Supporting Information


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Materials and methods

For the synthesis of the dl-AuFe$_3$O$_4$ NPs, gold acetate Au(ac)$_3$ (>99.9%, Alfa Aesar), iron pentacarbonyl Fe(CO)$_5$ (>99.99%, Sigma-Aldrich), dibenzyl ether (>98%, Merck), oleic acid (OA, >99%, Sigma-Aldrich), oleylamine (OLA, 70%, Sigma-Aldrich), 1,2-hexadecanediol (90%, Sigma-Aldrich), hexane (>95%, Sigma Aldrich) and ethanol absolute anhydrous (> 99.9%, Carlo Erba Reagents) were used. The mixture was heated using hemispherical heating mantle (model WiseTherm WHM 12112 from Witeg Labortechnik GmbH) connected to a temperature controller (J-KEM, model 310) with J-type Teflon thermocouple. Chitosan Glutamate (Protosan up G 113, 86% deacetylation, 45% glutamate content) was purchased from BioPolymer AS, NovaMatrix. All the other reagents were purchased from Sigma Aldrich and used as received. All aqueous solutions were prepared with deionized water obtained using an ultrafiltration system (Milli-Q, Millipore) with a measured resistivity above 18 MΩ/cm. Fourier transform infrared (FTIR) spectra were recorded on a Perkin-Elmer Spectrum 2000. Thermogravimetric analysis (TGA) was performed using a TA Instruments TGA-SDT 2960 on sample sizes from 1 to 3 mg, and the mass was recorded as a function of temperature. DLS measurements were performed on a Malvern Zetasizernano-S working with a 532 nm laser beam. ζ potential measurements were conducted in DTS1060C-Clear disposable zeta cells at 25 °C. TEM and STEM imaging combined with EDXS was performed by an aberration-corrected probe in a TEM (JEOL JEM-ARM200F), using the cold field emission source, equipped with EDXS (Centurio 100 mm$^2$, JEOL) system. The probe size was 0.1 nm, with a current of 20 pA and the convergence semi-angle of 24 mrad. The collection semi angle for HAADF detector was set between 45-180 mrad. The EDXS spectrum images were performed under continuous scanning mode with pixel dwell time of 25 micro seconds and by using probe currents of 250 pA, therefore degrading the spatial resolution to approaching values of 0.2
nm. Magnetic measurements were performed on a 10 mg of a dried sample with a vibrating-sample magnetometer (VSM) MicroSense model FCM 10 operated at room temperature.

**Synthesis of Chitosan-TGA (Chitosan-Thioglycolic Acid) and Chitosan-HCA (Chitosan-Hydrocaffeic Acid)**

Chitosan-TGA and Chitosan-HCA have been synthetized following two different methods reported in literature; briefly, a solution was prepared by dissolving Chitosan Glutamate (200 mg) in 20 ml of 1% (w/w) acid acetic solution.

After chitosan complete solubilisation, thioglycolic acid (200 µl, 70% w/w in water) and N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC, 200 mg, 1.04 mmol) were added to the reaction mixture. The pH of reaction mixture was adjusted to 6 with NaOH solution (0.1 M) and the resulting solution was stirred over night at room temperature. Then the solution was dialyzed against water (MWCO 3500 Da) and stored at -20 °C.¹

Chitosan-HCA was synthetized by dissolving Chitosan Glutamate (200 mg) in MES Buffer (10% w/v). Meanwhile, a different solution was prepared by dissolving the hydrocaffeic acid (HCA, 236 mg, 1.30 mmol) in a water/ethanol solution (50:50) with the N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC, 373.24 mg, 1.95 mmol). The final HCA solution was added drop wise to the chitosan solution and the reaction mixture was stirred overnight at room temperature. Then the solution was dialyzed against water (MWCO 3500 Da) and stored at -20 °C.²

**Synthesis of dl-AuFe₃O₄ NPs**

dl-AuFe₃O₄ NPs were prepared by using a modification of the procedure reported in the literature.³ Iron pentacarbonyl (Fe(CO)₅, 0.15 mL) and gold acetate (Au(ac)₃, 0.18 g) were dissolved in 20 mL of dibenzyl ether that contained surfactants oleic acid (0.1 mL) and oleylamine (0.1 mL). Finally, reducing agent (1,2-hexadecanediol, 350 mg) was added to the flask and the mixture was homogenized by stirring at room temperature under argon atmosphere for 1 hour. Then the temperature was raised to 250°C (in 45 minutes) and the reaction mixture was refluxed for 1 h to form dl-AuFe₃O₄ NPs. Subsequently, the reaction mixture was cooled to room temperature and NPs were precipitated by the addition of ethanol. NPs were purified with centrifugation (6000rpm/10min/3cycles) and finally dispersed in hexane. TEM images of as-synthesized dl-Au/Fe₃O₄ NPs taken at different magnifications (50,000-300,000x) are shown in **Fig. S1**, where the dumbbell structure is evidently shown.
Figure S1. TEM images of as-synthesized dl-Au/Fe₃O₄ NPs taken at different magnifications a) 50.000x, b) 80,000 and c) 300,000x.

**Synthesis of dl-AuFe₃O₄@Chit**

dl-AuFe₃O₄@Chit has been synthetized by oil-in-water method following a recent study reported in literature.² In particular, chitosan-TGA (16 mg) and chitosan-HCA (16 mg) was dispersed in 10 mL of water and the solution was stirred over night to allow the complete dissolution of the polymers. Then dl-AuFe₃O₄ NPs (20 mg, dried from hexane in nitrogen flux) have been dissolved in 1 ml of chloroform and the obtained suspension has been added dropwise to the chitosan water solution. The mixture was sonicated for 3 minutes by using a Branson sonicator 450 equipped with a microtip (600 W input, 80% ampl). Organic solvent was removed under vacuum and the obtained solution was purified by magnetic decantation washing with water and concentrated into centrifugal filter devices (Amicon Ultra, Ultrace membrane with 100.000 NMWL, Millipore, USA) until a final volume of 5 mL.

Figure S2. DLS analysis of dl-AuFe₃O₄@Chit; the graphs shows the results about hydrodynamic diameter (a) and ζ-potential (b).
Figure S3. dl-AuFe$_3$O$_4$@Chit size and $\zeta$-potential variations against pH.

Figure S4. FTIR analysis comparing dl-AuFe$_3$O$_4$ NPs and dl-AuFe$_3$O$_4$@Chit spectra.

Figure S5. TGA analysis comparing dl-AuFe$_3$O$_4$ NPs (a) and dl-AuFe$_3$O$_4$@Chit (b) samples.
Figure S6. UV-Vis spectrum of dl-AuFe₃O₄ NPs (red line) and dl-AuFe₃O₄@Chit (blue line).

Figure S7. Magnetic properties of the dl-AuFe₃O₄@Chit sample. The obtained saturation magnetization value for the dl-AuFe₃O₄@Chit is found to be $M_s = 4.3$ emu/g. This value is lower than for the pure Fe oxide NPs of similar size, which is expected due to the dilution of the magnetic phase by the presence of the non-magnetic components in the sample, Au and chitosan.

Lower magnification TEM images shown in Fig. S8 indicate that high number of dl-Au/Fe₃O₄@Chit capsules is formed with the size of 60-100 nm. In dark-field TEM image (Fig. S8a) the objects with bright contrast correspond to dl-Au/Fe₃O₄ NPs and it can be seen that are arranged in a clusters. In a bright-field TEM image (Fig. S8b) also the chitosan matrix that surrounds the dl-Au/Fe₃O₄ clusters is visible. No agglomeration of dl-Au/Fe₃O₄@Chit capsules was detected and a representative image of two separated dl-Au/Fe₃O₄@Chit capsules is shown Fig. S8c.
Figure S8. Low-magnification a) dark-field and b) bright-field TEM image of dl-Au/Fe₃O₄@Chit capsules and c) TEM image of two dl-Au/Fe₃O₄@Chit capsules at higher magnification.

In vitro ΔT measurements

The compound was disposed in a final volume of 100 µl at the desired concentration in a 96 wells sterile plate. The light source used in the experiments is a near-infrared fiber coupled diode laser LIMO25-F200-DL808 (from LIMO Lissotschenko Mikrooptik GmbH). It is integrated in power supply IB Photonics LDD80C which proved fine adjustment of the output power and thermal stabilization of the laser. The center wavelength of the output radiation is 808 nm and the maximum output power is 25 W. In this experiment it is used with an optical fiber with 400 µm core diameter. The output beam is collimated with aspherical lens with focus distance of 11 mm (Thorlabs C220TM-B). In current optical setup the laser spot diameter at the surface of sample holder is around 4.2 mm, measured at 1/e² intensity high. Three different irradiations conditions were applied: 15 W/cm² (≈ 2.1 W/well), 5 W/cm² (≈0.8 W/well), 1 W/cm² (≈0.15-0.09 W/well). The time of irradiation for all the powers was of 1 minute. The heating effect of the laser on the sample and reached equilibrium temperature was measured by thermal imaging infrared camera Flir InfraCam. It has a temperature range from -10 °C to +350 °C and sensitivity better than 0.2 °C. The resolution of the sensor is 120x120 pixels. In particular, t=0 was recorded before irradiation and t=1 just after the heating. Each point was analysed in triplicate and representative images taken with the thermocamera are reported.
Figure S9. Images taken with the thermocamera before and after laser irradiation.

**Photo-acoustic experiments in vitro**

The PA analyses were performed on a VEVO-2100 LAZR (VisualSonics - Fujifilm System) equipment, a fully Engineered Photoacoustic dual modality system, integrating photoacoustics with high-frequency ultrasound. The Vevo LAZR system is equipped with a 20 Hz tunable laser with a wavelength range of 680 - 970 nm and a spatial resolution of 40 µm. The transducer device used for the in vitro experiments was an integrated PA and US transducer (LZ-250) which operates between 13 - 24 MHz, being the peak frequency applied during our experiments of 21 MHz. The PA transducer probe setting parameters were: power output 100%, B-Mode gain 18 dB and photoacoustic gain 40 dB with high sensitivity and persistence equal to 6, for all image acquisitions.

With the aim of optimizing the in vitro scan, a suitable phantom for US and PA imaging was assembled. The phantom for photo-acoustic in vitro tests consisted of polyurethane tubing (outer diameter 0.9 mm; internal diameter 0.6 mm) submerged in a vinyl and borax gel (Figure S11).

PA Spectra were recorded on dl-AuFe\textsubscript{3}O\textsubscript{4}@Chit NPs test solution and on samples diluted with Phosphate Buffered Saline (PBS) solution within a wavelength range of 680 - 970 nm. Analyses were performed at concentrations ranging between 8.0 and 4.9 mM (Figure S10). Standard deviation values for PA intensity were in the range from 0.15 to 0.19. Fit trend behaviour of all spectra showed an exponential decay with an R\textsuperscript{2} value of 0.97.
**Figure S10.** Graph for PA intensity signal recorded at 680 nm for dl-AuFe$_3$O$_4$@Chit solutions at different concentrations (a) and different wavelengths (b). PA spectra for the dl-AuFe$_3$O$_4$@Chit solution at a concentration of 8 mM and a fitting curve expressing an exponential decay (inset).

**Figure S11.** Phantom structure used for US-PA in vitro experiments (left) and phantom US image: axial tube section. The shadow cones projecting under the tubes are visible (right).

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