanoparticles encapsulated in thermosensitive liposomes (TSL), before and after heating with a HIFU device. Red arrows show changes in T2* at the HIFU focal point corresponding to permeabilization of the thermosensitive liposome upon heating at or above Tm.

DETAILED DESCRIPTION OF THE INVENTION

By applying a pulse of radiofrequency energy at the precise precession (Larmor) frequency of the magnetic moment of a sample, precessing nuclei in the lower energy state are converted to precessing nuclei in the higher energy state thereby achieving resonance. When the radiofrequency pulse is turned off, the excited nuclei relax to achieve the initial equilibrium state. This relaxation can be achieved in two ways. In the first process termed spin-lattice relaxation (also longitudinal or T1 relaxation), the excited nucleus gives off its energy to the surrounding environment (lattice) at a particular rate characterized by a time-constant T1 and in the second process, termed spin-spin relaxation (also transverse or T2 relaxation), a nucleus in the high energy state exchanges energy with a nucleus in the low energy state at a particular rate characterized by a time-constant T2. The values for T1 and T2 depend upon the chemical and magnetic environment in which a particular nucleus is situated and different structures and tissues within the body have different T1 and T2 values (Tilcock *et al.* Adv Drug Delivery Reviews, 1999;37:33-51).

It is principally the relative magnitude of these two relaxation processes that generates the contrast between different tissues in the MR image and, depending upon the details of the pulse sequence used (*i.e.* the echo time TE, which is the time between the pulse and the top of

the echo, the repetition time TR, which is the time between pulses) image can be weighted to show mainly differences in T1 or T2.

Water proton relaxation processes can be “catalyzed” by the use of magnetic resonance (MR) contrast agents, thus shortening the longitudinal (T1) and transverse (T2) relaxation times and, therefore, increasing the intensity of the MR signal in the regions where they are distributed (S. Aime, *et al.*, Biopolymers 2002;66:419-428). The efficacy of a MR contrast agent is commonly evaluated in terms of its impact on the proton relaxation rates, or relaxivities, r_1 and r_2 . They are determined from the linear relationship:

5 $1/T_{i,\text{meas}} = 1/T_{i,\text{medium}} + r_i \times [\text{CA}]$ (with $i=1$ or $i=2$), where $T_{i,\text{meas}}$ is the relaxation time measured, $T_{i,\text{medium}}$ is the relaxation time of the region of interest without contrast agent, and [CA] refers to the contrast agent concentration (Martina *et al.*, J. Am. Chem. Soc. 2005; 127:10676-10685). Relaxivities are then expressed in s^{-1} per contrast agent concentration. Contrast agents increase both longitudinal and transverse relaxivities but to varying degrees, 15 depending on their nature, the applied magnetic field and the pulse sequence used (“T1-weighted” or “T2-weighted” images).

There are two main classes of contrast agents for MRI: paramagnetic complexes and superparamagnetic iron oxide nanoparticles.

20 The first class includes mainly chelates of ions presenting a high number of unpaired spins, *i.e.* Mn(II), Mn(III) and Gd(III) ions, conferring them a paramagnetic behavior. Gadolinium-based agents are the most commonly used. Paramagnetic agents increase r_1 and r_2 by similar amounts, but are best visualized using T1-weighted images since the percentage change in r_1 in the tissue is much greater than that in r_2 (Kosłowska *et al.*, Adv. Drug Deliv. Rev. 25 2009;61:1402–1411).

The second class of contrast agents is constituted of superparamagnetic iron oxide nanoparticles, iron oxide being maghemite ($\gamma\text{-Fe}_2\text{O}_3$) or magnetite (Fe_3O_4) crystalline structure (De Cuyper *et al.* Methods in Enzymology, 2003;373:175-198).

30 Superparamagnetism occurs when the size of the crystals is smaller than ferromagnetic domains (approximately 30 nm) and, consequently, they do not show any magnetic remanence (*i.e.* restoration of the induced magnetization to zero upon removal of the external

magnetic field), unlike ferromagnetic materials. Each crystal is then considered to be a fully magnetized single magnetic monodomain, and can be considered to be a monomagnet which is a direct consequence of the spinel structure of the crystal, allowing strong magnetic coupling and consequently perfect alignment of the individual magnetic spins (Corot *et al.* Adv. Drug Deliv. Rev. 2006;58:1471-1504).

Superparamagnetic contrast agents generally lead to a much larger increase in r_2 than in r_1 and are best seen with T2-weighted scans (Kosłowska *et al.* Adv. Drug Deliv. Rev. 2009;61:1402-1411). This predominant effect on the T2 relaxation time does not prevent the use of the properties of these agents on the T1 relaxation time when appropriate imaging sequences are chosen (C. Chambon, *et al.*, Magn. Reson. Imaging 1993;11:509–519; E. Canet, *et al.*, Magn. Reson. Imaging 1993;11:1139–1145).

Due to their high magnetic susceptibility, superparamagnetic nanoparticles also generate local magnetic field inhomogeneities thus leading to a loss of phase coherence among spins oriented at an angle to the static magnetic field. This phenomenon results in another type of relaxation time, $T2^*$, which is commonly due to a combination of magnetic field inhomogeneities and spin-spin transverse relaxation, with the result of rapid loss in transverse magnetization. $T2^*$ is defined as: $1/T2^* = 1/T2 + 1/T_{\text{inhomogeneities}} = 1/T2 + \gamma \Delta B_0$, where γ represents gyromagnetic ratio, and ΔB_0 the difference in strength of the locally varying field.

Superparamagnetic contrast agents are then highly sensitive to $T2^*$ -weighted scans.

Paramagnetic agents directly affect water protons in their close vicinity and are highly dependent on local water flux. Hence, the influence of these agents is very local and they should ideally be in contact with water with adequate exchange rates. In contrast, superparamagnetic agents affect the magnetic field independent of their environment and thus their influence in terms of contrast extends well beyond their immediate surroundings (G.M. Lanza, *et al.*, J. Nucl. Cardiol. 2004;11:733-743). On conventional spin-echo MR images, the presence of T2 (superparamagnetic) agents leads to a dark (hypointense) appearance of tissue, whereas T1 agents cause the opposite (hyperintensity).

The thermosensitive liposomes used in the context of the present invention can be administered in a subject by different routes such as local (intra-tumoral (IT) for example), subcutaneous, intra venous (IV), intra-dermic, intra-arterial, airways (inhalation), intra peritoneal, intra muscular and oral route (*per os*).

As used herein, the term "subject" means any organism. The term need not refer exclusively to human being, which is one example of a subject, but can also refer to animals, in particular warm-blooded vertebrates, typically mammals, and even tissue cultures.

- 5 Inventors herein provide a method of monitoring, in particular *in vivo*, the liposome membrane permeabilization and the incidental release of a product of interest, the liposome comprising a thermosensitive lipidic membrane encapsulating superparamagnetic nanoparticles the "electrostatic surface charge" (also herein identified as "charge" or "surface charge") of which is below -20 mV or above + 20 mV when measured in an aqueous medium
10 at physiological pH (between 6 and 8).

In a typical embodiment of the present invention, the method comprises the following steps of:

- a) measuring $T2^*$,
15 b) heating the liposome at T_m or above T_m ,
c) measuring $T2^*$ after step b),
d) obtaining the r_{2^*} values from the $T2^*$ values obtained from step a) and step c),
e) determining the ratio of r_{2^*} before the heating step at T_m or above T_m / r_{2^*} after the heating step at T_m or above T_m , a ratio above 1.5 being indicative of the liposome
20 membrane permeabilization and of the incidental release of the product of interest, thereby monitoring the liposome membrane permeabilization.

As mentioned above, the r_{2^*} ratio varies in response to the liposome membrane permeabilization, *i.e.*, in response to the product of interest release from the liposome. More
25 specifically, the r_{2^*} ratio varies from 1.2 up to 10, preferably the r_{2^*} ratio is above 1.5.

In a particular embodiment, the method further comprises measuring $T2^*$, or T_2 and $T2^*$, during the heating step b).

30 In a second embodiment, the method comprises the following steps of:

- a) measuring $T2^*$ and T_1 ,
b) heating the liposome at T_m or above T_m ,
c) measuring $T2^*$ and T_1 after step b),
d) obtaining r_{2^*} and r_1 values from, the $T2^*$ and T_1 values obtained from steps a) and c),

e) determining the ratio of r_{2^*}/r_1 before and after the heating step at T_m or above T_m , a ratio of r_{2^*}/r_1 before the heating step b) and of r_{2^*}/r_1 after the heating step b) above 2 being indicative of the liposome membrane permeabilization and of the incidental release of the product of interest, thereby monitoring the liposome membrane permeabilization.

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As mentioned above, the r_{2^*}/r_1 ratio varies in response to the liposome membrane permeabilization, *i.e.*, in response to the product of interest release from the liposome.

More specifically, the r_{2^*}/r_1 ratio varies from 1.2 up to 20, preferably the r_{2^*}/r_1 is above 2, more preferably above 5.

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In a particular embodiment, the method further comprises measuring T_2^* and T_1 , and optionally T_2 , during the heating step b).

The term “measuring” refers to a complete acquisition, or to a real-time acquisition of T_2 , T_2^* or T_1 in the context of real-time imaging techniques.

15

Typically, when performed *in vivo*, the heating step b) at T_m or above T_m of herein described methods is performed locally in order to monitor the liposome membrane permeabilization and the incidental release of a product of interest in a specific area (corresponding to the heat-treated area) of a subject. Such a locally performed method allows the assessment of spatial distribution of the released product.

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Inventors herein provide a thermosensitive liposome with a T_m (gel-to-liquid crystalline phase transition temperature). This liposome comprises a thermosensitive lipidic membrane encapsulating nanoparticles the electrostatic surface charge of which is below -20 mV or above + 20 mV when measured in an aqueous medium at physiological pH (typically between pH 6 and pH 8). These nanoparticles are usable as a diagnostic agent to monitor the delivery of thermosensitive liposomes on a target site and the efficient monitoring of the products of interest release from said thermosensitive liposomes when said liposomes are activated by heat. Preferably, inventors thus herein provide a liposome comprising a thermosensitive lipidic membrane encapsulating nanoparticles the electrostatic surface charge of which is below -20 mV or above + 20 mV when measured in an aqueous medium between pH 6 and 8, for use in a method of monitoring the liposome membrane permeabilization and the incidental release of a product of interest (the product of interest being originally present in addition to

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nanoparticles in said liposome). As further explained below, the electrostatic charge is typically determined by zeta potential measurements in an aqueous medium between pH 6 and 8, for a concentration of nanoparticles in suspension varying between 0.2 and 8 g/L.

5 The term "liposome" refers to a spherical vesicle composed of at least one bilayer of amphipathic molecules which forms a membrane separating an intravesicular medium from an external medium. The intravesicular medium constitutes the internal aqueous core of the liposome. Hydrophilic molecules or components can be encapsulated inside the internal aqueous core of the liposome via active methods of encapsulation known by the skilled
10 person and further herein below described. Hydrophobic molecules or components can be entrapped inside the membrane.

The amphipathic molecules constituting the bilayer are lipids, more particularly phospholipids. The amphipathic characteristic of phospholipid molecules lies in the presence of a hydrophilic head, constituted of a phosphate group and of a glycerol group, and a
15 hydrophobic tail, constituted of one or two fatty acids.

In an aqueous medium, phospholipids tend to self-assemble to minimize contact of the fatty acyl chains with water, and they tend to adopt different types of assembly (micelles, lamellar phase, etc) according to their chemical structure. More particularly, phosphatidylcholines are
20 known to form a lamellar phase consisting in stacked bilayers undergoing a "spontaneous" curvature and finally forming vesicles (Lasic *et al.* Adv. Colloid. Interf. Sci. 2001;89-90:337-349). The phospholipidic lamellar phase constitutes a thermotropic liquid crystal. This means that the ordering degree of the amphipathic molecules depends on the temperature. Indeed, a phospholipidic bilayer demonstrates a main phase transition temperature T_m (for temperature
25 of "melting"), corresponding to a transition between the "gel-like" lamellar phase L_β to the "fluid-like" lamellar phase L_α . In the "gel" phase, strong hydrophobic interactions between the carbonated chains of the fatty acids provoke a crystalline ordering of phospholipid molecules: the bilayer is only permeable to small ions. In the "fluid" phase, the hydrophobic tails are moving due to the thermal motion which causes a loss of ordering of the
30 phospholipid molecules and leads to a "liquid crystalline" phase: the bilayer becomes permeable to molecules such as a drug.

The "gel-to-liquid crystalline" phase transition temperature T_m depends on the chemical structure of the phospholipid molecule: hydrocarbon chain length, unsaturation, asymmetry

and branching of fatty acids, type of chain-glycerol linkage (ester, ether, amide), position of chain attachment to the glycerol backbone (1,2- vs 1,3-) and head group modification.

In the case of phosphatidylcholines, the structure and conformation of fatty acyl chains is of particular relevance (Koynova *et al.*, Biochim. Biophys. Acta 1998;1376:91–145).

- 5 Increasing the chain length of fatty acids increases the main phase transition temperature. For example, it was shown that for saturated diacyl phosphatidylcholines with chain lengths ranging from 9 to 24 carbon atoms, T_m is linearly dependent on $1/n$ (n being the number of carbon atoms in the fatty acyl chains), with T_m increasing from *i.e.* 41°C for $n=16$ to 80°C for $n=24$.
- 10 The effect of unsaturation on the main gel-to-liquid crystalline phase transition temperature depends on the conformation (*cis* or *trans* type), the position in the fatty acyl chains and the number of double bonds. For example, introducing a single site of unsaturation of the *cis* type on the *sn*-2 chain only and in both chains of a phosphatidylcholine comprising 18-carbons can have the effect of lowering the chain melting transition temperature by 50°C (from 54.5°C to
- 15 3.8°C) and 75°C (from 54.5°C to -21°C), respectively. In contrast, when the double bond is of *trans* type, the effect is considerably lessened. Further, T_m depends critically on the position of the *cis*-double bond. Specifically, T_m is minimized when the double bond is located near the geometric center of the hydrocarbon chain, and progressively increases as the double bond migrates toward either end of the chain. These dependencies apply when the double bond is
- 20 present in the *sn*-2 chain only or in both chains of phosphatidylcholine. Concerning the influence of the number of double bonds, it was shown that by increasing the number of *cis*-unsaturation T_m is decreased. For example, when two or three sites of *cis*-unsaturation are introduced into both acyl chains of a phosphatidylcholine comprising 18-carbons, the chain melting transition temperature is lowered by an impressive 109°C (from 54.5°C to -55.1°C)
- 25 and 116°C (from 54.5°C to -61.5°C), respectively (Koynova *et al.*, Biochim. Biophys. Acta 1998;1376:91–145).

Mixed-chain phosphatidylcholines present different hydrocarbon chains lengths at the *sn*-1 and *sn*-2 positions. Empirical equations have been derived that allow for accurate prediction

30 of the transition temperatures of related phosphatidylcholines of defined structure. A normalized chain-length inequivalence parameter, $\Delta C/CL$, has been described, where ΔC ($=|n_1 - n_2| + 1.5$) is the effective chain-length difference, and n_1 and n_2 are the number of carbons in the chains at the *sn*-1 and *sn*-2 positions of the glycerol backbone, respectively. CL is the effective length of the longer of the two chains. For phosphatidylcholines demonstrating the

same number of total carbon atoms constituting the two chains ($n_1+n_2=\text{constant}$), the chain melting temperature decreases monotonically when the chain length inequivalence parameter $\Delta C/CL$ is increased to about 0.4. When $\Delta C/CL$ goes above ca. 0.4, packing perturbation, caused by the methyl ends of the acyl chains, becomes so overwhelming that the asymmetric phosphatidylcholine molecules adopt a new packing arrangement referred to as mixed interdigitation. Upon this rearrangement, T_m increases with chain length asymmetry.

For drug delivery purposes, a sterol component may be included to confer the liposome suitable physicochemical and biological behavior. Such a sterol component may be selected from cholesterol or its derivative e.g., ergosterol or cholesterolhemisuccinate, but it is preferably cholesterol.

Cholesterol is often used in lipidic formulation of liposomes because it is generally recognized that the presence of cholesterol decreases their permeability and protects them from the destabilizing effect of plasma or serum proteins.

The cholesterol molecule contains three well-distinguished regions: a small polar hydroxyl group, a rigid plate-like steroid ring, and an alkyl chain tail. When cholesterol intercalates into the membrane, its polar hydroxyl groups positioned near the middle of the glycerol backbone region of the phosphatidylcholine molecule (Kepczynski M. *et al.*, Chemistry and Physics of Lipids, 2008;155:7-15). Incorporation of modifiers as cholesterol into the lipid bilayers changes greatly the structural or physical properties of the liposomal membrane such as its organization, free volume, thickness, fluidity (viscosity) and polarity (hydrophobicity).

The bilayer's viscosity depends on the position of cholesterol within the bilayer which influences the free volume of the membrane and on the temperature. The effect of cholesterol on the bilayer's microviscosity is rather complex. It is well-known that cholesterol increases the apparent microviscosities (reduces fluidity) of membranes being in liquid phase (Cournia *et al.* J.Phys.Chem.B, 2007;111:1786-1801).

Papahadjopoulos *et al.* showed that the protective effect of cholesterol for liposomes depends on the physical state, *i.e.* "gel" or "fluid", of the lipidic membrane when in contact with serum or plasma. In the gel state, the presence of cholesterol affects the ordering parameter of the phospholipid acyl chains within the bilayer and enhances the release of entrapped molecule. In the fluid state, cholesterol stabilizes the liposomes and prevents leakage of encapsulated material (Papahadjopoulos *et al.* Pharm. Research, 1995;12(10):1407-1416).

Upon addition of cholesterol in a concentration above 25 molar percentage (mol %), there is a dramatic influence on the gel-to-liquid crystal lipid-phase transition. A new thermodynamically stable region of coexistence between the liquid-disordered (fluid) and solid-ordered (gel) phase is described: the liquid-ordered phase (Cournia *et al.* J. Phys. Chem. B, 2007;111:1786-1801; Polozov *et al.*, Biophysical Journal, 2006;90:2051-2061). This new phase is characterized by a fluidity which is intermediate between the fluidity of the gel phase and the fluidity of the fluid phase formed by the pure lipids. Recently, it has been proposed that the liquid-ordered phase is formed when cholesterol associates with saturated, high-melting lipids, such as dipalmitoylphosphatidylcholine (DPPC) and sphingomyelin, to create dynamic complexes in model membranes, so-called "lipid-rafts". Cholesterol promotes a phase separation in model membranes where cholesterol-rich and cholesterol-poor microdomains are formed (Radhakrishnan *et al.* Proc. Natl. Acad. Sci., 2000;97:12422-12427; Mc Connell *et al.* Biochim. Biophys. Acta, 2003;1610:159-173). Indeed, Gaber *et al.* (Pharm. Research, 1995;12(10):1407-1416) showed that two lipidic formulations containing 33 mol % of cholesterol dipalmitoylphosphatidylcholine (DPPC), hydrogenated soybean phosphatidylcholine (HSPC) and cholesterol, in the molar ratio of 100:50:75 and 50:50:50 respectively, do not present a phase transition temperature between 30°C and 65°C, as demonstrated by differential scanning calorimetry measurements. Liposomes with such a formulation are called « non-thermosensitive » liposomes.

Typical "thermosensitive" liposomes usable in the context of the present invention (*i.e.* liposomes with a main phase transition temperature T_m typically comprised between 39°C and 55°C, preferably between 39°C and 50°C, even more preferably between 39°C and 45°C) comprises at least a phosphatidylcholine.

The phosphatidylcholine may be selected from dipalmitoylphosphatidylcholine (DPPC), distearylphosphatidylcholine (DSPC), hydrogenated soybean phosphatidylcholine (HSPC), monopalmitoylphosphatidylcholine (MPPC), monostearylphosphatidylcholine (MSPC) and any mixture thereof.

In a preferred embodiment the thermosensitive liposome further comprises distearylphosphatidylethanolamine (DSPE), distearylphosphatidylethanolamine(DSPE)-methoxypolyethylene glycol (PEG) (DSPE-PEG).

In a preferred embodiment, cholesterol is added in a molar ratio inferior to 25 mol %.

A preferred thermosensitive lipidic membrane comprises dipalmitoylphosphatidylcholine (DPPC), hydrogenated soybean phosphatidylcholine (HSPC), cholesterol and distearylphosphatidylethanolamine(DSPE)-methoxypolyethylene glycol (PEG), for example PEG2000 (DSPE-PEG2000).

In a particular embodiment, the molar ratio of the previously identified compounds is preferably 100:50:30:6 or 100:33:27:7

Another preferred thermosensitive lipidic membrane comprises dipalmitoylphosphatidylcholine (DPPC), monopalmitoylphosphatidylcholin (MPPC) and distearylphosphatidylethanolamine(DSPE)-methoxypolyethylene glycol (PEG), for example methoxypolyethylene glycol-2000 (DSPE-PEG2000).

In a particular embodiment, the molar ratio of the previously identified compounds is preferably 100:12:5.

Another preferred thermosensitive lipidic membrane comprises dipalmitoylphosphatidylcholine (DPPC), monostearylphosphatidylcholine (MSPC), and distearylphosphatidylethanolamine(DSPE)-methoxypolyethylene glycol (PEG), for example methoxypolyethylene glycol-2000 (DSPE-PEG2000).

In a particular embodiment, the molar ratio of the previously identified compounds is preferably 100:12:5.

Depending on the mode of preparation, the size and the degree of lamellarity of the vesicles can be tuned. Several methods for preparing unilamellar lipidic vesicles have been described in the literature: reverse phase evaporation (Szoka *et al.*, PNAS, 1978; 75(9):4191-4198), ethanol injection (Pons *et al.* International Journal of Pharmaceutics, 1993;95(1-3):51-56), heating method (Mozafari *et al.*, Journal of Biotechnology, 2007;129:604-613), but the most simple is the lipid film hydration method (Bangham *et al.*, J. Mol. Bio., 1965;13:238-252).

Briefly, in the lipid film hydration method, lipids are solubilized in an organic solvent such as chloroform. After homogenization of the solution, the organic solvent is evaporated under a nitrogen stream. The as-obtained dried lipid film is then hydrated by an aqueous medium at a temperature above the main phase transition temperature T_m , leading to the formation of multilamellar vesicles with sizes ranging from 100 to 800 nm (Mills J.K. *et al.* Methods in Enzymology 2004;387:82-113). Cycles of dehydration and rehydration, by respectively

freezing (in liquid nitrogen) and thawing the solution (at a temperature above T_m), allow increasing the aqueous internal volume by forming unilamellar vesicles. A process allowing vesicles size calibration is then applied to obtain a homogeneous size distribution. Sonication produces Small Unilamellar Vesicles (SUV) with size ranging from 20 to 50 nm, whereas
5 extrusion process through a filter membrane produces Large Unilamellar Vesicles (LUV) with size ranging from 50 to 500 nm depending on the size of the filter pores. Both processes, sonication and extrusion, have to be performed at a temperature above T_m .

The largest size of the thermosensitive liposomes according to the present invention is
10 typically comprised between 50 and 500 nm, preferably between 50 and 250 nm, for example between about 50 nm and about 150 nm.

Thermosensitive liposomes used in the present invention preferably comprise a biocompatible coating to ensure or improve their biocompatibility and specific biodistribution.

15 The biocompatible coating allows or favours the liposomes stability in a biocompatible suspension, such as a physiological fluid (blood, plasma, serum, etc.), any isotonic media or physiologic medium, for example media comprising glucose (5%) and/or NaCl (0.9 %), which is required for a pharmaceutical administration.

Such a biocompatible coating is obtained by treating the liposome with a surface treating
20 agent.

Stability may be confirmed by dynamic light scattering of the liposomes in biocompatible suspension.

Said coating advantageously preserves the integrity of the liposome *in vivo*, ensures or improves its biocompatibility, and facilitates its optional functionalization (for example with
25 spacer molecules, biocompatible polymers, targeting agents, proteins, etc.).

The coating can be non-biodegradable or biodegradable. Both options can be used in the context of the present invention.

Examples of non-biodegradable coatings are one or more materials or surface treating agents
30 selected in the group consisting of sugar (agarose for example), saturated carbon polymers (polyethylene oxide for example), reticulated or not, modified or not (polymethacrylate or polystyrene for example), as well as combinations thereof.

Examples of biodegradable coatings are for example one or more materials or surface treating agents selected from the group consisting of a biological molecule, modified or not, natural or not and a biological polymer; modified or not, of natural shape or not. The biological polymer may be a saccharide, an oligosaccharide or a polysaccharide, polysulfated or not, for example dextran.

The aforementioned materials, compounds or surface treating agents can be used alone or in combinations, mixtures or assemblies, composite or not, covalent or not, optionally in combination with other compounds.

Thermosensitive liposomes according to the present invention can further comprise a surface component enabling specific targeting of biological tissues or cells. Such a surface component is preferably a targeting agent allowing the liposome interaction with a recognition element present on the target biological structure.

Such targeting agents can act only once the liposomes are accumulated in the tumor.

As the conformation of the targeting agent will be responsible for its interaction with the target, the density of said targeting agent is to be controlled carefully according to methods known by the skilled person. A high density thereof can indeed perturb the targeting agent conformation and in consequence its recognition by the target cell (see for example J. A. Reddy *et al.* Gene therapy 2002;9:1542; Ketan B. Ghaghada *et al.* Journal of Controlled Release 2005;104:113). In addition, a high target agent density may favour liposomes clearance by the Reticulo Endothelial System (RES) during circulation in the vasculature.

The targeting agent may be selected from an antigen, a spacer molecule, a biocompatible polymer. The targeting agent can be any biological or chemical structure displaying affinity for molecules present in the human or animal body. For instance it can be a peptide, oligopeptide or polypeptide, a protein, a nucleic acid, a hormone, a vitamin, an enzyme and the like and in general any ligand of molecules (for example receptors, markers, antigens, etc.). Ligands of molecules expressed by pathological cells, in particular ligands of tumor antigens, hormone receptors, cytokine receptors or growth factor receptors. Said targeted agents can be selected for example in the group consisting in LHRH, EGF, a folate, anti-B-FN antibody, E-selectin/P-selectin, anti-IL-2R α antibody, GHRH, etc.

The coating can also contain different functional groups (or linker segments), allowing any molecule of interest to bind to the surface of the liposome, such as a surface component enabling specific targeting of biological tissues or cells.

- 5 The term “nanoparticle” refers to a particle or aggregate of particles, said nanoparticle comprising a core (or central core) and a coating, the largest dimension of the core being less than about 100 nm. Typically, the largest dimension of the core of the nanoparticle is the diameter of a nanoparticle of round or spherical shape, or the longest length of a nanoparticle of ovoid or oval shape.
- 10 The terms “size of the nanoparticle” and “largest size of the nanoparticle” herein refers to the “largest dimension of the core of the nanoparticle”.
The “core” can designate a single particle (crystal or crystallite) or an aggregate of particles (aggregate of crystal or crystallites).
Transmission Electron Microscopy (TEM) or cryoTEM can be advantageously used to
15 measure the size of the core of the nanoparticle especially when the core consists in a single particle (see Figure 2). As well, Dynamic Light Scattering (DLS) can be used to measure the hydrodynamic diameter of the core of the nanoparticle in solution when said core consists in a particle or in an aggregate of particles. These two methods may further be used one after each other to compare size measures and confirm said size.
- 20 The material is an inorganic material.

The electronic surface charge of the herein used nanoparticules is below – 15 mV or above + 15 mV, for example between - 15 mV and - 20 mV or between + 15 mV and + 20 mV, typically below - 20 mV or above + 20 mV, when determined by zeta potential
25 measurements, performed on nanoparticles suspensions with concentration varying between 0.2 and 8 g/L, nanoparticles being suspended in aqueous medium at pH comprised between 6 and 8.

The nanoparticle’s shape can be for example round, flat, elongated, spherical, ovoid or oval,
30 and the like. The shape can be determined or controlled by the method of production, and adapted by the person of the art according to the desired applications.

As the shape of the particles can influence their “biocompatibility” once delivered on the targeted site, particles having a quite homogeneous shape are preferred. For pharmacokinetic

reasons, nanoparticles being essentially spherical, round or ovoid in shape are thus preferred. Spherical or round shape is particularly preferred.

The largest size of the nanoparticles, i.e. the largest dimension of the core of the nanoparticles, used in the context of the present invention is typically comprised between 1 and 50 nm.

It is advantageously comprised between about 2 nm and about 50 nm, for example between about 2 nm and 30 nm or between about 2 nm and about 20 nm, preferably between about 2 nm and about 10 nm, typically between about 5 nm and about 10 nm, between about 4 nm and about 8 nm or between about 30 nm and 50 nm.

The nanoparticle used in the context of the present invention comprise a core and a coating, said coating being responsible for the presence of an electrostatic surface charge below -20 mV or above $+20$ mV when measured in an aqueous medium at physiological pH.

The electrostatic coating is advantageously a "full coating" (complete monolayer). This implies the presence of a very high density of molecules creating an appropriate and homogeneous charge on the all surface of the nanoparticle.

The coating can be an inorganic or organic surface coating.

When inorganic, the coating may be selected from the group consisting of an oxide, an hydroxide, and an oxyhydroxide. The inorganic coating may comprise for example silicium, aluminium, calcium and/or magnesium.

An inorganic agent selected from the group consisting of, for example magnesium and calcium, will bring a positive charge (above $+20$ mV) to the surface of the nanoparticle at pH 7.

In another embodiment, a silicium group may be used to bring a negative charge (below -20 mV) to the surface of the nanoparticle at pH 7.

When organic, the coating is prepared with a molecule capable of interacting, through covalent binding or electrostatic binding, with the nanoparticle surface and of giving surface properties to said nanoparticle.

The surface coating organic molecule has two groups, R and X. The function of X is to interact with the nanoparticle surface and the function of R is to give the nanoparticle's surface its specific properties.

X may be selected for example from a carboxylate (R-COO^-), a silane (R-Si(OR)_3), a phosphonic (R-PO(OH)_2), a phosphoric (R-O-PO(OH)_2), a phosphate (R-PO_4^{3-}) and a thiol (R-SH) group.

R brings at least the electronic surface charge to the nanoparticle in aqueous suspension at a physiological pH.

When R brings a positive charge to the nanoparticle's surface, R may be an amine ($\text{NH}_2\text{-X}$).

When R brings a negative charge to the nanoparticle's surface, R may be phosphate ($\text{PO}_4^{3-}\text{-X}$) or a carboxylate ($\text{COO}^-\text{-X}$).

Organic coating conferring a positive charge (above + 20 mV) to the nanoparticles surface may be selected from for example aminopropyltriethoxysilane, polylysine or 2-aminoethanethiol.

Organic coating conferring a negative charge (below - 20 mV) to the nanoparticles surface may be selected from for example a polyphosphate, a metaphosphate, a pyrophosphate, etc.,

or for example from citrate or dicarboxylic acid, in particular succinic acid.

Again, the electrostatic coating is advantageously a "full coating".

This electrostatic coating and especially amino or carboxylic moieties can further be used to link any group or agent on the nanoparticle's surface.

Optionally, the nanoparticle surface can be functionalized using a steric group. Such a group may be selected from polyethylene glycol (PEG), polyethylenoxide, Polyvinylalcohol, Polyacrylate, Polyacrylamide (poly(N-isopropylacrylamide)), Polycarbamide, a biopolymer or polysaccharide such as Dextran, Xylan, cellulose, collagene, and a switterionic compound such as polysulfobetain.

This steric group increases the nanoparticles stability in a biocompatible suspension, such as a physiological fluid (blood, plasma, serum, etc.), any isotonic media or physiological media, for example media comprising glucose (5%) and/or NaCl (0.9%).

The inorganic material constituting the core is a superparamagnetic material.

Superparamagnetic materials include for example iron, nickel, cobalt, gadolinium, samarium, neodymium, preferably in the form of an oxide, an hydroxide or a metal, and any mixture thereof.

In specific examples, the material forming the core is selected from the group consisting of ferrous oxide and ferric oxide. In a preferred embodiment of the present invention, the oxide nanoparticles are made of magnetite or maghemite.

Mixed material can also be used to optimize interactions between a magnetic field and nanoparticles. Solid solution forms (well known by the man skilled in the art as random mixtures of several materials) such as CoFe_2O_4 for example can be used as a mixed material. Solid solution form in demixed phases, such as Fe_2O_3 / Co for example, can further be used.

Products of interest in the context of the present invention include any compound (for example a drug) with therapeutic or prophylactic effects. It can be a compound that affects or participates in tissue growth, cell growth, cell differentiation, a compound that is able to invoke a biological action such as an immune response, or a compound that can play any other role in one or more biological processes.

A non-limiting list of examples includes antimicrobial agents (including antibacterial, in particular antibiotics, antiviral agents and anti-fungal agents); anti-tumor agents, in particular anticancer chemotherapeutic agents such as cytostatic(s), cytotoxic(s), and any other biological or inorganic product intended to treat cancer such as a therapeutic nucleic acid, in particular a micro RNA (miRNA), a short-hairpin RNA (shRNA) and/or a small interfering RNA (siRNA).

Drugs in the present invention can also be prodrugs.

Any combination of such products of interest may further be used.

The compound(s) of interest may be present in the inner, the outer, or both of the compartments of the carrier, e.g. in the cavity and/or in the shell of a liposome.

The present disclosure further provides kits comprising any one or more of the herein-described thermosensitive liposomes comprising superparamagnetic nanoparticles and a compound of interest to be delivered and released on a target site, or compositions comprising such liposomes. For example, the kit comprises at least one thermosensitive liposome or population of thermosensitive liposomes as herein described and also one or more containers filled with one or more of the ingredients of the diagnostic compositions herein described.

A labelling notice providing instructions for using the products can further be provided for using the thermosensitive liposome, population of thermosensitive liposomes, or compositions according to the present methods of the invention.

Other aspects and advantages of the invention will become apparent in the following examples, which are given for purposes of illustration and not by way of limitation.

5 EXPERIMENTAL SECTION

EXAMPLE 1: Preparation of 5 nm-sized nanoparticles

10 Iron oxide nanoparticles with a size distribution centered on 5 nm are synthesized by coprecipitation of ferrous and ferric ions adapted from (US4329241; Bacri *et al.*, J. Magn. Magn. Mat., 1986;62:36-46).

Briefly, the reacting medium, with controlled ionic strength, consists in a solution of sodium nitrate 3M maintained at pH 12. A 3M sodium nitrate solution of ferrous and ferric ions in a molar ratio Fe(III)/Fe(II) equal 2 is prepared and slowly added to the reacting medium under
15 mixing. It rapidly turns black. The whole solution is then aged during one night at ambient temperature under mixing.

Ferrite nanoparticles are then sedimented on a magnet and the supernatant is removed in order to eliminate the reacting medium. Then peptization (acidification) and oxidation of the nanoparticles surface is performed by diluting the pellet in a solution of nitric acid HNO₃ 2M
20 at room temperature under vigorous mixing.

Oxidation of the nanoparticles core is performed by incubation of the pellet in a solution of ferric nitrate at elevated temperature (>90°C) under vigorous mixing.

The nanoparticles are then washed by centrifugation.

The pellet is finally diluted in acidic water in order to reach a concentration of 150 g/L in iron
25 oxide. The solution is homogenized by sonication and pH is then adjusted to pH 2.

The morphology (size and shape) of nanoparticles was observed by Transmission Electron Microscopy (Figure 1). The crystalline structure of iron oxide nanoparticles was confirmed by X-ray diffraction analysis.

EXAMPLE 2: Surface treatment of nanoparticles - Functionalization of nanoparticles with sodium hexametaphosphate (HMP)

Suspension of sodium hexametaphosphate is added to the suspension of iron oxide nanoparticles from example 1 (the amount of sodium hexametaphosphate added being below LD50/5) and the pH of the suspension is adjusted to a pH comprised between 6 and 8

Electronic surface charge (< -20 mV) is determined by zeta potential measurements on a Zetasizer NanoZS (Malvern Instruments), using a 633 nm HeNe laser, performed on nanoparticles suspensions with concentrations varying between 0.2 and 2 g/L, nanoparticles being suspended in aqueous medium at pH comprised between 6 and 8.

EXAMPLE 3: Co-encapsulation of iron oxide nanoparticles and doxorubicin inside liposomes

3.1 Preparation of thermosensitive liposomes (TLS) encapsulating iron oxide nanoparticles:

The thermosensitive liposomes encapsulating iron oxide nanoparticles are prepared using the lipidic film re-hydration method (Bangham *et al.*, J. Mol. Bio., 1965;13:238-252; Martina *et al.*, J. Am. Chem. Soc., 2005;127:10676-10685):

a) Lipids are solubilized in chloroform. Chloroform is finally evaporated under a nitrogen flow. Re-hydration of the lipidic film is performed at 55°C with 2mL of the iron oxide solution described in Example 2, so that the lipidic concentration is 50mM.

The following lipidic composition was used: dipalmitoylphosphatidylcholine (DPPC), hydrogenated soybean phosphatidylcholine (HSPC), cholesterol (Chol) and pegylated distearylphosphatidylethanolamine (DSPE-PEG2000) in the molar ratio 100:33:27:7 (DPPC:HSPC:Chol:DSPE-PEG2000).

b) Freeze-thaws cycles are then performed 20 times, by successively plunging the sample into liquid nitrogen and into a water bath regulated at 55°C.

c) A thermobarrel extruder (LIPEXTM Extruder, Northern Lipids) was used to calibrate the size of the nanoparticles-containing liposomes under controlled temperature and pressure. In all cases, extrusion was performed at 55°C, under a pressure ranging from 2 to 20 bars.

d) Separation of non-encapsulated particles is performed by size exclusion chromatography on a Sephacryl S1000 filtration gel.

e) Elution profile is determined by quantification of magnetic nanoparticles by UV-visible spectroscopy (Cary 100 Varian spectrometer) via a ferrous ions/phenanthroline colorimetric

reaction (Figure 2) adapted from Che *et al.*, Journal of Chromatography B, 1995;669:45-51, and Nigo *et al.*, Talanta, 1981;28:669-674. Liposomes-containing fractions are collected (Figure 2, first peak). The concentration of iron oxide in liposomes ranges from 1 to 2.5 g/L. T_m of the composition is of 43°C.

5

3.2 Loading of thermosensitive liposomes (TLS) encapsulating iron oxide nanoparticles with doxorubicin

An active loading of doxorubicin (DOX) inside iron oxide-containing liposomes was used, via pH gradient. Once trapped in the internal aqueous core of liposome, doxorubicin leakage is prevented by complexation of the anthracycline molecule with an ammonium salt:

10

a) Iron oxide-containing liposomes (prepared as explained in example 3.1) are incubated in a solution of ammonium hydrogen phosphate $(\text{NH}_4)_2\text{HPO}_4$ 120mM, at pH around 5 during 1 hour.

15

b) Dialysis steps are performed in HEPES buffer saline (HEPES 20mM, NaCl 140mM, pH 7.4) to eliminate external $(\text{NH}_4)_2\text{HPO}_4$: once over 1 night at 4°C, then twice during 3 h at ambient temperature (final dilution factor of 250*250*250).

c) 284μL of a doxorubicin stock solution at 7mg/mL in NaCl 140mM is added to 1mL of iron oxide-containing liposomes solution to obtain a doxorubicin concentration of 1.55mg/mL. The composition is incubated during 1 night at 37°C.

20

d) Non-encapsulated doxorubicin is then eliminated by size-exclusion chromatography on a Sephadex G50 filtration gel (column height: 5 cm; eluant: HEPES buffer saline). The volume of doxorubicin loaded liposomes deposited onto the gel is 700μL. Fractions of 500μL are collected.

25

e) Elution profile is performed by quantifying doxorubicin concentration in each fraction (by measurement of absorbance at 480nm). Liposomes/doxorubicin-containing fractions are collected.

f) Final liposomal doxorubicin concentration is determined by measuring absorbance of doxorubicin at 480 nm by UV-visible spectroscopy.

30

3.3 Preparation of “Non-thermosensitive liposomes” (NTSL) encapsulating iron oxide nanoparticles

The previously described procedure (see example 3.1) was followed except that, in step a) the following lipidic composition was used: hydrogenated soybean phosphatidylcholine (HSPC),

cholesterol (Chol) and pegylated distearylphosphatidylethanolamine (DSPE-PEG2000) in the molar ratio 100:65:7 (HSPC:Chol:DSPE-PEG2000).

In steps a), b) and c), re-hydration of the lipidic film, thaw cycles and extrusion process are performed at 62°C.

5

EXAMPLE 4: Kinetic of drug release from thermosensitive liposomes encapsulating both doxorubicin and iron oxide nanoparticles

Figure 3 shows the release kinetics profile of doxorubicin (DOX)-loaded iron oxide-containing thermosensitive liposomes (TSL) as prepared in example 3.2. Doxorubicin release, as a function of temperature and time, is determined by fluorescence measurements.

The doxorubicin release profile is characterized using the autoquenching properties of doxorubicin (Jeffrey K. Mills *et al.* Biochimica et Biophysica Acta 2005; 1716:77-96). Briefly, fluorescence intensity is quenched when DOX is highly concentrated inside liposomes, but increases when DOX is released and then diluted in the external medium.

Fluorescence measurements are performed with a Cary Eclipse (Varian) spectrophotometer with $\lambda_{\text{excitation}} = 480\text{nm}$ and $\lambda_{\text{emission}} = 590\text{nm}$. Liposomes are diluted 200 times in phosphate buffer saline (PBS) supplemented with 50% volume fetal bovine serum (FBS). Initial fluorescence intensity I_0 was measured at time $t=0$ and at $T_0=25^\circ\text{C}$. The diluted samples are plunged in a water bath to reach temperature T (37°C , 41°C , 43°C , 45°C). At different time points, a sample is collected and plunged in an ice bath to reach the initial temperature (T_0). The fluorescence intensity I_{released} is then measured. 0.1% volume of Triton TX100 (10% vol in distilled water) is then added in order to break apart the lipidic membrane to obtain I_{total} corresponding to the total amount of doxorubicin. The external % of DOX present at $t=0$ and $T_0=25^\circ\text{C}$ is calculated as I_0/I_{total} and the released % of DOX (function of temperature) is calculated as $I_{\text{released}}/I_{\text{total}}$.

As shown in Figure 3, release of doxorubicin is maximal when the thermosensitive liposomes solution is heated above 43°C (this corresponds to T_m which is the main gel-to-liquid crystalline phase transition temperature of this liposome composition). At this temperature the lipidic membrane is in a fluid state and its permeabilization allows for the release of the drug out of the liposome.

30

EXAMPLE 5: MRI monitoring of membrane permeabilization (and subsequent drug release) of thermosensitive liposomes encapsulating iron oxide nanoparticles upon heating at or above T_m .

5 Determination of r_1 and r_2^* relaxivities was done thanks to measurements of relaxation times T_1 and T_2^* performed on a 1.5 Tesla clinical MRI Philips Achieva, using a head SENSE coil, for thermosensitive liposomes (TSL) (example 3.1) and non-thermosensitive liposomes (NTSL) (example 3.3), both loaded with 5 nm-sized iron oxide nanoparticles, and for non-encapsulated iron oxide nanoparticles (free NP) (example 1). A range of dilution for each
10 sample was performed corresponding to 40, 20, 10 and 5 $\mu\text{g/mL}$ in iron oxide respectively. Dilutions were performed in agarose gel (4%). Samples were heated using a water bath first at 37°C and then at 45°C, and relaxation times T_1 and T_2^* of each dilution were measured at 37°C and at 45°C.

For T_1 measurements, inversion recovery sequences were used
15 (TR / TI first / ΔTI = 2000 / 50 / 100 ms, TE = 10 ms, NEX = 2, FOV = $(1 \times 1 \times 3 \text{ mm})^3$, matrix = 128×168 , with TR = repetition time, IT = inversion time, TE = echo time, NEX = number of excitations, FOV = field of view.

Fast gradient echo sequences were used for T_2^* measurement, with flip angle = 40°, 10 echoes, TR / TE first / ΔTE = 300 / 10 / 10 ms, NEX = 4.

20 Figure 4 shows the modification of relaxivity values r_1 (Figure 4A) and r_2^* (Figure 4B) upon heating at 45°C corresponding to a temperature above the main gel-to-liquid crystalline phase transition temperature T_m (at this temperature the lipidic membrane is then in a fluid state).

Non-encapsulated iron oxide nanoparticles (free NP) and iron oxide-loaded NTSL
25 demonstrated no significant evolution of the longitudinal relaxivity r_1 before and after heating at 37°C and 45°C respectively. On contrast, the longitudinal relaxivity r_1 of iron oxide-loaded TSL presents a 10-fold increased upon heating (from $r_1 = 0.1 \text{ mM}^{-1} \cdot \text{s}^{-1}$ at 37°C to $r_1 = 1.68 \text{ mM}^{-1} \cdot \text{s}^{-1}$ at 45°C).

30 Non-encapsulated iron oxide nanoparticles (free NP) and iron oxide-loaded NTSL demonstrated no significant evolution of the transverse relaxivity r_2^* before and after heating at 37°C and 45°C respectively. On contrast, the transverse relaxivity r_2^* of iron oxide-loaded TSL is decreased by about 2-fold upon heating (from $r_2^* = 208.7 \pm 23.4 \text{ mM}^{-1} \cdot \text{s}^{-1}$ at 37°C to $r_2^* = 109.4 \pm 0.2 \text{ mM}^{-1} \cdot \text{s}^{-1}$ at 45°C).

This example demonstrates the influence of the permeabilization of the lipidic membrane of TSL on the longitudinal and transverse relaxation times of protons in the environment of nanoparticles. This phenomenon is well highlighted by presenting the results as an evolution of the ratio r_2^*/r_1 (see Table 1) before and after heating of the thermosensitive liposomes solution above T_m .

Table 1: Evolution of the ratio r_2^*/r_1 upon heating for TSL, NTSL and free NP

Temperature (°C)	TSL	NTSL	Free NP
37°C	1000	150	33
45°C	67	128	28
$k^{(*)}$	15	1,2	1,2

^(*) k represents the ratio between r_2^*/r_1 values at 37°C and 45°C respectively.

A surprisingly marked decrease of r_2^*/r_1 values upon heating is demonstrated for TSL, whereas no significant evolution for NTSL and free NP is observed (see k value).

Figure 5 shows MR imaging of iron oxide-containing samples (TSL, NTSL and free nanoparticles) for different dilutions factors (40, 20, 10 and 5 µg/mL in iron oxide). Experiment was performed on a Philips Achieva 1.5 Tesla, using a fast gradient echo sequence ($TR / TE = 300 / 10$ ms, $NEX = 4$, $FOV = (1 \times 1 \times 3 \text{ mm})^3$, matrix = 128×168 , flip angle = 40°). For a concentration in iron oxide of 40 µg/mL, the modification of MRI contrast for thermosensitive liposome (TSL) is clearly visible upon heating: a “hyposignal” is observed at 37°C whereas a “hypersignal” is obtained at 45°C. No significant signal variation was observed on both non-thermosensitives liposomes (NTSL) and iron oxide nanoparticles (free NP).

This major modification of the ratio r_2^*/r_1 upon heating at a temperature above T_m constitutes a highly sensitive “probe” of the permeabilization of the lipidic membrane of liposomes. The subsequent drug release that occurs when a drug is co-encapsulated with the iron oxide nanoparticles inside the liposome (see example 4.1) can be monitored via detection of this marked MRI signal modification.

Furthermore, monitoring of lipidic membrane permeabilization of the thermosensitive liposome may be observed by a T2*-mapping prior and after heating of the thermosensitive liposomes solution at or above T_m.

In figure 6, ultrasound experiments were performed with an in-house-designed, single-channel focused ultrasound transducer (Imasonic SA) incorporated in the bed of the 1.5-T MRI system. The transducer has a focal length of 80 mm, with sinusoidal signal of 1.5 MHz. Heating was performed with acoustic power of 20 W during 60 seconds.

TSL containing iron oxide nanoparticles (20 µg/ml), included in agarose gel (4% - 2% silica), were used.

Upon heating, thermosensitive liposomes showed punctual MR signal and T₂* relaxation time enhancement, with variations clearly visible at the HIFU focal point (red arrow). On average, the positive enhancement was of about 51%. Before heating, T₂* was 4.9 ± 0.4 ms, and increased to 54 ± 31.4 ms after HIFU heating (red arrow).

The result demonstrates that real-time imaging of drug release is now possible.

CLAIMS

1. A liposome comprising a thermosensitive lipidic membrane encapsulating superparamagnetic nanoparticles the electrostatic surface charge of which is below -20 mV or above + 20 mV when measured in an aqueous medium between pH 6 and 8, for use in a method of monitoring the liposome membrane permeabilization and the incidental release of a product of interest, the method comprising:

- a) measuring $T2^*$,
- b) heating the liposome at T_m or above T_m ,
- c) measuring $T2^*$ after step b),
- d) obtaining the r_{2^*} values from the $T2^*$ values obtained from step a) and step c),
- e) determining the ratio of r_{2^*} before the heating step at T_m or above T_m / r_{2^*} after the heating step at T_m or above T_m , a ratio above 1.5 being indicative of the liposome membrane permeabilization and of the incidental release of the product of interest, thereby monitoring the liposome membrane permeabilization.

2. A liposome comprising a thermosensitive lipidic membrane encapsulating superparamagnetic nanoparticles the electrostatic surface charge of which is below -20 mV or above + 20 mV measured in an aqueous medium between pH 6 and 8, for use in a method of monitoring the liposome membrane permeabilization and the incidental release of a product of interest, the method comprising:

- a) measuring $T2^*$ and $T1$,
- b) heating the liposome at T_m or above T_m ,
- c) measuring $T2^*$ and $T1$ after step b),
- d) obtaining r_{2^*} and r_1 values from the $T2^*$ and $T1$ values obtained from steps a) and c),
- e) determining the ratio of r_{2^*}/r_1 before and after the heating step at T_m or above T_m , a ratio of r_{2^*}/r_1 before the heating step b) and of r_{2^*}/r_1 after the heating step b) above 2 being indicative of the liposome membrane permeabilization and of the incidental release of the product of interest, thereby monitoring the liposome membrane permeabilization.

3 The liposome according to claim 1, wherein the liposome is for use in a method as defined in claim 1 further comprising at least one step of measuring $T2^*$, or $T2$ and $T2^*$, during the heating step b).

4. The liposome according to claim 2, wherein the liposome is for use in a method as defined in claim 2 further comprising at least one step of measuring T2* and T1, and optionally T2, during the heating step b).

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5. The liposome according to anyone of the preceding claims, wherein Tm is between 39°C and 45°C.

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6. The liposome according to anyone of the preceding claims, wherein the thermosensitive lipidic membrane comprises at least a phosphatidylcholine.

7. The liposome according to claim 5, wherein the thermosensitive lipidic membrane further comprises cholesterol.

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8. The liposome according to claim 6 or 7, wherein the thermosensitive lipidic membrane further comprises distearylphosphatidylethanolamine-methoxypolyethylene glycol.

9. The liposome according to anyone of the preceding claims, wherein the largest size of the liposome is between 50 and 500 nm, preferably between 50 and 250 nm.

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10. The liposome according to anyone of the preceding claims, wherein the nanoparticles are covalently or electrostatically fully coated with an agent selected from a carboxylic acid, a phosphate and an amine agent.

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11. The liposome according to anyone of the preceding claims, wherein the nanoparticles are prepared from iron oxide such magnetite and/or maghemite.

12. The liposome according to anyone of the preceding claims, wherein the nanoparticle largest size is between 2 and 30 nm, preferably between 2 and 20 nm.

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13. The liposome according to anyone of the preceding claims, wherein the product of interest is selected from a therapeutic nucleic acid, a cytostatic compound, and a cytotoxic compound.

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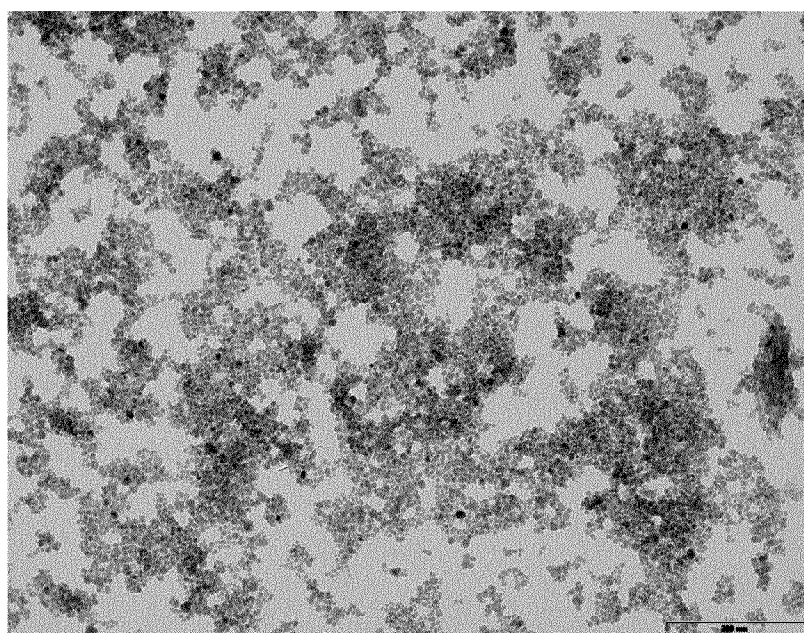


Figure 1

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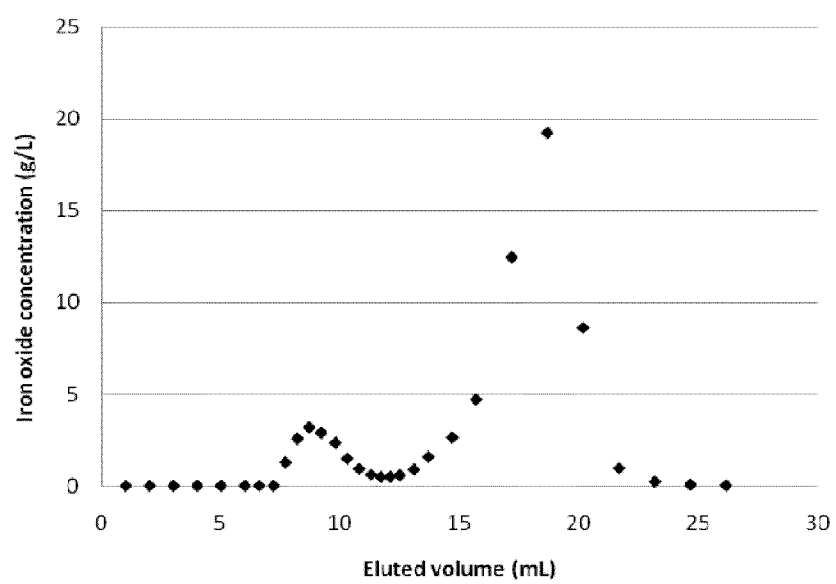


Figure 2

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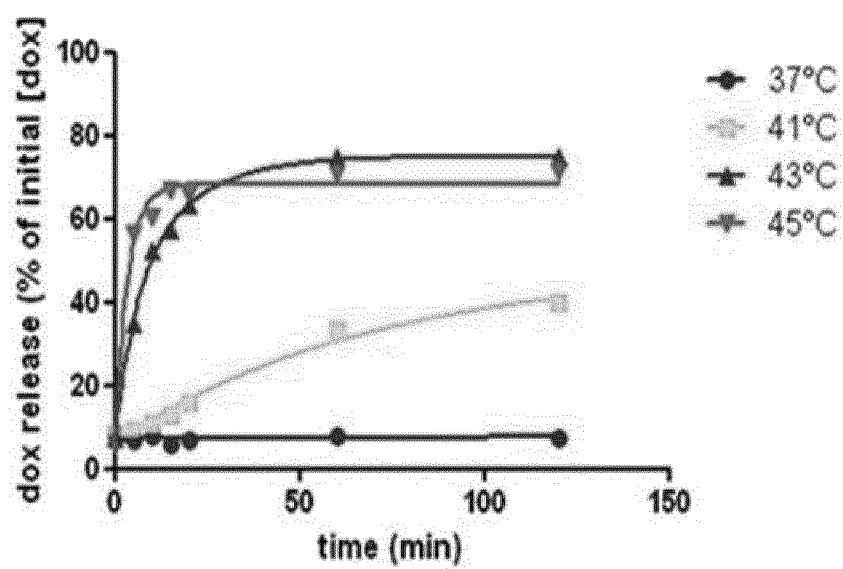


Figure 3

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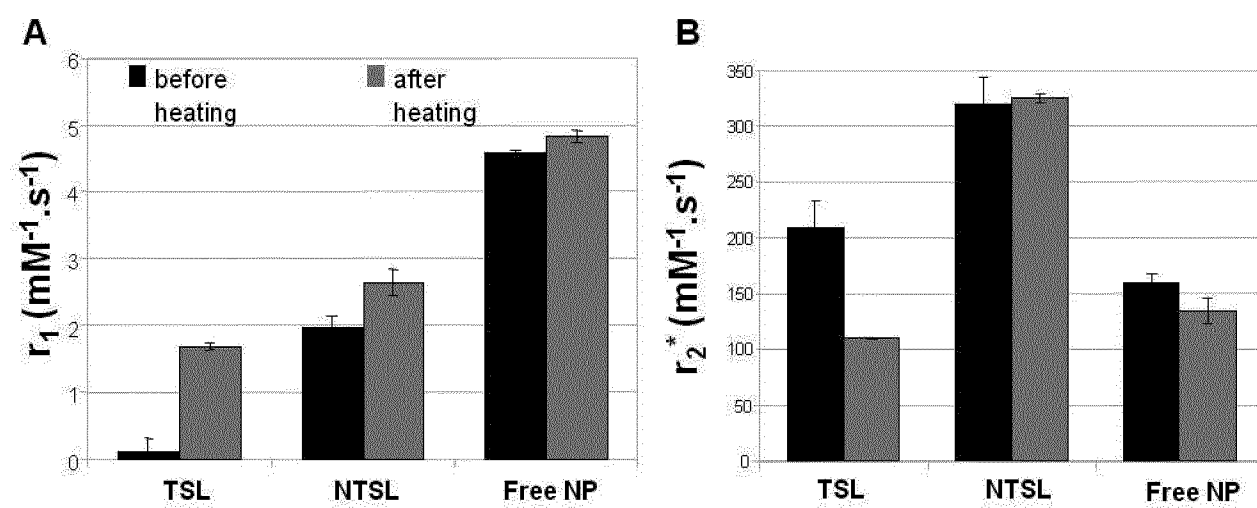


Figure 4

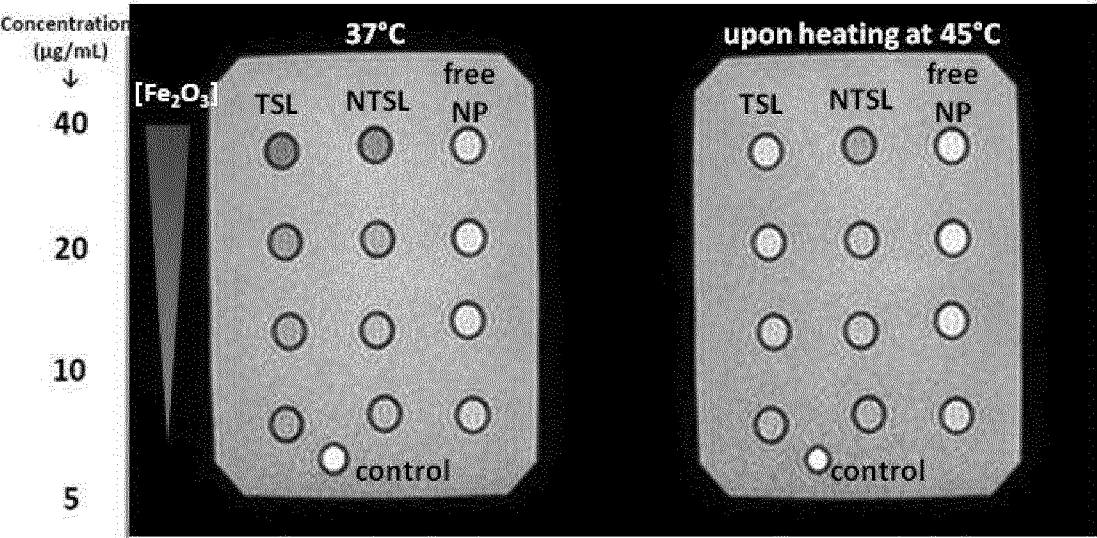


Figure 5

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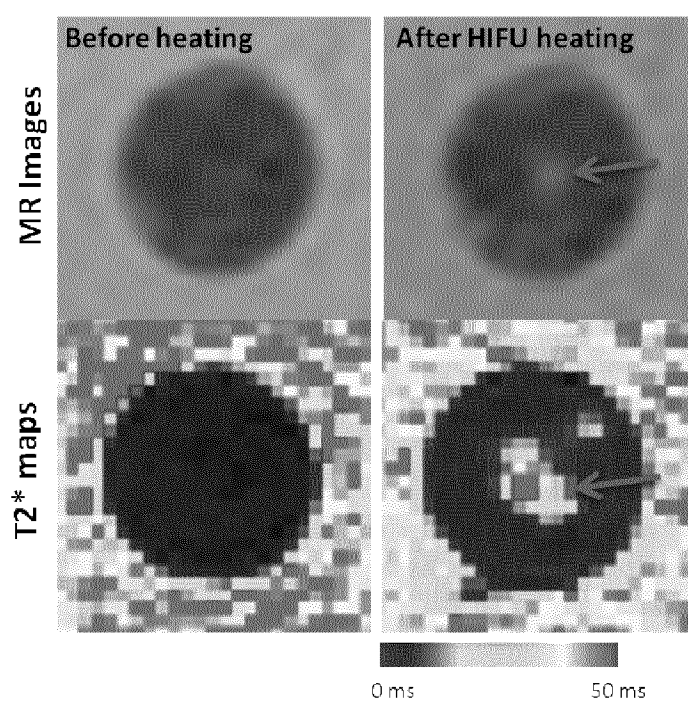


Figure 6