Multi-photon excited luminescence of magnetic FePt core-shell nanoparticles

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Abstract: We present magnetic FePt nanoparticles with a hydrophilic, inert, and biocompatible silico-tungsten oxide shell. The particles can be functionalized, optically detected, and optically manipulated. To show the functionalization the fluorescent dye NOPS was bound to the FePt core-shell nanoparticles with propyl-triethoxy-silane linkers and the fluorescence of the labeled particles were observed in ethanol (EtOH). In aqueous dispersion the NOPS fluorescence is quenched making them invisible using 1-photon excitation. However, we observe bright luminescence of labeled and even unlabeled magnetic core-shell nanoparticles with multi-photon excitation. Luminescence can be detected in the near ultraviolet and the full visible spectral range by near infrared multi-photon excitation. For optical manipulation, we were able to drag clusters of particles, and maybe also single particles, by a focused laser beam that acts as optical tweezers by inducing an electric dipole in the insulated metal nanoparticles. In a first application, we show that the luminescence of the core-shell nanoparticles is bright enough for in vivo multi-photon imaging in the mouse neocortex down to cortical layer 5.

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1. Introduction

Since the development of colloid chemical routes for nanoparticle synthesis, metallic nanoparticles greatly raised expectations in view of their biomedical applications. This is due to the magnetic properties of some metallic particles but also due to novel possibilities of bio-functionalization [1,2]. For biomedical purposes, the first shell’s functionality comprises mainly two aspects. On one hand it provides good dispersibility in aqueous media and on the other hand chemical binding sites for synthetic coupling reactions. One suitable compound that can be employed for coating nanoparticles are polyoxometalates, which are inorganic cluster molecules synthesized in aqueous solution. Polyoxometalates offer a high degree of thermodynamic stability [3] and have been shown to cover the surface of gold nanoparticles in water via electrostatic interaction [4,5]. They also coat magnetic FePt nanoparticles during synthesis in non-polar organic solutions when modified to match the required solubility. The metal-oxide backbone of polyoxometalates provides a hydrophilic nanoparticle surface even after a thermal modification to a porous W-O network in the shell. Moreover, the hydrophilic surface allows ligation of the nanoparticles with bio-organic or organic molecules.

Another important and useful feature of nanoparticles is fluorescence. In general, if the particles are fluorescent their biochemical effects and specific accumulation properties in and around living cells can be studied in vitro but also in vivo. Some particles are intrinsically...
fluorescent like semiconductor quantum dots while others need additional coating with organic fluorescent dyes. This fluorescence can be excited by a 1-photon process but also by a 2-photon [6] or multi-photon process using infrared femtosecond (fs) -pulsed lasers. Multi-photon fluorescence excitation can be used for imaging by scanning a focused infrared fs-laser beam through the sample. Multi-photon microscopy [7] is less sensitive to light scattering than 1-photon microscopy due to infrared excitation, nonlinear absorption, and scanning of the sample. Additionally, multi-photon microscopy allows sectioning, and reduces photo-toxicity. These advantages make multi-photon microscopy in combination with genetic encoded or synthetic functional fluorescent dyes one of the most important imaging techniques with optical resolution in biology, and specifically in basic neuroscience [8].

Interestingly, not only fluorescent dyes but also noble metal surfaces and gold nanoparticles can be excited by multi-photon absorption to emit light. When absorbing simultaneously two or more infrared photons a fluorescent dye molecule is electronically and vibrationally excited, relaxes on the picosecond-scale to an electronically-excited, vibrational ground state, before returning to the electronic ground state by emission of a photon on the nanosecond scale. On noble metal surfaces [9] and in gold nanoparticles [10] multi-photon absorption generates an electron-hole pair, which, after energy loss due to electron and hole scattering processes, recombines by emission of a photon. In nanoparticles and rough surfaces of noble metals the luminescence is increased by resonant coupling with surface plasmons.

Focused light can also have another effect on particles in the size from tens of nanometers to tens of micrometers (for review see [11]): The electric field of the focused light polarizes the particles and traps them in its electric gradient. Mainly dielectric materials are used for optical trapping but also metallic and therefore reflecting nanoparticles with dimensions in the Rayleigh regime (particle size \(<<\) wavelength of light) can be successfully trapped [12].

Here we present a novel nanoparticle that combines all above described features: A core-shell magnetic nanoparticle system with a magnetic FePt core and an annealed, hydrophilic SiW<sub>x</sub>O<sub>y</sub> first shell. The SiW<sub>x</sub>O<sub>y</sub> first shell also enables functionalization as we proof by labeling with a fluorescent dye. We characterize the luminescence and fluorescence of the particles under 1-photon and multi-photon excitation and show that clusters of particles can be dragged by a focused laser beam. To prove the particle’s viability and visibility for biomedical applications we image the particles and clusters of particles in the cerebral cortex of mice through a cranial window. The nanoparticles used in this paper are ferromagnetic as will be shown elsewhere.

2. Materials and methods

2.1 Nanoparticles

The bimetallic FePt core-shell precursor nanoparticles were synthesized in a one-pot reaction from iron-(III)-acetylacetonat and platinum-(II)-acetylacetonat in the presence of modified Keggin-poly silico tungstate as described before [3] followed by high-temperature annealing in a reducing gas atmosphere at 700 °C for 3 hours. To prevent agglomeration during annealing, the nanoparticles were incorporated into a sodium-chloride matrix prior to the high temperature treatment, which was dissolved and washed out again using high-purity de-ionized water afterwards. The nanoparticles were dispersed in EtOH. The precursor nanoparticles where checked for monodispersity prior to the annealing procedure.

2.2 Fluorescent dye labeling of FePt nanoparticles

The magnetic core-shell nanoparticles were fluorescence-labeled using the N-(2,5-bis(dimethyllethyl)phenyl)-N´-(3-(trithoxysilyl)propyl)-Perylene-3,4,9,10-tetra-carboxylic acid (German: Säure) diimide (NOPS, previously called MPD), a home-made poly-aromate which was synthesized as described by Blechinger et al. [13]. The labeling reaction was initiated by adding 10 µl NOPS solution (10 mg/ml in chloroform) to 0.4 ml of nanoparticles.
(10-15 mg/ml dispersed in hexane). The solvents were subsequently evaporated in a N₂ gas stream and the residue was re-dispersed in 2 ml of EtOH by ultrasonication. The dispersion was heated under vigorous stirring in a closed screw-cap vial placed in an oil bath at a temperature of 140°C for 17 hours. After cooling to room temperature, the labeled nanoparticles were isolated by centrifugation (5400g, 15 min), washed four times with EtOH (each wash: re-dispersion in 1 ml EtOH, centrifugation at 5400g for 15 min) and finally stored in EtOH.

2.3 Electron microscopy and small-angle x-ray scattering

A FEI Titan 80-300 transmission electron microscope operated at an electron acceleration voltage of 300 kV was used to image the FePt nanoparticles in the high angle annular dark field mode. Due to the correction of residual aberrations a lateral resolution of 0.08 nm was achieved. The sample was prepared by drying in the particles dispersed in EtOH on ultrathin silicon windows. The magnetic FePt nanoparticles were individualized by oleylamine and oleic acid to prevent agglomeration due to magnetic interaction.

To determine the size distribution small-angle x-ray scattering was done on a commercial instrument (NanoStar, AXS Bruker GmbH).

2.4 One-photon fluorescence spectroscopy

Fluorescence spectra of the labeled nanoparticles were measured with a F900 luminescence spectrometer (Edinburgh Analytical Instruments, Livingston, UK), using an excitation wavelength of 488 nm.

2.5 Multi-photon fluorescence imaging

Multi-photon imaging was done using a custom built multi-photon microscope (MOM, Sutter Instruments) running ScanImage software [14]. The excitation wavelength was 900 nm or 1000 nm of a ultrafast Ti:sapphire laser (Vision II, Coherent). A 25 × /1.05 numerical aperture water-immersion objective (Olympus) and two GaAsP photomultiplier-tubes (Hamamatsu) were used. Fluorescence was detected simultaneously in the green (490 - 560 nm bandpass filter, Chroma) and red (570 - 640 nm bandpass filter, Chroma). The laser power at the objective front lens was 0.4 mW.

The dragging of particle clusters was done on a commercial multi-photon microscope (LSM710, Zeiss) with pulsed Ti:sapphire laser (Chameleon, Coherent) and a 40x/1.0 water immersion objective (Zeiss) with focal laser power of 10 to 30 mW. Image analysis, like particle selection, was done with ImageJ (NIH) and Igor Pro (WaveMetrics).

2.6 Multi-photon spectroscopy

Multi-photon excited fluorescence spectra were measured on a commercial multi-photon microscope (LSM710, Zeiss) in lambda-mode where an array of photomultiplier tubes collect photons with a spectral resolution of 9.7 nm. A 40x/1.0 NA water-immersion or 20x/0.5 NA water-immersion objective (Zeiss) was used for excitation and collection of particle luminescence. The particles were dried in from an EtOH dispersion and then water or EtOH was added to measure the luminescence of particles and clusters of particles. Care was taken to avoid saturation of the detectors. For spectral analysis the images were background corrected and corrected for wavelength-dependent instrument sensitivity by calibrating the setup with standardized spectra of DAPI and SR101 [15]. Laser power was measured at the objective front lens. Spectral analysis was done with ImageJ (NIH) and Igor Pro (WaveMetrics).

2.7 In vivo experiments

All animal experiments were approved by the O.I.S.T. Institutional Animal Care and Use Committee (IACUC). At first mice underwent a local gene manipulation to allow multi-
photon imaging of neurons. This procedure was described before [16-18]. In short: A 0.5 mm craniotomy was made above barrel cortex (1.5 mm posterior of bregma, 2.5 mm lateral) in two mice under ketamine/xylazine anesthesia. Through this craniotomy an adeno-associated virus (AAV) delivering the gene of the green fluorescent protein (GFP) under the control of the human synapsin promoter (University of Pennsylvania Vector Core Facility) was injected at a depth of 500 µm with a quartz pipette beveled to a 10 µm opening. 70 nl of viral vector stock with a titer of 5.5 \(10^{13}\) genetic copies/ml were injected. The skin was closed again and the animal recovered.

4 days after AAV injection a 4 mm craniotomy was made under ketamine/xylazine anesthesia and 140 nl of NOPS-labeled magnetic nanoparticles dispersed in PBS were injected in a depth of 400 µm about 500 µm off the center of the previous virus injection. After particle injection a chronic cranial window [19] was mounted. After the surgery the anesthetized mice were used for imaging with the above described custom build multi-photon microscope (MOM). Stacks of images were recorded with images every 1 µm in z-direction and averaged 4 times, except imaging at 700 µm where 100 averages were taken. The wavelength was 1000 nm and the laser power at the objective front lens varied with imaging depth between 7 and 112 mW.

3. Characterization and functionalization of FePt core-shell nanoparticles

The nanoparticles consist of a magnetic FePt core with an average diameter of 2.3 nm (Fig. 1(a)) as shown by high resolution scanning transmission electron microscopy (HR-STEM). A hydrophilic, annealed first shell of silicone-tungsten-oxide surrounds the core and increases the average diameter to 3 nm as revealed by averaging many HR-STEM single-particle images. Small-angle X-ray scattering (SAXS) experiments have shown a size distribution of the annealed particles from 3 nm to several tens of nanometers. The larger particles are most likely nanoparticles which clustered due to magnetic interaction.

To show the possibility of FePt core-shell nanoparticle functionalization we labeled the particles with NOPS [13] by propyl-triethoxy-silane linkers to the silico-tungsten oxide shell (Fig. 1(b)).

![HR-TEM image of magnetic FePt core-shell nanoparticles, however, the shell is not visible without averaging (a). Tungsten oxide covering the surface of the crystalline nanoparticles causes dispersibility in polar solvents like EtOH and water but also allows functionalization as, for example, labeling with the fluorescence dye NOPS as illustrated schematically in (b).](image)

The estimated hydrodynamic diameter of these labeled core-shell nanoparticles is 8 nm assuming a double hydrate shell and full lateral extension of the NOPS molecules attached to the nanoparticles.
The size of the FePt core-shell and the functionalized particle are very small in comparison to many other nanoparticles [20] and also smaller than the previously published non-annealed particles which are 4.7 nm in diameter [3]. The particles are of similar size as proteins like the green fluorescent protein that has a diameter of 3 nm and a length of 4 nm [21]. However, due to their ferromagnetic properties agglomerates are expected to form.

4. One-photon excited fluorescence of NOPS-labeled FePt core-shell nanoparticles

The fluorescence of NOPS labeled particles was measured in cuvettes with dispersions of particles. In EtOH the labeled particles show two emission bands with maxima at 540 nm and 570 nm (Fig. 2) in agreement with 1-photon-emission spectra recorded for other NOPS-labeled nanoparticles [13]. However, the fluorescence is quenched once the dye-labeled nanoparticles are dispersed in de-ionized water. We also could not detect any fluorescence from the unlabeled or labeled nanoparticles by 1-photon excitation with a confocal microscope. Upon re-dispersion of the NOPS-labeled FePt core-shell nanoparticles in EtOH we again observe the regular fluorescence spectrum of NOPS.

These experiments show that the functional group, in this case NOPS, is covalently bound to the FePt core-shell nanoparticle. However, neither the unlabeled nor the labeled FePt core-shell nanoparticles are visible in aqueous solutions with 1-photon excitation.

Fig. 2. Fluorescence of the NOPS-labeled FePt nanoparticles is observed upon 1-photon excitation at a wavelength of 488 nm when dispersed in EtOH (red). The fluorescence of the labeled nanoparticles is fully quenched in H2O (blue) but recovers after re-dispersion in EtOH (green).

5. Multi-photon excited luminescence of magnetic FePt core-shell nanoparticles

Despite the quenching of fluorescence upon 1-photon excitation we tested the labeled and unlabeled particles with multi-photon microscopy. For these experiments the particles were immobilized by drying the EtOH suspension on a glass cover slip for fixation before adding solvent for imaging. With excitation by the ultrafast laser oscillator we find bright red and slightly weaker green fluorescence of the NOPS-labeled particles (Fig. 3(a), 3(b)). The images show a wide range of cluster sizes. If the smallest detected dots are single particles is not clear. Surprisingly, also the unlabeled magnetic FePt particles show bright luminescence under multi-photon excitation in EtOH and water (Fig. 3(c), 3(d)).
Fig. 3. FePt nanoparticles or clusters of particles immobilized on a glass slide show bright luminescence in the two wavelength intervals (green: bandpass 490 nm to 560 nm, red: bandpass 570 nm to 640 nm) using multi-photon excitation. The fluorescence emission was evident for both, dye-labeled (a, b) and non-labeled nanoparticles (c, d) in EtOH (a, c) and in H2O (b, d). The excitation wavelength was 900 nm and the laser power 0.4 mW with a 25x/1.05N.A. water immersion lens. The image pairs are normalized to the intensity of the red channel.

To study the particle luminescence and fluorescence in more detail we measured the multi-photon-excited emission spectrally resolved with a resolution of 9.7 nm in EtOH (Fig. 4(a)) and in water (Fig. 4(b)). The spectra are average spectra of all particles in the field of view. With the unlabeled particles we find bright luminescence which increases slightly from 400 nm to 700 nm in both solvents. The spectrum of the NOPS-labeled particles is composed of the luminescence spectrum of the unlabeled particles and the NOPS fluorescence. However, the emission peaks are shifted to 550 nm and 600 nm, compared to 540 nm and 570 nm with one-photon excitation. Also, the peak amplitude ratio at 550 nm and 600 nm changed to about 1:1 compared the ratio of 2:1 at 540 nm and 570 nm in the 1-photon measurement in EtOH. Additionally, the red spectral tail continues much further than with 1-photon excitation. The fluorescence and luminescence is independent of the excitation wavelength in the tested range between 800 nm and 1000 nm with a laser power of 1 mW. The intensity ratios at 1 mW focal laser power excitation at 800 nm, 900 nm, and 1000 nm are 1:0.7:0.7.

Surprisingly, the luminescence intensity increased with the power of 3.8 to 3.2 in the emission range from 433 nm (40 nm spectral width) to 672 nm (40 nm spectral width) of the excitation laser power below 3.2 mW (Fig. 4(c)). This indicates that a multi-photon excitation process causes the luminescence of the FePt core-shell nanoparticles in the full spectral range and that for lower wavelength, and so higher energy, more photons are necessary. Most likely, the larger size of nanoparticles compared to dye molecules increases the probability of multi-photon absorption.

In some spectra also the backscattered or back reflected second harmonic light was observed at half the excitation wavelength (Fig. 4(a), 4(b)) as described before for rough noble metal surfaces [9].
Fig. 4. Average multi-photon-excited fluorescence/luminescence spectra of NOPS-labeled and unlabeled magnetic FePt core-shell particles immobilized on glass slides in EtOH (a) and H₂O (b) excited with 1.5 mW at 800 nm, 900 nm, 1000 nm. Spectra were normalized to the intensity at 507 nm. The difference of the two spectra resembles the emission spectrum of NOPS but shows significant differences compared to the 1-photon emission spectrum in EtOH (Fig. 2). In some spectra the backscattered or reflected second harmonic signal is visible (arrows). The intensity of unlabeled magnetic FePt core-shell nanoparticles, immobilized on a glass slide with water added and excited at 1000 nm, increases wavelength-dependent by the power of 3.8 to 3.2 up to an excitation power of 3.2 mW (c). Background-corrected data was fitted by $y(x) = ax^b$. Values of $b$ are listed ± standard deviation.

The broad band luminescence of the FePt core-shell nanoparticles and the increasing luminescence with a power between 3 and 4 are in accordance with multi-photon generation of an electron/hole pair and luminescence after electron and hole scattering processes. The bright luminescence is most probably caused by resonant coupling of the electron/hole pair to surface plasmons as shown before from noble metal particles and rough surfaces. The wide spectral range of multi-photon excitability and huge emission wavelength range makes these particles unique: These nanoparticles can be combined for imaging with any other fluorescent dye because they can be excited at any Ti:sapphire wavelength. At the same time the particles’ luminescence overlaps and exceeds the emission range of any other fluorescent dye. By dual channel recording the particles can be easily detected and separated from other sources of emission if one channel detects the dye fluorescence overlaid with particle luminescence and the second channel just detects the particle luminescence (see in vivo imaging example below).

Further investigations will be necessary to understand why the particles show bright multi-photon excited luminescence and no one-photon excited luminescence.
6. FePt core-shell nanoparticles can be manipulated with optical tweezers

When using laser power of more than 10 mW the FePt core-shell nanoparticles or clusters of nanoparticles were dragged through the bath by the scanning laser beam (Fig. 5). In these phenomenological experiments the particles are dragged and imaged at first in direction of the fast scan direction before being left behind by the laser focus. When scanning the following line the laser catches and images the particle again and pulls it to the current scan line and in the direction of the fast scan line. This process is repeated and results in a diagonal track pattern of the particles traveling through the imaging field. Rotation of the scan direction at the same imaging location also changed the direction of the particle movement (Fig. 5(a), 5(b), 5(c)). For bi-directional scanning where the beam moves at the same speed from left to right as from right to left the particles are pulled parallel to the slow scan direction (data not shown).

![Fig. 5. FePt core-shell nanoparticles or clusters of particles are dragged by the scanning focus of a fs-infrared laser beam when the laser power exceeds 10 mW. White arrows indicate the scan direction of the laser beam through the sample. The yellow arrows indicate the main movement direction of the particles. Three scanning direction (a, 0°; b, 90°; c, 180°) in the same imaging location were tested.](image)

The rotation of the particle movement with the rotation of the scan direction proves that the particle movement only depends on the direction of the laser but not on any flow in the chamber.

The dragging is due to the induced electrical dipole within the nanoparticles in combination with the gradient of the electrical field generated by the laser focus. This suggests that the presented particles can also be positioned with optical traps. The laser-induced electrical dipole moment can be attributed to the dielectric silico-tungsten oxide shell but also to the metallic core due to its size within the Rayleigh regime [12]. Due to their magnetic field the FePt particles with their insulating oxide shell agglomerate and behave like a dielectric cluster. We propose that materials made from such nanoparticles will have a nanoparticle-size dependent susceptibility.

7. In vivo multi-photon imaging of NOPS-labeled magnetic FePt core-shell nanoparticles in the barrel cortex of mouse

As a first example of an application, we image the nanoparticles with multi-photon microscopy in vivo (Fig. 6) to show their visibility. We genetically targeted a subset of neurons with the green fluorescent protein (GFP) by gene transfer through a viral vector in the murine somatosensory cortex. GFP acts as a reference for the particle luminescence. To do this an adeno-associated virus (AAV) was injected through a 1 mm craniotomy into the cortex. After the expression of GFP was high enough for visualization with two-photon microscopy the NOPS-labeled magnetic FePt nanoparticles were injected under deep
Fig. 6. **In vivo** imaging of NOPS-labeled magnetic nanoparticles or clusters of particles in the barrel cortex of an anesthetized mouse. To label a subset of neurons in barrel cortex at first a viral vector is injected delivering the DNA of a fluorescent protein. 4 days later magnetic FePt nanoparticles were injected and imaged through a chronic cranial window with multi-photon microscopy.

Anesthesia through a craniotomy about 500 µm away from the injection site of the viral vector to avoid dense overlay of neuronal labeling and particle luminescence. Then a window was mounted on the craniotomy to seal the skull. After a 4 hour waiting period the brain region where the particles were injected was imaged in the still anesthetized animal. Stacks of images were recorded with images every 1 µm in z-direction starting from the dura mater to a depth of 700 µm.

We can clearly image the particles in the green and red channel (Fig. 7(a), 7(b)) as expected from the particle imaging in water (Fig. 3(d)) while the genetically modified neurons expressing GFP are only visible in the green channel. The reconstruction starts at the dura (top), followed by a 30 µm-wide dark gap between dura and pia and then 670 µm imaging into cortex. The overlay shows the particles yellow as they emit fluorescence in the green and red channel while GFP is only detected in the green channel (4 averages, Fig. 7(b), except last image). With 100 averages we can still see the particles 700 µm below the dura (Fig. 7(b), last image).

The intensity of the fluorescence is comparable to that of neuronal processes labeled with GFP in the same experiment.

The distribution of fluorescence intensity of the particle indicates a wide range of nanoparticle agglomerates and maybe also single particles (Fig. 8). As single particles are hydrophilic they should not pass cell membranes and so they underlie hindered diffusion in the extracellular space which has a width of 38-64 nm [22]. With an expected diffusion coefficient of about 150-200 µm²/s [23] the degree of hindrance will determine if single particles or small clusters of a few particles can be seen in averaged images as shown in Fig. 7 and 8.

The bright fluorescence under **in vivo** conditions allows tracking the nanoparticles over time using multi-photon microscopy. The particle injection can be monitored and treatment or clearance can be followed. Chronic cranial windows allow observations of a brain region over months. The combination of these features will allow optimizing parameters in animal models.
Fig. 7. Multi-photon imaging in the mouse barrel cortex in vivo after nanoparticle injection. A stack of images was recorded with two channels starting from the dura and then reconstructed to show the side view (a). Clusters of NOPS-labeled magnetic FePt core-shell nanoparticles and maybe also single particles are visible in the green (left) and red (middle) channel while the axons and dendrites of GFP expressing neurons are only visible in the green, as can be seen in the overlay (right). Particles or clusters of particles can be detected in xy images between axons (majority of processes) and dendrites down to a depth of 700 µm (b). Depth of imaging is indicated in the upper left corner corresponding to the arrows in (a). For the image at 700 µm in (b) the contrast and averaging was increased in comparison to the images at lower depth. The dura is visible due to backscattering of the second harmonic signal of collagen. The excitation wavelength was 1000 nm.
Fig. 8. In vivo imaging as in Fig. 7 but at higher magnification shows nanoparticles (yellow) and axons of cortical pyramidal neurons (green) in cortical layer 1, 90 µm below the brain surface. The bright green spots along the axons are synaptic boutons.

The images show the viability of multi-photon imaging of the particles in the intact brain. This will allow controlling the particles with optical resolution while testing the magnetic properties in living organisms. While the nanoparticles were injected extracellularly, electron microscopic investigations will be required to clarify whether the nanoparticles remain extracellular due to their hydrophilic shell and their clustering or whether they enter cells by endocytosis. With a diameter of 3 nm for single unlabeled particles or 8 nm for single NOPS-labeled particles, even small clusters fit well between cells in the brain where the extracellular space has a width of 38-64 nm [22].

The small size of single particles and the chance of small clusters is also a good basis for localized hyperthermia treatment of brain tumors. Localized hyperthermia applications are based on guiding the particles at first to the target region by magnetic fields. Then high-frequency magnetic fields are used to induce an oscillation of the magnetic nanoparticles in the tissue and so locally raise the tissue’s temperature [2,24,25]. For specificity the nanoparticles can be equipped with marker ligands that bind to malignant tumor cells and so overheat and kill the targeted cells.

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