

Supporting Information

For the characterization of size and shape of prepared MNPs, transmission electron microscopy (TEM, Hitachi H-7500) was used to acquire their images required for the analysis.

Field emission scanning electron microscope (FESEM, JSM-6700F, JEOL, Tokyo, Japan) was used to obtain the images required for the morphological investigations. NIH Image J software (1.46r/Java 1.6.0_20 (32-bit)) was used for the image analysis.

Fourier Transform Infrared Spectroscopy (FT-IR) through spectra analysis in the range of 400-4000 cm^{-1} provided the opportunity to chemically investigate the integrity of the samples.

The instrument operated to measure the hydrodynamic diameters of the samples was a ZetaSizerNano ZS90 particle-sizing instrument (Malvern, Worcestershire, UK). Prior to measurements, samples were equilibrated at 25°C for 5 min.

The iron concentration of the samples was determined using atomic absorption spectroscopy (AAS) at 248.3 nm. The AAS instrument used was a SpectrAA-10 Plus spectrometer (Varian, France).

Crystallinity of the samples was verified through X-ray diffraction (XRD). The instrument operated was a Panalytical X'PERT MPD X-ray diffractometer. The diffractometer was equipped with a copper anode and it was able to generate high intensity Cu K α radiation ($\lambda = 1.54065 \text{ \AA}$) from 10° to 90° as the range of 2 θ .

The changes in chemical and physical characteristics of the samples were analysed using thermogravimetric analysis via a thermogravimetric analyser (Shimadzu, modelo TG-50) under certain adjustments: heating rate of 10°C/min, N₂ flowing rate of 50 ml/min and temperature ranging from 25 to 800°C.

SAR is known as the magnitude of the heat generated per unit gram of a magnetic material per unit time. 500 μl of MNPs-PEG-TRA at different concentrations (0.05, 0.1, 0.2, 2 and 10 mg/ml) was used to measure the SAR values at different concentrations. Via a sample holder, the samples which were already transferred to 0.5 ml micro-centrifuge tubes were then located in the center of an 8-turn copper coil (40 mm inner diameter). The coil was water-cooled and connected to an induction power supply (2.4 kW, 150-400 kHz, EASYHEAT, Ambrell, USA) producing AMF with a constant frequency of 393 kHz and amplitude of up to 33.5 kA/m. To add another thermal barrier, a jacket within the coil through which a coolant of 25 °C was in circulation prior to the initiation of AMF (33.5 kA/m) exposure. Increment in temperature was recorded in intervals of 1 s using a fiber optic temperature sensor (FluoTemp GT-ST Temperature Converter, Photon Control, Canada) connected to FluoSoft software (version 2.9) for data

plotting. The assembly of the system used to carry out the SAR is schematically shown in figure 1.

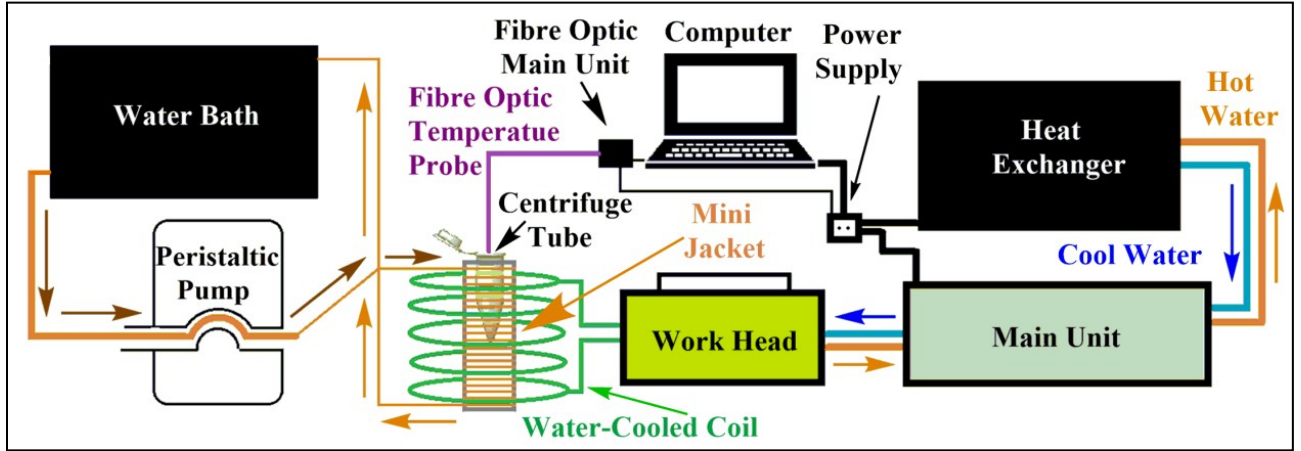


Figure 1. The schematic assembly of the experimental set-up for induction heating to carry out SAR and *in vitro* hyperthermia.

The SAR values of the samples were obtained through the equation (1):

$$SAR = \left(\frac{C \cdot V_s}{m} \right) \times \left(\frac{dT}{dt} \right) \quad (1)$$

where C is the specific heat capacity of the medium which is water ($C_w = 4.185 \text{ J/g } ^\circ\text{C}$), V_s is the volume of the sample, m is the iron mass present in the sample and dT/dt is the initial slope of the temperature-time curve ¹.

The Bradford assay was performed using Coomassie protein assay reagent² to determine concentration of immobilized trastuzumab in surface of MNPs-PEG-TRA. In order to quantify the efficiency of trastuzumab immobilization, the amount of unbound trastuzumab after immobilization experiment in the supernatant was determined via the Bradford assay after the magnetic separation. Then from the known amount of trastuzumab used in the immobilization experiment, the amount of immobilized trastuzumab calculated.

“Trastuzumab immobilization efficiency” is defined as follows:

$(\text{amount of trastuzumab after conjugation} / \text{weight of trastuzumab taken for conjugation}) \times 100\%$

Based on the Bradford assay result, the trastuzumab immobilization efficiency was about 80% and no appreciable trastuzumab was released after extensively washing the conjugated pellets.

For blood biocompatibility test, the blood sample was obtained from a male donor (29 years old, no history of extreme disease) at Health Centre of Universiti Teknologi Malaysia (UTM) and transferred to a tube containing citrate to avoid the blood sample from coagulating. Informed consent was obtained from the donor for the use of their blood and the dissemination of information about their blood in the scientific record. Medical advisory committee and the clinical ethic committee of Sultanah Aminah healthcare hospital Berhad, Malaysia in association with Ministry of Health (MOH) accepted the procedure as satisfactory. PBS solution (pH 7.4) was used to dilute the samples (0.2 ml) and the diluted samples were incubated together with 500 μ l plasma of fresh human blood under a certain condition: 30 min, 37°C and in a transparent plastic tube. Eventually, the PT, TT, APTT and FB were measured thrice for each of the samples ³. Moreover, the hard clotting time test was performed on glass slides to determine the real blood clotting time in the presence and absence of the nanoparticles. Briefly, samples were incubated with a drop of blood on the glass slide at 37° C. Blood coagulation started when fibrin threads attached to the needle. Through manual dipping in a hook (stainless-steel and coated with silicone) into the solution for detection of fibrin threads, monitoring of the hard clot formation was performed and once the first sign of fibrin formation appeared on the hook, clotting time was recorded. The test was repeated thrice for each sample to ensure reliable results. In order to carry out haemolysis study, 100 μ l of washed RBCs together with 900 μ l of each of the samples dispersed in saline was incubated for 2 h followed by the centrifugation: 5 min and 1500 rpm. Subsequently, to the collected supernatant, 900 μ l of saline was added and via a UV spectrophotometer (Carywin UV) the absorbance was recorded at 541 nm ⁴. Samples concentration was 250 μ g/ml of Fe for all tests.

Subsequently, for *in vitro* hyperthermia, the cells were grown to reach 80% confluence in T-25 cell culture flasks. Then, cells underwent incubation (4h) with MNPs-PEG-TRA dispersed in 7 ml of cell culture media (250 μ g Fe/ml). Subsequent to the incubation, washing with PBS was accomplished and the detachment of loose MNPs-PEG-TRA was performed using 0.25% trypsin/EDTA. Then, cells were resuspended

immediately in culture media for counting after which 5×10^6 cells were centrifuged (1000 rpm, 5 min) to catalyze the cell pellet formation. Over the course of hyperthermia, 0.1 ml of media containing the cell pellets in micro-centrifuge tube (0.5 ml) was placed in the center of an 8-turn water-cooled copper coil while the temperature of the water jacket inside the coil embracing the tube was maintained at 37 °C.

Prior to exposure to the AMF, samples were equilibrated at 37 °C. Then, an AMF of 230 kHz was exposed to the treated cells. The increment in temperature was recorded at 1s intervals via a fiber optic temperature sensor (FluoTemp GT-ST Temperature Converter, Photon Control, Canada) positioned at the center of the pellets and connected to FluoSoft software (version 2.9) for data plotting. Targeted temperatures (40 °C, 42 °C or 45 °C) were maintained for 20 min by adjusting the power of the induction power supply (2.4 kW, 150-400 kHz, EASYHEAT, Ambrell, USA) and then the pellets were allowed to cool down to 37 °C. Upon the dismissal of the exposure, the pellets were washed via PBS. Next, resuspension of the pellets in their related media was carried out prior to seeding them in 96-well plates (1×10^5 cells/well). The seeded cells were incubated for 24h, 48 h, 72h, 96 h and 120 h. Finally, the cell growth inhibition rate was gauged through MTT assay as mentioned earlier in our previous study⁵.

The temperature of water flow in the mini jacket was maintained using an insulating paper. The cells were transferred into micro-centrifuge tubes and placed at the center of the coil while temperature probe was used to record the temperature at intervals of 1s. The whole unit was placed under safety cabinet for anti-septic purposes.

In order to investigate the morphological alterations of cells by fluorescence microscope, the cells were prepared and treated in the same manner practiced in inverted microscopy study. Subsequent to hyperthermia, cells were allowed to re-grow for 24 h and then trypsinized and centrifuged at 1500 rpm for 5 min. Once the supernatant was removed, cold PBS (50 µl, pH 7.4) was added to the pellets. The staining procedure was carried out through using 2 µl of 1x working solution of nuclear stains. The working solution was prepared by mixing 500 µg of EB and 100 µg of AO in 1 ml of PBS. The stained cells were carefully observed under fluorescent microscope (AXIOVERT A1).

The presence of tumor cells in DMBA-induced mice was confirmed using H&E staining method. Upon necropsy, DMBA-induced tumor tissues were removed and fixed using 10% formalin immediately. Processing of the tissues was then accomplished. The processed tissues were paraffin-embedded and then sectioned at 5 µ and placed on glass slides to stain them using hematoxylin and eosin (H&E). The presence of tumor cells was confirmed under the inverted microscope (Nikon Eclipse Ti-S examination).

For quantitative biodistribution studies, mice were injected via tail vein with 100 μ l of MNPs, MNPs-PEG and MNPs-PEG-TRA (10 mg/kg body weight) and sacrificed at 4, 12 and 24 h time points. The blood was collected by cardiac puncture and after euthanasia, the heart, liver, spleen, lungs, kidneys, stomach, muscle and tumor were collected, weighted and dissolved completely by adding 2 ml of HCl and 1 ml of HNO₃ at 70°C for 6h. The solution was diluted with deionized water and filtered with 0.45 μ m Teflon filter. The samples were analyzed for iron using an atomic absorption spectroscopy (AAS) (VARIAN, model AA240FS). The uptakes of the iron in the organs were calculated as a mean percentage of injected doses per gram of organ tissues (%ID/g). Each sample and time point included six independent repetitions.

For qualitative biodistribution studies, DMBA-induced live models were injected via tail vein with 100 μ l of SPIONs-PEG-TRA (10 mg/kg) and sacrificed at 24 h after injection. To determine the SPIONs-PEG-TRA biodistribution in major organs including heart, liver, spleen, lungs, kidneys, stomach, muscle and tumor, these tissues were collected and washed with PBS and then were fixed in 10% paraformaldehyde and embedded in paraffin. Ultrathin sections prepared by an ultramicrotome were placed on 200 mesh copper grids for TEM analysis and stained with 2% osmium tetroxide (dissolved in distilled water) for 30 min according to the standard procedure⁶. All the stained sections were observed under a Hitachi HT7700 Bio-Medical TEM microscope.

For in vivo hyperthermia treatment, solution of MNPs-PEG-TRA (100 μ l, 10 mg/kg) in PBS was intravenously injected into DMBA tumor-bearing live models. A day later, live models belonged to group IV were anesthetized and laid inside the coil such that the position of the tumor was at the center of the coil. Once the live models were correctly positioned, the exposure to AMF was initiated. The AMF was provided by a 3-turn horizontal coil (inner diameter: 7 cm; length: 7 cm) connected to 2.4 kW, 150-400 kHz, EASYHEAT, Ambrell, USA. The frequency of the magnetic field was 230 kHz. Exposure to the AMF with the details mentioned above was performed for 20 min once a week for four weeks. Using a fiber optic temperature sensor (FluoTemp GT-ST Temperature Converter, Photon Control, Canada), the temperatures of tumor and rectum were gauged.

Figure S1 shows that the work-head of the induction heating system and the temperature probe were located under the safety cabinet for anti-septic reasons.

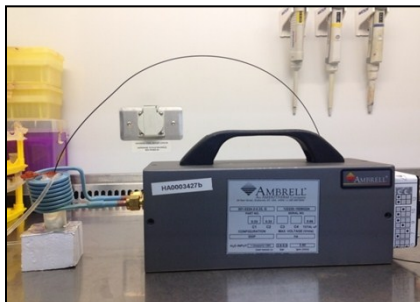


Figure S1. The 8-turn water-cooled coil connected to the work-head of the induction heating system.

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