Supplementary Information

Biofunctionalization of Magnetite Nanoparticles with Stevioside: Effect on the Size and Thermal Behaviour for use in Hyperthermia Applications.

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Characterization Studies

The crystalline structure and the approximate size of the particles were determined by Bruker X-ray diffraction (XRD) system using Cu K\(\alpha\) radiation with a wavelength of 1.5404 Å and operated at 40 kV and 25 mA. A coarse scan from 20 to 80° (2θ) was performed at a scan rate of 0.020 to acquire an overall spectrum to identify the crystal structure. The mean diameter of the particles were also calculated from the XRD spectra according to the linewidth of the (311) plane refraction peak using Scherrer equation (1), given as:

\[
D = \frac{K\lambda}{b\cos\theta}
\] (1)

Where \(\theta\) is the angle at which the reference peak occurs, \(\lambda\) is the X-ray wavelength (1.5418 Å), \(b\) is the FWHM of the XRD peak and \(K\) is the shape factor whose value is 0.9 for magnetite.

Transmission electron microscopy (TEM) was done to study the morphology and size of the nanoparticles using JEOL JEM 2100 transmission electron microscope operating at the acceleration voltage of 200 KV. A drop of diluted nanoparticle suspension in methanol (100 \(\mu\)g/ml) was casted onto a 300 mesh formvar-carbon coated copper grids and air-dried before measurements. The hydrodynamic diameter of the magnetite nanoparticles were measured using Malvern Zetasizer Nano-ZS (\(\lambda = 632.8\) nm, \(T = 25^\circ\)C). The concentration of each sample analysed was 10 \(\mu\)g/ml DMSO.

Fourier transform infrared spectroscopy (FTIR) analysis was done using Agilent Technologies Cary 600 series spectrometer. The spectrum for all samples was recorded in the wavelength range of 400-4000 cm\(^{-1}\). The powder samples of the coated nanoparticles were ground with KBr and compressed into a pellet using manual hydraulic press. These generated pellets were used to obtain the spectrum. The spectrum of neat OA and P-80, and pure STE powder was recorded as standard reference for the coated nanoparticles. To further confirm the STE coating onto the surface of Fe\(_3\)O\(_4\) nanoparticles, FTIR spectra of bare Fe\(_3\)O\(_4\) nanoparticles mixed with pure STE powder was also compared with that obtained from STE-coated Fe\(_3\)O\(_4\) nanoparticles.

The magnetic measurements of the coated and uncoated Fe\(_3\)O\(_4\) nanoparticles were performed with a Quantum Design Dynacool PPMS magnetometer in the temperature range 5–300 K and magnetic fields up to 5 T. The zero-field-cooled (ZFC) curves were obtained by cooling the magnetite samples from 300 to 5 K in the absence of an external magnetic field, followed by the magnetization measurement under a magnetic field of 100 Oe as the temperature was raised back to room temperature. The field-cooled (FC) measurements were carried out in a similar way, except for the cooling process, which was performed under an external magnetic field of 100 Oe. The hysteresis loop measurements were
performed at 300 K and 5 K under magnetic fields of up to 5 T.

Table 1: Observed particle sizes of synthesized magnetite nanoparticles by various characterization techniques.

| Sample          | XRD (nm) | DLS (nm)     | TEM (nm)   |
|-----------------|----------|--------------|------------|
| Fe₃O₄           | 12.4     | 447.56 ± 15.97 | 21.66 ± 5.14 |
| P-80-Fe₃O₄     | 9.9      | 191.5 ± 5.72  | 13.70 ± 2.52 |
| OA-Fe₃O₄       | 10.4     | 270.4 ± 7.25  | 17.05 ± 1.57 |
| STE-Fe₃O₄ (0.5g)| 7.1      | 56.83 ± 10.76 | 4.75 ± 1.01  |
| STE-Fe₃O₄ (1.0g)| 6.7      | 49.77 ± 6.98  | 3.78 ± 0.79  |

Table 2 - Magnetic properties of MNPs.

| Sample          | Mₛ (emu/g) | Mᵣ (emu/g) | Hᵥ (T) |
|-----------------|------------|------------|--------|
|                 | 300 K      | 5 K        | 300 K  | 5 K        | 300 K  | 5 K        |
| Fe₃O₄           | 60.8577    | 65.8695    | 11.8426 | 19.4084    | 0.0141 | 0.0388    |
| P-80-Fe₃O₄     | 66.5413    | 74.0601    | 1.2026  | 18.6381    | 0.00036 | 0.0293    |
| OA-Fe₃O₄       | 36.6132    | 41.8764    | 0.7687  | 11.8919    | 0.00063 | 0.0389    |
| STE-Fe₃O₄ (1g) | 53.9951    | 65.3753    | 7.8975  | 13.2925    | 0.0059  | 0.0105    |
| STE-Fe₃O₄ (0.5g)| 58.7179    | 71.2820    | 6.9939  | 14.0035    | 0.0043  | 0.0105    |

To understand the adsorption mechanism of the surfactant moieties onto the surface layer of magnetite nanoparticles, FTIR analysis were performed on bare Fe₃O₄ nanoparticles; OA-, P-80- and STE-coated Fe₃O₄ nanoparticles; neat drop of OA, P-80 and STE powder (Figure S1). In Figure S1(A), the FTIR study confirms the successful stevioside coating on Fe₃O₄ nanoparticles due to the presence of characteristics peaks of pure stevioside onto the STE-coated Fe₃O₄ nanoparticles, which are absent in the spectrum obtained from bare Fe₃O₄ nanoparticles. The spectrum of stevioside exhibits intense peaks at 1033 cm⁻¹ and 1072 cm⁻¹ corresponding to carboxylic acid esters. The peak at 1398 cm⁻¹ can be attributed to O-H bending and the peaks at 1642 cm⁻¹ and 1729 cm⁻¹ corresponds to >C=O.25 The peak at 2925 cm⁻¹ corresponds to C=C-H and the O-H stretching band appears at 3396 cm⁻¹. Bare Fe₃O₄ nanoparticles and STE-coated Fe₃O₄ nanoparticles also exhibited the characteristic peak of Fe-O bond vibrations at 556-586 cm⁻¹. STE coating onto Fe₃O₄ nanoparticles was further validated by carrying out FTIR analysis of
mixture of bare Fe₃O₄ nanoparticles and pure STE powder that gave similar spectra as that of STE-coated Fe₃O₄ nanoparticles. In Figure S1(B), FTIR spectra of bare Fe₃O₄ nanoparticles and pure OA and P-80 was compared with that of OA- and P-80-coated Fe₃O₄ nanoparticles respectively which confirmed the successful generation of OA- and P-80-coated magnetite nanoparticles. The FTIR spectrum of pure OA shows its characteristics peaks. The peak at 1286 cm⁻¹ corresponds to the presence of existence of C=O stretch. The two sharp peaks at 2858 cm⁻¹ and 2930 cm⁻¹ correspond to the symmetric and asymmetric CH₂ stretch respectively. The bands corresponding to O-H out-of-plane and in-plane appeared at 939 cm⁻¹ and 1467 cm⁻¹ respectively.¹⁷

It is worth to note that the band present at 1706 cm⁻¹ corresponding to C=O stretching of the carboxyl group in the pure OA FTIR spectra was absent in the corresponding spectra for OA-coated nanoparticles. Rather a new band characteristic of the symmetric νₛ(COO⁻) stretch appeared at 1639 cm⁻¹ in the coated nanoparticles spectra which suggests the bonding pattern of carboxylic acids onto the surface layer of magnetite nanoparticles as a combination of molecules bonded both symmetrically and at an angle to the surface.

Similarly, FTIR spectra analysis of P-80 exhibited characteristic peaks at 723 cm⁻¹ corresponding to in-plane bending or rocking of CH₂ group and 1107 cm⁻¹ corresponding to C-O bond.¹⁸ The peaks in the region 1150-1351 cm⁻¹ can be attributed to out-of-plane bending/wagging and out-of-plane bending/twisting of wCH₂ and xCH₂ groups of P-80. The peak at 1461 cm⁻¹ corresponds to in-plane bending or scissoring of CH₂. The C=O stretching band appeared at 1735 cm⁻¹. The bands at 2852 cm⁻¹ and 2923 cm⁻¹ corresponds to symmetric and asymmetric stretching of CH₂. For all the coated nanoparticles, a slight shift in the position of the characteristic signature peaks of the surfactant moieties and lower peak intensities can be attributed to the interaction of these molecules with the surface layer of magnetic nanoparticles.
Figure S1. FTIR analysis of (A) Fe₃O₄, STE, STE-Fe₃O₄ and STE mixed Fe₃O₄; (B) bare Fe₃O₄, P-8.
Zeta potential measurements

![Zeta potential measurements](image)

Figure S2: Zeta potential measurements of the nanosystems generated.

The surface charge of all the MNPs synthesized in the study was evaluated using Malvern Zetasizer Nano-ZS. The bare Fe₃O₄ nanoparticle exhibited surface charge of \(-12 \pm 0.66\) mV while for the coated MNPs it was found to be \(-16.4 \pm 2.82\) mV (P80-MNP), \(-13.8 \pm 1.2\) mV (OA-MNP), \(-11.0 \pm 0.53\) mV (STE-MNP (0.5g)) and \(-11.5 \pm 0.23\) mV (STE-MNP (1.0g)).

Chemotherapeutic effect of stevioside

C6 cells when treated with varying concentration of stevioside (25-1000 \(\mu\)M) for a period of 24-72 h (Figure S3). After treatment, cell viability was calculated by MTT assay. As seen, the cell viability was found to reduce with increasing concentration of stevioside and time of incubation. The cell viability was found to reduce to approximately 60% after treatment with 1000 \(\mu\)M for 72 h. Hence, it confirms the anti-tumor potential of stevioside alone.
Figure S3: Chemotherapeutic effect of stevioside on Rat C6 glioma cells.

**Biocompatibility assay of STE-MNP (0.5g) on normal murine NIH3T3**

Figure S4: Biocompatibility test of STE-coated MNPs (0.5 g) on murine normal fibroblasts cell line (NIH3T3).
Effect of MNP concentration on SAR value

The SAR value with particle concentration has been evaluated by using the method suggested by Natividad et al 2013 [1], using the equation (Table 3A):

\[ \text{SAR} \left( \frac{W}{g} \right) = \frac{C}{m_{\text{MNP}}} \frac{\Delta T}{\tau} \]

Where \( \Delta T \) is maximum rise in temperature achieved; \( \tau \) is the relaxation time of the nanosystems calculated from the complete T-vs-t curve fit to the exponential trend characteristic of isoperibol conditions.

Table 3A: SAR value variation with increasing particle size (calculated using relaxation time) on application of AMF of strength 168 Oe at \( f = 405 \) kHz for 10 mins.

| S.No | Concentration (mg/mL) | SAR for STE-Fe\(_3\)O\(_4\) (0.5 g) | SAR for STE-Fe\(_3\)O\(_4\) (1.0 g) |
|------|-----------------------|--------------------------------------|--------------------------------------|
|      |                       | (W/g MNP)                            | (W/g Fe)                             | (W/g MNP) | (W/g Fe) |
| 1    | 0.5                   | 133.99                               | 6472.94                             | 90.04     | 2297.13 |
| 2    | 1.0                   | 58.26                                | 2931.88                             | 73.18     | 1867.01 |
| 3    | 2.5                   | 38.69                                | 1878.74                             | 76.23     | 1944.71 |
| 4    | 5.0                   | 33.69                                | 1627.54                             | 4.46      | 113.94  |
| 5    | 10.0                  | 50.26                                | 2428.01                             | 14.70     | 375.20  |

The effect of increasing nanoparticle concentration on SAR values was further evaluated for both STE-coated MNPs (Table 3B) using the conventional initial slope method for SAR value calculations. The following equation was used:

\[ \text{SAR} \left( \frac{W}{g} \right) = \frac{C}{m} \times \frac{dT}{dt} \]

Where \( dT/dt \) is the initial slope of the temperature versus time graph taking into consideration first few seconds only.
Table 3B: SAR value variation with increasing particle size (calculated by initial slope method) on application of AMF of strength 168 Oe at $f = 405$ kHz for 10 mins.

| S.No | MNP Concentration (mg/mL) | SAR for STE-Fe$_3$O$_4$ (0.5 g) (W/g MNP) | SAR for STE-Fe$_3$O$_4$ (1.0 g) (W/g MNP) |
|------|--------------------------|------------------------------------------|------------------------------------------|
| 1    | 0.5                      | 196.50                                   | 205.73                                   |
| 2    | 1.0                      | 214.70                                   | 302.56                                   |
| 3    | 2.5                      | 105.72                                   | 66.73                                    |
| 4    | 5.0                      | 91.42                                    | 65.93                                    |
| 5    | 10.0                     | 65.87                                    | 50.85                                    |

As seen, the SAR value increases with increasing particle concentration only until it reaches the optimal particle concentration. Beyond this concentration, SAR value starts decreasing with further increase in particle concentration. This effect could be attributed to increased relaxation time of the nanosystems due to enhanced dipolar interaction and agglomeration with increasing particle concentration. Similar trend in SAR variation with particle concentration has also been reported in various other studies, both experimental [2] and theoretical [3-4].
Figure S5: Temperature-versus-time fitting curves for SAR value calculations (‘σ’ denotes the standard error for thermal relaxation time calculations).
Table 4: Relaxation time of MNPs calculated from (A) $T$-vs-$t$ fitting (Hyperthermia data) and (B) MT fitting (magnetic data).

| Sample name       | Concentration of MNP in ferrofluid | Relaxation time ($\tau$; seconds) |
|-------------------|------------------------------------|-----------------------------------|
| Bare MNP          | 1 mg/mL                            | 294.30                            |
| P80-MNP           | 1 mg/mL                            | 966.07                            |
| OA-MNP            | 1 mg/mL                            | 658.56                            |
| STE-MNP (0.5g)    | 1 mg/mL                            | 647.82                            |
| STE-MNP (1.0g)    | 1 mg/mL                            | 653.34                            |
| STE-MNP (0.5g)    | 0.5 mg/mL                           | 361.86                            |
|                   | 1.0 mg/mL                           | 647.39                            |
|                   | 2.5 mg/mL                           | 722.21                            |
|                   | 5.0 mg/mL                           | 647.68                            |

*Figure S6: Temperature versus time graph fitting for increasing concentration of nanomaterials in the ferrofluid for (A) STE-MNP (0.5g) and (B) STE-MNP (1.0g).*
| STE-MNP (1.0g) |          |        |
|---------------|----------|--------|
| 10 mg/mL      | 427.44   |        |
| 0.5 mg/mL     | 278.55   |        |
| 1.0 mg/mL     | 365.53   |        |
| 2.5 mg/mL     | 339.96   |        |
| 5.0 mg/mL     | 5053.81  |        |
| 10 mg/mL      | 1185.11  |        |

References

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