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In vitro hyperthermia with improved colloidal stability and enhanced SAR of magnetic core/shell nanostructures

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ABSTRACT

Magnetic core/shell nanostructures of Fe_3O_4 nanoparticles coated with oleic acid and betaine-HCl were studied for their possible use in magnetic fluid hyperthermia (MFH). Their colloidal stability and heat induction ability were studied in different media viz. phosphate buffer solution (PBS), saline solution and glucose solution with different physiological conditions and in human serum. The results showed enhanced colloidal stability in these media owing to their high zeta potential values. Heat induction studies showed that specific absorption rates (SAR) of core/shells were 82–94 W/g at different pH of PBS and concentrations of NaCl and glucose. Interestingly, core/shells showed 78.45 ± 3.90 W/g SAR in human serum. The cytotoxicity of core/shells done on L929 and HeLa cell lines using 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide and trypan blue dye exclusion assays showed >89% and >80% cell viability for 24 and 48 h respectively. Core/shell structures were also found to be very efficient for *in vitro* MFH on cancer cell line. About 95% cell death was occurred in 90 min after hyperthermia treatment. The mechanism of cell death was found to be elevated ROS generation in cells after exposure to core/shells in external magnetic field. This study showed that these core/shells have a great potential to be used in *in vivo* MFH.

1. Introduction

Nanotechnology has become a key word of public interest, since people have realized the social and economic power of nanotechnology development and its applications [1–6]. In recent years, engineered nanoparticles started to become the most important components in nanotechnology. Heated under a high-frequency magnetic field, magnetic nanoparticles (MNPs) trigger drug release or produce hyperthermia/ablation of tissues [7], currently reported as magnetic fluid hyperthermia (MFH). In particular, oxide-based spinel ferrites [8] are considered very promising for MRI and hyperthermia treatment. Iron oxides are good candidates for MFH due to their well-known biocompatibility. The successful implication of MNPs for cancer hyperthermia therapy depends on both the intrinsic magnetization properties of MNPs and their biophysical properties such as biocompatibility, colloidal stability and specific absorption rate (SAR) at physiological pH. These attributes of MNPs can be achieved through their surface functionalization using chemical modifications [9–12].

Suspensions of superparamagnetic Fe_3O_4 nanoparticles have van der Waals forces and magnetic dipole–dipole interactions generated from residual magnetic moments, which tend to agglomerate the particles. Therefore, to form a stable dispersion, repulsive forces are required to keep each particle discrete and prevent it from amassing as larger and faster setting agglomerates. Steric hindrance plays an important role in stabilizing suspensions, which is accomplished by the protective shields on the oxide surface produced by molecules or polymers. Zeta potential is considered a key parameter to investigate the surface charge of Fe_3O_4 nanoparticles. The authors have some recent reports with Fe_3O_4 nanoparticles coated with organic biopolymers like chitosan, acrypol, oleic acid (OA) and betaine-HCl (BTH), improving their colloidal stability and heat induction ability with lowered cytotoxicity [13–17]. These coated MNPs were found to be better candidates for their use in hyperthermia application than the bare Fe_3O_4 MPs. The research on Fe_3O_4 MNPs based hyperthermia has been started because of their biocompatibility. The surface functionalization of the MNPs needs to be evaluated for their safe use in the biomedical field. In our recent publication we have synthesized OA–BTH coated Fe_3O_4 nanoparticles (OA–BTH–Fe_3O_4) and done their structural and biocompatible study for their successful application in cancer hyperthermia treatment [14].

In present manuscript, OA–BTH–Fe_3O_4 MNPs are thoroughly studied for their plausible use in MFH cancer therapy. Their colloidal stability is checked in water, NaCl, phosphate buffer solution (PBS) and glucose as base media. Concentrations and pH of each media is varied to study their effects on colloidal stability of MNPs. All these media are used to study heat induction ability of MNPs in order to get hyperthermia temperature profiles in each medium. Additionally, for the first time in literature, colloidal stability and heat induction ability of MNPs in human

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serum are studied and reported in this manuscript. MNPs were also studied for their cytotoxicity on HeLa and L929 cell lines with varying concentrations and exposure time. Finally, *in vitro* study of the coated MNPs was done and their efficiency of killing cancer cells after hyperthermia treatment was checked with respect to mechanism of cell death and ROS generation by confocal laser scanning microscopy (CLSM) using multiple staining of fluorescein isothiocyanate (FITC), propidium iodide (PI) and 4′, 6-diamidino-2-phenylindole (DAPI). There is no report in the literature till date on OA–BTH–Fe_3O_4 with their thorough study on colloidal stability, cytotoxicity, heat induction ability and *in vitro* hyperthermia study.

2. Materials

Ferrous chloride ($FeCl_2 \cdot 4H_2O$), hydrochloric acid (HCl), methanol, acetone, potassium hydrogen phosphate (K_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4), sodium chloride (NaCl), glucose and sodium hydroxide (NaOH) were procured from HiMedia, India. OA and BTH were purchased from Sigma-Aldrich, USA. Double distilled water was used throughout the procedure. Serum samples were obtained from a healthy donor with help of a local pathology laboratory.

3. Experimental

3.1. Synthesis of OA–BTH–Fe_3O_4 nanoparticles

MNPs were prepared using $FeCl_2 \cdot 4H_2O$ and bare MNPs were further functionalized with BTH as per the protocol reported earlier [13,14]. In brief, the obtained bare Fe_3O_4 MNPs were coated with OA. 1 g of bare Fe_3O_4 MNPs were dispersed in 100 mL of methanol and then heated to 80 °C with continuous stirring on magnetic stirrer. To this 10 mL of OA was added drop wise. The mixture was kept at 80 °C till all methanol get evaporated. The remaining mixture was filtered to remove excess of OA and washed several times with double distilled water and finally with acetone. The OA coated MNPs were collected and dried at 50 °C.

The OA coated nanoparticles (200 mg) were dispersed in 25 mL of 1% BTH with continuous stirring for 6 h. After that, coated MNPs were separated out using external magnetic field and washed several times with double distilled water. The coated MNPs were dried at 50 °C.

3.2. Characterizations

3.2.1. Colloidal stability

Zeta potential and hydrodynamic diameter (HDD) of particles were measured using PSS/NICOMP 380 ZLS particle sizing system (Santa Barbara, CA, USA) with a red He–Ne laser diode at 632.8 Å in fixed angle 90° plastic cell. The zeta potential measurements were performed at 25 °C after homogenization of 5 min. The measurements were carried out in different physiological media. At least three measurements were conducted for each parameter set for reproducibility. The instrument calibration was checked before each experiment using a latex suspension of known zeta potential (i.e. -55 ± 5 mV). The DLS graphs were shown as number weighted particle size distributions.

3.2.2. MTT assay

The issue of cytotoxicity was addressed for functionalized MNPs to be used for biomedical applications. The viability of the HeLa and L929 cell lines in presence of MNPs was assessed relative to cells in the control experiment (no MNP present) using 3-(4,5- dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT) assay and trypan blue dye exclusion (TB) assay. The cell lines were obtained from the National Centre for Cell Sciences, Pune, India and the detailed toxicity study was done in the National Toxicology Centre Pune, India (ISO 10993/ USP 32 NF 27) by MTT assay. The cells were grown in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% *v/v* fetal bovine serum, kanamycin (0.1 mg/mL), penicillin G (100 U/mL) and sodium bicarbonate (1.5 mg/mL) at 37 °C in a 5% CO_2 atmosphere. The cells were incubated with the concentration of 1×10^4 cells/mL in the medium for 24 h in a 96-well microtiter plate. After 24 h, the old media was replaced by fresh media and different proportions of sterile MNPs (0.1, 0.5, 1.0, 1.5 and 2.0 mg/mL of cultured media) were added. The total medium was then incubated at 37 °C in a 5% CO_2 atmosphere for 24 h (and 48 h). After 24 h (and 48 h), 10 μL MTT solution was added into each well including control wells. The plates were incubated for 3 h at 37 °C in a 5% CO_2 atmosphere for metabolization of MTT with the nanoparticles and cell media. The total medium was then removed by flicking the plates and only anchored cells remained in the wells. The cells were then washed with PBS and any formazan formed was extracted in 200 μL acidic isopropanol and finally the absorbance is read at 570 nm and from it, the cell viability is calculated. The experiments were replicated three times and the data was graphically presented as the mean. The same protocol was followed for L929 cell line.

3.2.3. TB assay

TB assay was done by following the procedure reported by Prasad et al. [18]. In brief, the cells were incubated with the concentration of 1×10^5 cells/mL for 24 h in a 96-well microtiter plate. After 24 h, the old media replaced by fresh media and different proportions of sterile coated MNPs (0.1, 0.5, 1.0, 1.5 and 2.0 mg/mL of cultured media). Then, the total medium is incubated at 37 °C in a 5% CO_2 atmosphere for 24 h and 48 h. After incubation of coated MNPs with HeLa cells, the well plates have been washed thrice with PBS to remove the MNPs from the cell surface. Both the attached and unattached cells have harvested and combined after trypsinization (0.020% trypsin, 25 min). Then, the cells stained with trypan-B dye and counted with a hemocytometer (Bright-Line, Hauser Scientific and Horsham, PA) to determine the percentage cell viability. The relative cell viability (%) compared with control well containing cells without MNPs are calculated by the equation: $-[A_{count}]_{tested} / [A_{count}]_{control} \times 100$. The same protocol was followed for L929 cell line.

3.2.4. In vitro hyperthermia study

Induction heating of Fe_3O_4 nanoparticles for hyperthermia application was performed in a plastic micro centrifuge tube (1.5 mL) using an induction heating unit (Easy Heat 8310, Ambrell; UK) with a 6 cm diameter (4 turns) heating coil. To keep the temperature of the coil at ambient temperature, a provision of water circulation in coils was provided. MNPs (2 mg as earlier reported suspended in 1 mL of distilled water) were placed at the centre of the coil and the applied frequency was 265 kHz. Particles were dispersed in water with ultrasonication for 20 min to achieve a good dispersion of the MNPs in carrier fluid. Samples were heated for 5 min with the desired current (400 A). For the conducted experiments, the magnetic field was calculated from the relationship:

$$H = \frac{1.25ni}{L} Oe$$

where n, i and L denote the number of turns, applied current and the diameter of the turn in cm, respectively. Calculated value of the magnetic field (H) at 400 A was 335.2 Oe (equivalent to 26.7 kA/m). Temperature raise of the system was measured using an optical fiber probe with accuracy 0.1 °C.

SAR is a measure of heat generated by MNPs in AC magnetic field and is calculated by using the following relation.

$$SAR = c\frac{\Delta T}{\Delta t}\frac{1}{m_{magn}}$$

where c is the sample-specific heat capacity, which was calculated as a mass weighted mean value of MNPs and water. The heat-capacity of both samples is negligible because of its low concentration, and thus a