Preparation and characterization of manganese ferrite-based magnetic liposomes for hyperthermia treatment of cancer

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Abstract

Comparative evaluation of two different methods of magnetic liposomes preparation, namely thin film hydration (TFH) and double emulsion (DE) with different molar ratios of egg-phosphatidyl choline (egg-PC) and cholesterol using lauric acid coated manganese ferrite-based aqueous magnetic fluid, is reported. TFH was found to be a better method of encapsulation and TFH 2:1 (egg-PC: cholesterol) magnetic liposomes showed the highest encapsulation efficiency and comparable heating ability to that of magnetic fluids. Stealth TFH 2:1 magnetic liposomes containing DSPE-PEG\textsubscript{2000} were three-fold more cytocompatible as compared to the magnetic fluid. Stealth TFH 2:1 manganese ferrite-based magnetic liposomes might be useful for hyperthermia treatment of cancer.

Keywords: Magnetic fluid; Magnetic liposome; Thin film hydration; Heating ability; Cytocompatibility

1. Introduction

Magnetic nanoparticle-based hyperthermia has emerged as a promising therapeutic approach to cancer treatment [1,2]. For hyperthermia, superparamagnetic as well as ferrimagnetic particles such as Fe\textsubscript{3}O\textsubscript{4} or $\gamma$-Fe\textsubscript{2}O\textsubscript{3} have been explored extensively [3–5]. However, the recent challenges are to develop the magnetic nanoparticles with high heating ability (measured by specific absorption rate (SAR)) and to control the temperature of magnetic nanoparticles in vivo. In this context, several groups have investigated the feasibility of use of substituted ferrite systems for hyperthermia application [6–8]. Besides engineering the magnetic particle to an appropriate size and narrow size distribution, SAR can be enhanced by choosing the magnetic particles of high magnetization at applied AC field. In our previous paper, we reported that ferrite systems like Fe\textsubscript{1–x}Mn\textsubscript{x}Fe\textsubscript{2}O\textsubscript{4}, where a certain degree of substitution of Fe(II) ions has been done by divalent Mn ions, have increased the magnetic moment and thereby Fe\textsubscript{0.6}Mn\textsubscript{0.4}Fe\textsubscript{2}O\textsubscript{4} has shown higher SAR than the Fe\textsubscript{3}O\textsubscript{4} [8]. One of the crucial steps for hyperthermia application is to control the temperature of the magnetic nanoparticles in vivo to avoid the damage to the surrounding normal tissue due to over heating. A possible approach to control the temperature is to design the materials in such a way that it exhibits temperature sensitive magnetic properties. The use of a suitable Curie temperature ($T_C$\textasciitilde42–56 °C) of a material would be the smartest way to control the in vivo temperature. Below the Curie temperature it will generate heat. Once the temperature exceeds the Curie
particular Zn concentration. Among these ferrites, we exploited substituted ferrites system like $A_{1-x}Zn_xFe_2O_4$ [$A = \text{Mn, Fe(II), Co}$] for this purpose. It is important to mention that these systems have favorable $T_C$ at a particular Zn concentration. Among these ferrites, $Mn_0.8Zn_{0.2}Fe_2O_4$ is more promising as it has desired $T_C$ and its SAR can be optimized by choosing a suitable particle size (data to be published soon).

Biocompatibility and site-specific delivery are the two major issues for these substituted ferrites to be successfully used for hyperthermia application. Recently, we reported the biocompatibility and heating ability (SAR) of two substituted ferrites i.e. $\text{MnFe}_2\text{O}_4$ and $\text{CoFe}_2\text{O}_4$, and compared them to that of $\text{Fe}_3\text{O}_4$ [9]. We found that lauric acid coated $\text{MnFe}_2\text{O}_4$ has a similar biocompatibility and heating ability as that of $\text{Fe}_3\text{O}_4$. There is a necessity of an efficient delivery system for these ferrites to be successfully delivered at the cancer tissue. Magnetic liposomes (magnetic nanoparticles encapsulated in phospholipid bilayers) appear to be a versatile delivery system due to biocompatibility, chemical functionality and its potential for combination of drug delivery and hyperthermia treatment of cancers [10]. In order to achieve optimum efficacy, magnetic liposomes with high encapsulation efficiency, stability and long circulating (stealth) ability are desired. Moreover, for hyperthermia application, magnetic liposomes should not affect the heating ability (SAR) of the magnetic fluid unfavorably. Hence, the present investigation was chalked out to prepare magnetoliposomes encapsulating lauric acid coated water-based manganese ferrite ($\text{MnFe}_2\text{O}_4$) magnetic fluid (taken as a model for water-based magnetic fluids) with different molar ratios of egg-phosphatidyl choline (egg-PC) and cholesterol by two different methods (thin film hydration (TFH) and double emulsion (DE)) and thereby to optimize their ratios for the best formulation of magnetoliposomes for hyperthermia application.

2. Materials and methods

Lauric acid coated manganese ferrite-based magnetic fluid was prepared by co-precipitation technique in $N_2$ atmosphere [9]. For this, manganese (II) chloride ($\text{MnCl}_2$, $H_2O$) and iron (III) chloride ($\text{FeCl}_3$, $H_2O$) at 1:2 molar ratio were dissolved in deionized $N_2$ purified water. The metal ion solution (0.2 M) was quickly added into the base solution ($NH_4OH$) with vigorous stirring (3000 rpm). The pH of the resultant reaction mixture was adjusted to 10–11. The pH was monitored by a pH meter (Toshcon, India). The slurry was aged between 90 and 95 °C for 15–20 min. The precipitate was repeatedly washed with 10% $NH_4OH$ solution. Then the lauric acid (30–40 wt% of ferrite) (Sigma-Aldrich, USA) and water were added. This mixture was kept on a hot plate and heated (~100 °C) for 10 min with stirring to form magnetic fluid. For purification, magnetic fluid was filtered through a Whatman 2 (qualitative) paper. Then the magnetic fluid was filtered through a Whatman 2 (qualitative) paper followed by dialysis against deionized water using a cellulose membrane (12.4 kDa, Sigma-Aldrich, USA) for 72 h. The resultant magnetic fluid was centrifuged at 3500 g for 15 min at room temperature and purified magnetic fluid was used for further characterization. Magnetic phase purity of the magnetic fluid was determined by X-ray Diffraction (XRD) studies using a Philips powder diffractometer PW1710 with CuK$\alpha$ radiation. Samples were scanned in continuous mode from 20 to 80° with a scanning rate of 0.02°/15. The size and morphology of the coated particles in the magnetic fluid were observed using a transmission electron microscope (TEM) (CM 200, Philips). For TEM observation, magnetic fluid was diluted 10 times and the sample was deposited dropwise onto a copper grid coated with carbon film and dried. Room temperature magnetization measurement of the magnetic fluid was done with a vibrating sample magnetometer (VSM, Lake Shore, Model-7410). The hydrodynamic diameter ($d_h$) of magnetic particles was determined using photon correlation spectroscopy (PCS) by Zeta Plus (Brookhaven Instrument Corporation, USA).

Magnetic liposomes were prepared by two different methods i.e. TFH and DE. Five different molar ratios [1:0,1:1,1:2,2:1,3:2] of egg-PC (Sigma Aldrich, USA) and cholesterol (Sigma Aldrich, USA) were taken for magnetic liposomes preparation. Required amount of egg-PC and cholesterol was dissolved in a solvent containing chloroform/methanol (2:1 v/v) in a round bottomed flask. Then, the solvent was evaporated under vacuum at room temperature in a rotary evaporator (rotavap) to form a thin lipid film. The magnetic fluid was added to the flask and hydration was done for 30 min at 40 °C. The sample was sonicated in a bath type of sonicator (20 kHz, 250 W, Vibronics Pvt. Ltd., India) for 15 min to form unilamellar magnetic liposomes. 0.9% NaCl solution was added to the samples at 1:1 (v/v) ratio for precipitating unencapsulated magnetic nanoparticles. This is based on the fact that only unencapsulated particles got precipitated after mixing with 0.9% NaCl solution due to ionic interactions. After addition of normal saline solution (0.9% NaCl solution), centrifugation was done at 1000 g for 10 min at 4 °C. Then supernatant was taken and the precipitate was discarded as it contained unencapsulated particles. The samples were stored at 2–4 °C until further characterization.

DE was performed following a reported method [11]. In brief, the required amount of egg-PC and cholesterol was dissolved in the same solvent as TFH and magnetic fluid was added to the lipid solution. The primary
emulsion was formed by sonication for 15 min in a sonication bath. Deionized water was added slowly to the primary emulsion and again subjected to sonication for 15 min to form secondary emulsion. A rotary evaporator operating under vacuum at room temperature was used to remove the solvent from the solution. The same procedure was followed as TFH for separation of unencapsulated magnetic nanoparticles from encapsulated one. All the magnetic liposomes were characterized for size, polydispersity, zetapotential by PCS by Zeta Plus (Brookhaven Instrument Corporation, USA). Cryo-Transmission Electron Microscopy (Cryo-TEM, Philips, TECHNA G² 120) was done to visualize the morphology of the best magnetic liposome formulation (TFH 2:1 magnetic liposomes) selected on the basis of smallest size and highest encapsulation efficiency. Tiron (4,5-dihydroxy- m-benzenedisulfonic acid, Sigma-Aldrich, USA) was used for spectrophotometric determination of iron concentration for magnetic fluid and magnetoliposome samples using a calibration curve. To obtain the calibration curve, known concentrations of aqueous solutions of 10 mM FeCl₃ were added to an equal volume of 100 mM Tiron and the absorbance of the mixture (pH < 3) was taken at 670 nm wavelength in a spectrophotometer (Lambda 25, Perkin Elmer, USA). The magnetic fluid and magnetoliposome samples were dissolved in the concentrated HCl and added to an equal volume of 100 mM T iron. The pH of the resultant solution was adjusted between 2.9 and 3 by adding NH₄OH solution. Then the absorbance was taken at 670 nm wavelength in the spectrophotometer. Concentration of iron was calculated from the equation

\[ y = 95.019x + 3.4953 \quad (R^2 = 0.9984) \]

obtained from the calibration curve, where \( x \) denoted absorbance at 670 nm and \( y \) denoted concentration in \( \mu g/ml \). Encapsulation efficiency of magnetic liposome samples was calculated using the formula \( W_1/W \times 100\% \), where \( W \) is initial concentration of Fe (mg/ml) and \( W_1 \) is encapsulated concentration of Fe (mg/ml).

The heating ability of the magnetic fluid and magnetic liposomes was measured by the time-dependent calorimetric measurements of specific absorption rate (SAR) at 300 kHz frequency and 15 kA/m AC field. For this measurement, 3 ml of fluid sample was taken in a glass sample holder with accessory arrangement of insulation to minimize the heat loss. The increase in temperature was measured by an optical fiber probe interfaced with a computer. The temperature was plotted with the corresponding time and the initial slope of the time-dependent temperature curve was determined. The SAR was calculated using the following equation \[9,12\]:

\[
\text{SAR} = C \frac{\Delta T}{\Delta t m_{Fe}},
\]

where \( C \) is the combined specific heat of sample holder and sample (\( C \) is calculated as mass weighted mean value of magnetic particles, surfactant and water). \( \Delta T/\Delta t \) is the initial slope of the time-dependent temperature curve. \( m_{Fe} \) is the mass of iron in gram.

Stealth liposomes were prepared by incorporating 5% (molar) diestearoyl-sn-glycero-3-phosphoethanolamine- \( N \)-[amino (polyethylene glycol) 2000] (DSPE-PEG₂₀₀₀) was incorporated into the bilayer of the magnetic liposome i.e. TFH 2:1 (optimized on the basis of encapsulation efficiency). Cytotoxicity of liposomes and free magnetic fluids was compared using L929 (mouse fibroblast) cell lines purchased from National Centre for Cell Science (NCCS), Pune, India. The cells were grown in Dulbecco’s modified eagle medium (DMEM, Sigma Aldrich, USA) supplemented with 10% fetal bovine serum (FBS Sigma Aldrich, USA) and 1% antibiotic antimycotic solutions (Himedia, India) and incubated at 37 °C temperature under 5% CO₂ and saturated humid environment. Five different ferrite concentrations ranging from 0.05 to 0.6 mg/ml of culture medium for magnetic fluid and stealth TFH 2:1 magnetic liposomes were exposed to L929 cells up to 48 h. Then, Sulforhodamine B (SRB) assay was conducted similar to the procedure by Skehan et al. [13] with slight modifications to evaluate the cell growth. In brief, cells were fixed by adding 50 \( \mu \)l of ice-cold 50% trichloroacetic acid (TCA, Loba Chemie, India) slowly to the medium and incubated at 4 °C for 1 h. Then, the plates were washed five times with deionized water and dried in air. 100 \( \mu \)l of 0.4% sulforhodamine B (Sigma Aldrich Chemie, USA) dissolved in 1% acetic acid was added to the fixed cells and kept at room temperature for 20 min after which they were washed with 1% acetic acid to remove unbound dye. The plates were dried and hundred microliters of 10 mM Tris base (Sigma, USA) was added to each well and kept for 20 min to dissolve the dye. Thereafter, the plates were placed on a shaker to allow the mixing of the dye and the absorbance (optical density) of each well was read in a plate reader (Thermo Electron Corporation, USA) at 560 nm. Cell viability was measured as viability (%) = [absorbance of sample/absorbance at 0 mg/ml (control)] × 100. More than 90% cell viability was considered as cytocompatible.

Statistical analysis of the data was done by analysis of variance (ANOVA) and Newman–Keul’s test using Microsoft Excel 2000 software. Data were represented as mean±standard deviation (SD) for three replicates (\( n = 3 \)).

3. Results and discussion

The XRD patterns (result not shown here) of magnetic fluid confirmed formation of single-phase cubic MnFe₂O₄. Fig. 1 shows the TEM photograph of lauric acid coated MnFe₂O₄ particles in magnetic fluid. It is clear that the particles are nearly spherical in shape.
and are generally well dispersed. The mean Fe$_3$O$_4$ particle size is 10 nm which is determined by considering about 250 particles of the TEM image. This is in good agreement with the crystallite size measured from the X-ray line (3 1 1) broadening using Scherrer’s formula. However, the mean hydrodynamic diameter of the particles measured by PCS is 70 nm with a polydispersity index 0.169. Low polydispersity index was suggestive of uniform distribution of particles in the magnetic fluid as could be seen from TEM photographs also. In the magnetic fluid, there is a dipole–dipole interaction between individual magnetic particles. Thus, it is possible that the magnetic particles may form close rings or long open loop and cluster in the magnetic fluid [14]. These chain/loop/clusters have lower diffusion coefficient than single particle. The equivalent sphere diameter measured by light scattering is higher than the actual hydrodynamic diameter of coated particle or the particle size measured from the other technique like TEM. It is important to note that this magnetic fluid is stable for more than 12 months (no precipitation of particles and no change of hydrodynamic diameter ($d_h$) was observed during this time period). The room temperature (27 °C) magnetization curves of MnFe$_2$O$_4$ particles in magnetic fluid did neither show saturation behavior nor any remanence or coercivity, which are signature of the superparamagnetic nature. SAR of the magnetic fluid (MnFe$_2$O$_4$) was found to be 145 W/g of iron at 300 kHz frequency and 15 kA/m AC field.

Fig. 2 shows the mean hydrodynamic diameters of different magnetic liposome samples. From this figure, it is evident that magnetic liposome samples showed a wide range of mean hydrodynamic diameters depending on composition (ratio of egg-PC cholesterol) and methods of preparation (TFH/DE). Most of the TFH samples had a lower diameter than DE samples (except for egg-PC cholesterol 1:0 ratio). The smallest mean hydrodynamic diameter, i.e. 171.6 nm, was observed for TFH 2:1 egg-PC cholesterol sample. The size of the TFH 2:1 liposomes was significantly smaller than TFH 1:0 and TFH 1:2 ($p<0.05$; ANOVA and Newman–Keul’s test). However, the size was not significantly
smaller than TFH 3:2 and 1:1 (p > 0.05; ANOVA and Newman–Keul’s test). Smaller diameter liposomes have advantages over the larger ones as they can escape phagocytosis by the reticuloendothelial system and can easily extravasate in the interstitial space [15]. However, downsizing of liposomes can be done by further sonication or extrusion [16–17]. All the magnetic liposome samples have polydispersity index more than 0.2 indicating their broad size distribution. However, low polydispersity index of a liposome formulation can be achieved by optimum sonication or extrusion [17–18]. Generally low polydispersity index (narrow size distribution) of the liposomes is more desirable for an ideal formulation. All the samples have shown zeta potentials more negative than −30 mV at pH 7.2, which implies that all the samples are physically stable against aggregation [19].

Fig. 3 shows the Cryo-TEM photographs of TFH 2:1 blank and magnetic liposome samples. From these figures it is clear that magnetic fluid is encapsulated within the aqueous chamber of liposomes (seen as a dark interior in the magnetic fluid loaded liposomes, Fig. 3(b)). As our magnetic fluid is water-based fluid, it is located in the inner chamber of liposomes which is hydrophilic in nature. This was confirmed by the lack of this structure in the blank liposomes and the enhancement of magnetic particles on prolonged focusing of the electron beam on magnetic liposomes.

Encapsulation efficiency of all the samples is given in Fig. 4. From this bar diagram, it is evident that for 1:0, 2:1 and 3:2 molar ratios of egg-PC and cholesterol, TFH has shown significantly higher encapsulation efficiency than DE (p < 0.05; ANOVA and Newman–Keul’s test). This result is contradictory to Ref. [11], where DE, as a method, has been found to have higher encapsulation efficiency for hemoglobin than TFH. In our case, comparatively lower encapsulation efficiency for DE might be due to the loss of lauric acid coating during emulsion processes leading to aggregation of magnetic manganese ferrite and precipitation of particles before liposomal encapsulation. However, there is no significant difference in encapsulation efficiency for 1:1 and 1:2 molar ratios between TFH and DE methods (p > 0.05; ANOVA and Newman–Keul’s test). Also these two molar ratios (1:1 and 1:2) have shown comparatively lower encapsulation efficiency than 1:0, 2:1 and 3:2 molar ratios (significant for TFH, p < 0.05 but not significant for DE, P > 0.05). Decreased encapsulation efficiency for 1:1 and 1:2 egg-PC cholesterol ratios could be due to increased rigidity of the membrane from higher cholesterol (≥ 50 mol%) content of the liposomes. For both the methods 2:1 ratio has shown the highest encapsulation efficiency, which may be due to presence of 33 mol% of cholesterol (2:1) in the bilayer. Cholesterol molecules play an important role in phase transition of a bilayer made up of phospholipid. It induces a third phase, i.e. liquid order phase, when present in bilayer of a phospholipid instead of either solid ordered or liquid disordered phase. This is because, a cholesterol molecule interacts with two phospholipid molecules and prevents them from going through the gel to liquid crystalline phase. At an optimal concentration of 33 mol%, there is no apparent transition as all of the phospholipids of the membrane are in liquid order phase [20]. Similar phenomena have also been reported for dipalmitoylphosphatidylcholine (DPPC) and cholesterol [21]. So, for the ratio 2:1, optimum mol% of cholesterol, i.e. 33 mol%, may be responsible for optimum permeability resulting into higher encapsulation efficiency. Overall, TFH 2:1 egg-PC cholesterol has shown highest encapsulation efficiency (70%).

Fig. 2. Mean hydrodynamic diameters of different magnetic liposome samples. Results are expressed as mean ± SD; n = 3.
TFH 2:1 showed a SAR value of 135 W/gm of iron (at 300 KHz frequency and 15 kA/m AC field), which is not much lower than the SAR values of the free magnetic fluids (145 W/gm of iron). Hence, so far as heating ability is concerned, the TFH 2:1 is as good as the magnetic fluid it encapsulated. However, TFH 2:1 is a conventional magnetic liposome sample, which will be very easily phagocytosed in vivo. To make it long circulating, stealth magnetic liposomes were prepared by incorporating 5% DSPE-PEG2000 in the bilayer of the TFH 2:1 magnetic liposomes. Stealth TFH 2:1 magnetic liposomes showed mean hydrodynamic diameter of 188 ± 13 nm (n = 3) and were stable at pH 7.2 (zeta potential was –30.88 mV). Comparative encapsulation efficiency of TFH 2:1 conventional and stealth liposome is given in the inset of Fig. 4. Stealth TFH 2:1 also showed similar encapsulation efficacy as conventional ones.

Fig. 3. Cryo TEM photographs of TFH 2:1 (a) blank liposome (b) magnetic liposomes (bar-200 nm).

Stealth TFH 2:1 also showed similar encapsulation efficacy as conventional ones. Fig. 5 shows the comparative cytocompatibility of lauric acid coated MnFe$_2$O$_4$-based magnetic fluid and its stealth magnetic liposome formulation (TFH 2:1 with 5% DSPE-PEG$_{2000}$) with L929 cell lines after 48 h of incubation. It is observed that lauric acid coated MnFe$_2$O$_4$ is cytocompatible up to 0.2 mg/ml ferrite concentration, beyond which the cell viability is decreased more than 10%. Hence threshold cytocompatible concentration of lauric acid coated MnFe$_2$O$_4$ is 0.2 mg/ml with L929 cell lines. But, stealth magnetic liposome formulation of the same magnetic fluid does not show any cytotoxicity up to 0.6 mg/ml ferrite concentration (highest concentration tested here). This stealth magnetic liposome formulation has resulted in a three-fold increase in threshold cytocompatible concentration of lauric acid coated MnFe$_2$O$_4$. Biocompatibility of magnetic fluid is determined by the core and coating materials. Fe$_3$O$_4$ as a core material is already reported to
be biocompatible. But reports on biocompatibility of MnFe₂O₄ are scanty in literature. On the other hand, though dextran as a coating material has been found to be biocompatible, its use is restricted due to loss of coatings intracellularly in a short period to time [5]. Cytocompatibility of lauric acid coated MnFe₂O₄ found in this study is lesser than the dextran coated Fe₃O₄ reported in literatures (1–2 mg/ml) [5]. However, the threshold cytocompatible concentration of the magnetic fluid can be increased up to several fold (three-fold) by encapsulating within liposomes without compromising other relevant parameter like SAR. Hence, promising substituted ferrite like MnFe₂O₄ in the form of magnetic liposome can well be exploited for hyperthermia applications. Though the intracellular stability of both the lauric acid coated MnFe₂O₄ and its magnetic liposomes remains to be seen, use of the magnetic liposomes has several advantages over the use of

Fig. 4. Comparative encapsulation efficiency of different magnetic liposome samples. Inset shows the comparative encapsulation efficiency of TFH 2:1 conventional and stealth magnetic liposome (with 5 molar% DSPE-PEG). Results are expressed as mean ± SD; n = 3.

Fig. 5. Comparative cytocompatibility of lauric acid coated MnFe₂O₄ based magnetic fluid and its stealth magnetic liposome with L929 cell lines after 48 h of incubation. Results are expressed as mean ± SD; n = 3.
magnetic fluid. Magnetic liposomes can easily be fabricated to be long circulating and can simultaneously contain chemotherapeutic drugs so that they can be used for combination therapy of hyperthermia and chemotherapy. Also, magnetic liposomes can be functionalized for active targeting by incorporating folic acid tagged phospholipids in the bilayer without affecting the magnetic properties.

4. Conclusions

In conclusion, TFH is a better method of encapsulation of lauric acid-based magnetic fluid than DE method. Manganese ferrite-based stealth magnetic liposomes made up of egg-PC cholesterol 2:1 molar ratio prepared by TFH can be used for hyperthermia treatment of cancer. Moreover, this formulation can be further exploited for targeted thermo-chemotherapy of cancers.

Acknowledgments

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References