Chapter 2

Experimental details

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2.1. Materials

Ethylene glycol (EG) (Central Drug House, CDH, purity 98%), diethylene glycol (DEG) (CDH, 99%), ferric chloride hexa-hydrate ($FeCl_3.6H_2O$) (CDH, 98%), anhydrous ferric chloride ($FeCl_3$) (CDH, 97%), manganese chloride tetrahydrate $MnCl_2.4H_2O$ (CDH, 98%), sodium acetate trihydrate (NaOAc) (Fisher scientific, 99%), polyvinylpyrrolidone (PVP) K-30 (HIMEDIA) and sodium citrate tribasic dihydrate (NaCit) (Sisco research laboratories, 98%) were acquired and used for the synthesis of MNPs without any additional purification.

2.2. Synthesis procedure

2.2.1. γ-Fe_2O_3 magnetic nanoflowers

The synthesis of γ-Fe_2O_3 magnetic nanoflowers was carried out by microwave-assisted polyol process using a self-modified microwave refluxing system (frequency ~ 2.45 GHz, power ~ 700 W). Microwave assisted synthesis offers numerous advantages over conventional heating, such as instantaneous, rapid and selective heating as well as high temperature homogeneity. Its effectiveness depends on ability of the reaction mixture to efficiently convert the absorbed microwave energy into heat which is determined by the loss tangent (δ) parameter and varies with the choice of solvents used for the reaction. The higher the '$tan\delta$' value, the better the solvent is for microwave absorption and efficient heating. A series of six samples, namely S13, S14, S15, S16, S17 and S18, were prepared by varying the amount of NaOAc such that the ratio of Fe^{3+}: Na^+ were maintained at 1:3, 1:4, 1:5, 1:6, 1:7 and 1:8 respectively. Briefly for S13, a specific amount of anhydrous $FeCl_3$ (0.609 g) dissolved in 15 mL EG was added drop-wise to the solution of 25 mL EG and 1.54 g NaOAc.

The resulting mixture was stirred at 30 °C for 2 h and then transferred to a microwave refluxing system. Here it was irradiated with microwave energy for 90 min with 5 min ON and 2 min OFF condition. The precipitate obtained was washed several times with double distilled (DD) water and finally with ethanol and was then dried under vacuum at 70 °C overnight. The same procedure was followed to synthesise other samples by varying the amount of NaOAc.

2.2.2. $MnFe_2O_4$ nanoflowers

For the synthesis of $MnFe_2O_4$ magnetic nanoflowers, a high-temperature solvothermal method was employed following the steps described previously with some modification [136], [137]. The solvent used in the synthesis consisted of three parts of DEG and one part of EG. Briefly, a solution of stoichiometric amounts of salts viz. $FeCl_3$ (0.43 g) and $MnCl_2.4H_2O$ (0.26 g) in 15 mL solvent was added drop-wise into a solution of NaOAc (3.8 g) and PVP (1 g) in 25 mL solvent. The resulting solution was stirred for 2 h using a magnetic stirrer while maintaining the temperature in the range of 30-35 °C. Then the mixture was transferred in a Teflon lined autoclave and placed inside a vacuum oven maintained at 180 °C for 12 h. After completion, a black precipitate was obtained which was washed several times with DD water and finally with ethanol using a magnet. After washing, the precipitate was dried under vacuum at 70 °C for overnight to get a dry powder. The powder was then used for further study and various characterization.

2.2.3. Fe_3O_4 mesoporous nanoparticles

For the synthesis of meso-porous Fe_3O_4 magnetic nanoparticles, the solvothermal method as described above was employed with minor modification [136], [137]. The solvent used in the synthesis consisted of three parts of DEG and one part of EG. Briefly, a solution

of stoichiometric amounts of salt, $FeCl_3.6H_2O$ (1.07 g) and PVP (1 g) in 15 mL solvent was added drop-wise into a solution of NaOAc (3.8 g) in 25 mL solvent. The resulting solution was stirred for 2 h using a magnetic stirrer while maintaining the temperature in the range of 30-35 °C. The solution was then transferred to a Teflon lined autoclave which was heated at 200 °C for 15 h inside a vacuum oven. At the end, a black precipitate was obtained that was washed several times with double distilled (DD) water and finally with ethanol using a magnet. After washing, the precipitate was dried under vacuum at 70 °C overnight to get a dry powder. The powder was then used for further study and various characterization.

2.3. Magnetic ferrofluid

To study the heating behavior of MNPs with time-varying AMF and NIR irradiation, an aqueous ferrofluids of desired concentration was prepared following steps as defined henceforth [138]. Briefly, 50 mg of powder sample was washed with an aqueous solution of 0.01 M HCl and collected with the help of a magnet. It was then dispersed in 30 mL aqueous solution of 1 M sodium citrate dihydrate (NaCit) by ultra-sonication for 1 h. The washing with HCl creates a net positive charge on the surface of MNPs which facilitate the citrate coating through electrostatic attraction. After sonication the magnetic materials were collected using a magnet and washed twice using DD water to remove the excess NaCit. Finally, the material was re-dispersed in DD water (required volume) and sonicated for 1 h to get a stable aqueous ferrofluid. It was further diluted to get a desired concentration for different studies.

2.4. Materials characterisation

2.4.1. X-ray diffraction (XRD)

The XRD is a powerful technique frequently used to determine the crystal structure and phase of a material. In addition, it assists in determining several structural parameters including average grain size, crystallinity, strain, and crystal defects. The technique uses Bragg's law of diffraction which is satisfied by the condition:

$$n\lambda = 2d_{hkl}sin\theta \qquad 2.1$$

where n is a positive integer number, λ is the wavelength of the X-rays, d_{hkl} is the inter-planner spacing between diffracting planes and θ is the incident angle. The X-ray diffraction (XRD) patterns of the powder sample was acquired in 10-90 degree (2θ) range by BT- Rigaku Miniflex X-ray diffractometer equipped with Cu Kα energy of wavelength, λ = 1.54 Å (0.02° step size and 2°/min scan rate).

2.4.2. Mössbauer spectroscopy

Mössbauer spectroscopy is a great tool which provides useful information on the magnetic state, along with chemical nature of the system. It can be used effectively to characterize iron base materials as magnetic hyperfine patterns are distinctive for the different types of iron species. Moreover, isomer shift (δ) is sensitive to the oxidation state of iron and allows for identification and quantization of the magnetite or maghemite content in a sample. The room temperature Mössbauer spectrum of the powder sample was recorded using a ^{57}Co source of 10 mCi in the Rh matrix. The spectrum was collected with standard transmission geometry having a triangular waveform in constant acceleration mode. An enriched α-^{57}Fe metal foil was used for the velocity scale calibration. The line width (inner)

of calibration spectrum is 0.23 mms^{-1} and the isomer shift (d) values are relative to Fe metal foil (d = 0.0 mm/s).

2.4.3. Electron microscopy (EM)

The particle's size, morphology, and distribution were evaluated by bright-field, high-resolution and high-angle annular dark-field imaging (HAADF) transmission electron microscopy (TEM) images recorded on FEI Tecnai-G^2 200. The selected area electron diffraction (SAED) pattern of the sample was obtained with the same equipment. To prepare the TEM sample, few drops of ferrofluids were dispersed in 20 mL methanol by ultra-sonication. From this suspension, two drops in succession were released over the surface of a carbon-coated copper grid (200 mesh) and dried under IR lamp before observing under TEM. The samples' size distribution was determined manually by counting more than 150 particles from TEM bright-field micrographs, using Image J software.

EVO-Scanning Electron Microscope (MA15/18, Carl Zeiss) equipped with 51N1000–EDS System (Oxford Instrument) was used for the mapping of elements in $MnFe_2O_4$.

2.4.4. Thermo gravimetric analysis (TGA)

TGA was carried out from ambient temperature to 600 °C at a heating rate of 10°/min with the help of TGA-50 (Shimadzu Pte Ltd., Asia Pacific). This analytical technique is used to determine the thermal stability of the material prepared and the fraction of volatile components by monitoring the weight change of the sample on heating at a constant rate.

2.4.5. Magnetic measurements

The field-dependent magnetic properties of the powder samples were measured at a temperature of 300 K in ±50 kOe range using a SQUID (superconducting quantum

interference device) magnetometer (MPMS®5, Quantum design). For the measurement few grams of powder samples were loaded in an instrument specific Teflon tubes and magnetic data were collected up to the applied field of ±50 kOe. The temperature-dependent magnetization behavior was evaluated in zero-field cooled (ZFC) and field cooled (FC) conditions in the temperature range of 5 to 300 K with an exciting field of 250 Oe.

2.4.6. Dynamic Light Scattering (DLS) and Zeta potential

Zeta sizer (Malvern Instruments) capable of both particle size analysis and zeta-potential measurement was used to analyze the hydrodynamic size and colloidal stability of MNPs in aqueous ferrofluid. The experiments were carried out thrice at four different pH values of 2, 4, 6 and 8, respectively. For the experimentation the instrument was calibrated using a latex suspension of known zeta potential (i.e., ± 55 to ± 5 mV).

2.4.7. Heating ability

2.4.7.1. Magnetic fluid hyperthermia (MFH)

The temperature rise of ferrofluid under AMF was evaluated using MagneTherm in selected amplitude and frequency of fields (Nanotherics, UK). An optical fibre temperature sensor, LumaSense Technologies, was used to measure the increase in temperature. The heating efficiency of the material, i.e. intrinsic loss power (ILP) was obtained by dividing the SLP value by the square of amplitude (H^2) and frequency (f) of the applied AMF. The SLP value of the material was calculated using the following equation [139].

$$SLP(wg^{-1}) = C * \frac{V_s}{m} * \frac{dT}{dt} \qquad 2.2$$

where, dT/dt is the initial slope of temperature versus time curve, C is the solution's volumetric specific heat capacity, Vs is the sample volume, and m is the mass of magnetic material.

2.4.7.2. Photo-thermal therapy (PTT)

The photo-thermal heating was evaluated by exposing aqueous suspensions (0.5 mL) of different concentrations of MNPs (0.5, 0.25 and 0.1 mg/mL) to a NIR laser irradiation of 808 nm with a controllable power density of 0.66 and 0.33 Wcm^{-2} (MDL-H-808-3W) for a duration of five min. The FLIR E30bx infrared camera was used to monitor the rise in the temperature of the suspension.

2.4.8. Photoluminescence (PL) and vis-NIR absorption spectroscopy

Photoluminescence measurement was performed on Edinburgh fluorimeter F900, which is equipped with a microsecond xenon flash lamp of variable frequency spanning 1-00 Hz and M300 monochromator. Absorption of the suspensions was evaluated in the wavelength range of 500 to 900 nm by a multi-mode microplate reader (BioTek Synergy H1).

2.4.9. Photothermal transduction efficiency (η)

The photothermal transduction efficiency, η, was evaluated following the method described elsewhere, using the equation [140].

$$\eta = \frac{hA(T_{max} - T_{min}) - E_{in,sample}}{I(1-10^{-OD})} \qquad 2.3$$

where, h is the heat transfer coefficient, A is the sample well surface area, T_{max} is the maximum temperature of the aqueous suspension after laser exposure, T_{min} is the surrounding temperature, $E_{in,sample}$ is the energy input based on the heat generated by solvent and sample well, I is the laser power in watt and OD is the optical density. The product hA was calculated from decay time constant τ_s using relation $hA = {m.C}/{\tau_s}$, where m is the mass of material and C is specific heat capacity of solvent. The decay time constant τ_s was obtained from

relation, $t = -\tau_s \ln(\theta)$. Here, t is measurement time and θ is a dimension less parameter given as, $\theta = (T - T_{min})/(T_{max} - T_{min})$ [141].

2.4.10. In-Vitro studies

2.4.10.1. Cell culture

SiHa cells were cultured in a 5% CO_2 incubator maintaining the temperature at 37 °C. For this the cells were seeded in 25 mL culture flask containing Dulbecco's Modified Eagle Medium (DMEM, Himedia), 10% heat-inactivated fetal bovine serum (Himedia) and antibiotics (penicillin and streptomycin, Himedia). The culture flask was then placed in a 5% CO_2 which was maintained at 37 °C for the cells to grow.

2.4.10.2. Nanoparticle uptake by cells

For the study, SiHa cells were grown (till 70% confluence) on glass slides with 8 well attachments. After that MNPs suspension (250 mg/mL) was added to the media in selected wells and the cells were then incubated in the 5% CO_2 incubator. A 2% paraformaldehyde solution was used to fix the cells at different time intervals to monitor particles uptake which were imaged with the help of confocal microscopy. For confocal microscopy, cells were washed twice with PBS and stained with Alexa Fluor-488 tagged phalloidin (0.06 μM, Invitrogen) and propidium iodide (5 μg/mL, SRL chemicals) for 30 min. After washing thrice with PBS, glass slides were removed from the well attachments, dried and visualized under Zeiss LSM 700 laser scanning confocal microscope, using 40X lens. All experiments were done in triplicates.

2.4.10.3. Cell viability assays after PTT

The cell viability was evaluated after PTT to assess its impact in-vitro. For this SiHa cells (~1×10^5 cells/mL) were grown in a 48-well plates providing incubation for 24 h in the

5% CO_2 incubator. After 24 h when the cells confluence reached ~70%, the MNPs suspension was added to the media in selected wells to attain a concentration of 100, 250 or 0.5 mg/mL and incubated for 1 h (as significant MNPs uptake was achieved after 1 h incubation) in the 5% CO_2 incubator. The cells (without changing the media) were then exposed to the NIR laser (808 nm) of 0.66 Wcm^{-2} power density for a duration of 5 min. After treatment, the cell viability was estimated through MTT assay. Briefly, 100 µL of MTT (0.5 mg/mL) in DMEM was added to the wells and incubated at 37 °C for 2-3 h until the purple formazan crystals were visible. After that, the media was removed and 100 µL DMSO was added to each cell culture well to dissolve the formazan crystal. Samples were transferred to 1.5 ml tubes, centrifuged at 16000 g for 5 min to pellet down cell fragments and remaining MNPs. The purple supernatant was transferred to a clear 96 well plate and the absorbance was taken at 570 nm with Biotek Synergy H1 microplate reader. The relative cell viability of the samples is expressed in percentage values, with respect to the control (considered 100%). For live-dead staining a similar treatment (laser, 808 nm exposure) was given to the cells. After treatment the cells were washed with PBS and incubated with Calcein-AM (3 µg/mL final concentration) and propidium iodide (5 µg/mL final concentration) in PBS; and kept at 37 °C. After 30 min incubation, wells were washed twice with PBS. The cells were microscopically examined with the help of Biotek Cytation 5 cell imaging multi-mode reader, showing viable cells as green (stained by Calcein-AM) and damaged cells as red (stained by propidium iodide). All experiments were done in triplicates.