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Dual responsive PNIPAM–chitosan targeted magnetic nanopolymers for targeted drug delivery

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ABSTRACT

A dual stimuli sensitive magnetic hyperthermia based drug delivery system has been developed for targeted cancer treatment. Thermosensitive amine terminated poly-N-isopropylacrylamide complexed with pH sensitive chitosan nanoparticles was prepared as the drug carrier. Folic acid and fluorescein were tagged to the nanopolymer complex via N-hydroxysuccinimide and ethyl-3-(3-dimethylaminopropyl) carbodiimide reaction to form a fluorescent and cancer targeting magnetic carrier system. The formation of the polymer complex was confirmed using infrared spectroscopy. Gadolinium doped nickel ferrite nanoparticles prepared by a hydrothermal method were encapsulated in the polymer complex to form a magnetic drug carrier system. The proton relaxation studies on the magnetic carrier system revealed a 200% increase in the T1 proton relaxation rate. These magnetic carriers were loaded with curcumin using solvent evaporation method with a drug loading efficiency of 86%. Drug loaded nanoparticles were tested for their targeting and anticancer properties on four cancer cell lines with the help of MTT assay. The results indicated apoptosis of cancer cell lines within 3 h of incubation.

1. Introduction

Development of effective models for theragnosis applications has been intensively studied in the past decade [1]. A single system to both specifically detect and effectively treat cancerous tumors has been the main objective behind this research. Magnetic resonance imaging (MRI) has by far proven to be the most effective technique in detecting deep tissue cancers with contrast agents highlighting the tumor growth [2–5]. Currently gadolinium and manganese based contrast agents are being used for obtaining T1 weighted functional MR images, while superparamagnetic iron oxides are under consideration for T2 weighted images [5]. These properties in addition to magnetic hyperthermia heating and external control over the drug delivery system have imbibed the interest of researchers to use magnetic nanoparticles as suitable drug carriers [4–6]. Further it is well known that the use of magnetic hyperthermia for localized heating of cancer cells is an effective way to induce cancer apoptosis [7]. Although this technique has been effective on peripheral cancer tissues, the use of magnetic hyperthermia as a standalone therapy for deep tissue cancer has not been successful [7].

In this paper, magnetic hyperthermia based drug release system has been developed in order to selectively deliver drug to the required site and release drug at the required time. Polymer complex made from chitosan and poly N-(iso-propyl acrylamide) conjugated with folic acid (targeting agent) and fluorescein has been developed for this purpose. Gadolinium based magnetic nanoparticles with intrinsic MRI contrasting property have been prepared and introduced into the drug delivery system. Magnetic hyperthermia and relaxometric studies were conducted to understand the magnetic heating and MRI contrasting property respectively for the developed polymer nanoformulation. These formulations were loaded with curcumin and were tested for anticancer properties on four cancer cell lines.

2. Materials and methods

Ferrous sulfate, nickel acetate, gadolinium acetate, amine terminated poly N-(iso-propyl acrylamide) (NH-PNIPAM), N-hydroxysuccinimide (NHS), ethyl(dimethylaminopropyl) carbodiimide (EDC) and folic acid were purchased from Sigma Aldrich, USA. Triethylamine, chitosan, fluorescein, sodium tripolyphosphate (TPP) and

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curcumin were purchased from Sisco Laboratories, Chennai. All the chemicals were used without further purification for the synthesis of drug delivery system. De-ionized (DI) water was used as solvent in all the reactions.

Phosphate buffer saline (PBS), fetal bovine serum (FBS), 25 mm dialysis tubing sack, tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit and supplemented Dulbecco's modified eagles medium (DMEM) were purchased from Sigma Aldrich, USA for performing drug release and cytotoxicity studies. Cancer cell lines were kindly donated by Life Line Hospitals, Chennai.

2.1. Gadolinium doped nickel ferrite magnetic nanoparticles (MNPs)

Gadolinium doped nickel ferrites with the molecular formula $NiFe_{1.8}Gd_{0.2}O_4$ were prepared using a hydrothermal process similar to the previously reported procedure [8]. 60 mL aqueous solution containing 10 mM nickel acetate, 18 mM ferrous sulfate, and 2 mM gadolinium acetate precursor salts was titrated with 10% triethylamine solution until the pH reached 10. The reaction mixture was enclosed in a 100 mL Teflon coated autoclave and heated at 150 °C for 6 h. The autoclave was allowed to cool down naturally before it was opened. A dark brown magnetically susceptible precipitate was formed which was washed multiple times with DI water and ethanol and air dried at 60 °C overnight.

The structural properties of the MNPs were characterized by PANalytical Xpert Pro X-ray diffraction system (XRD) with Cu-kα source and morphological and composition analysis was characterized using JEOL 2100 high resolution transmission electron microscopy (TEM) and energy dispersive spectroscopy (EDS) at 100 keV beam voltage. The TEM analysis was performed by dropping an aqueous dilution of MNPs onto a copper grid and analyzing at 300 keV accelerating voltage.

2.2. Folic acid and fluorescein tagged NH-PNIPAM and chitosan polymer complex

The amine functional groups of NH-PNIPAM and chitosan were complexed with the help of 2% glutaraldehyde solution. A weight ratio of 1:4 chitosan and NH-PNIPAM was determined as the desired ratio for the preparation of thermosensitive polymer complex (not discussed here) with a lower critical solution temperature (LCST) of 45 °C. Thermosensitive polymer complex was prepared by adding 50 mg of chitosan/NH-PNIPAM (1:4 weight ratio) mixture to 10 mL of 2% acetic acid solution under continuous stirring for 2 h in the presence of Tween-80. Glutaraldehyde (5 mL) was slowly titrated into the polymer solution and stirred rigorously for 2 h. The resulting solution was neutralized with 1% sodium hydroxide solution and centrifuged at 4200*g*. The supernatant was discarded and the pellet was lyophilized and stored for further experiments.

Folic acid and fluorescein with carboxyl groups were added to the amine rich polymer chain using NHS-EDC reaction [9]. The evidence of folic acid and fluorescein tagged polymer complexes was established with the help of Perkin Elmer ALPHA FT-IR Infrared spectroscopy (FTIR).

A volume of 10 mL of 1 mg/mL polymer complex was mixed with 10 mg of MNPs in PBS suspension. The temperature of the solution during the addition of the nanoparticles was kept at 45 °C and quenched to 10 °C using an ice bath. The mixture was left for mechanical stirring overnight at 10 °C to form the magnetic polymer complex (MPC). The MPC was washed multiple times using centrifugation to remove any excess MNPs and free polymer complexes. The resulting pellet with 80% polymer binding efficiency was re-suspended in 10 mL of PBS.

The MPCs were characterized further for their morphological, hyperthermia heating and proton relaxation properties using a Quanta FEG 200 scanning electron microscope (SEM), Quantum Design magnetic property measurement system (MPMS), Ambrell hyperthermia heating system and Bruker Minispec Mq10 respectively. The SEM analysis was done by dropping an aqueous suspension of the MPC onto a carbon tape and air dried for a period of 3 h before imaging at 5 keV beam voltage.

2.3. Entrapment efficiency and drug loading

The entrapment efficiency (*E*) was calculated by determining the amount of curcumin loaded versus the total curcumin used. 10 mL of 3 mg/mL ethanolic solution of curcumin was added dropwise along with 10 mL of 6 mg/mL MPC into hot (45 °C) 1% TPP solution. The mixture was left to stir at this temperature for 6 h for the ethanol to evaporate and then cooled to 10 °C using an ice bath. The resulting mixture was filtrated to separate undissolved curcumin from the curcumin loaded MPC system. This was termed as the magnetic drug delivery system (DDS) which was used for further experiments. The filtrate was re-suspended in 10 mL ethanol to dissolve the unloaded curcumin. The DDS was dissolved with the help of dilute acetic acid in order to dissolve the loaded curcumin in a separate beaker and mixed with ethanol. The absorbance value for ethanolic solutions of curcumin were recorded at 425 nm and plotted against a standard absorbance plot (not shown here) to estimate the amount of loaded and unloaded curcumin. The DDS solution was centrifuged at 17,000*g*, lyophilized and stored at 4 °C for further analysis.

2.4. In vitro drug release studies

Dialysis bag diffusion method was used for understanding both passive and active in vitro drug release characteristics of DDS. In the passive drug release model, lyophilized DDS were re-suspended in 10 mL PBS in a dialysis bag (10 kDa cut off) and submerged completely into 50 mL PBS solution. The entire setup was placed in an incubator and maintained at 37 °C in order to mimic human body temperature. Sample of volume 1 mL were collected and replenished every 3 h upto 10 h from the solution.

In the active drug release model of DDS, a setup similar to the passive drug release model was used with only a change in the setup temperature. The setup was maintained at 45 °C using a magnetic hyperthermia setup discussed earlier. Samples of volume 1 mL were collected and replenished every 15 min from the sample till 2 h.

All the samples were analyzed with the help of a UV–VIS Spectrophotometer model 3000+ from LabIndia analytical.

2.5. In vitro cytotoxicity studies

Four cancer cell lines namely SK-OV-3 (human adenocarcinoma), MCF-7 (human breast cancer), DU-145 (human prostate cancer) and MD-MB-231 (human breast adenocarcinoma) were cultured in uncoated 24 well culture plates in DMEM (with 1000 mg/L glucose, L-glutamine and sodium bicarbonate with pyridoxine) supplemented with 10% FBS and 1% of penicillin/streptomycin/gentamycin. The cells were then incubated at 37 °C in a 5% CO_2 incubator. When the cells reached a confluence of 80–90% they were harvested using 0.1% trypsin and 0.02% EDTA in phosphate buffer saline.

The harvested cells were added to a 96-well plate with a cell count of 5000. Five different concentrations (0.5, 0.2, 0.1, 0.05, and 0.01 mg/mL) of DDS, MPC and MNPs were added to the 96-well plate. The cells were left to attach onto the plates for 3 h at 37 °C in a 5% CO_2 incubator before DDS dissolutions (subjected to magnetic

hypethermia) were injected into the culture plates along with appropriate amount of MTT. A compound microscope was used to evaluate the effect of DDS on the cell lines every 3 h upto 12 h. The extent of formazan formation was evaluated with the help of an ELISA plate reader at 560 nm.

3. Results and discussions

3.1. Characterization of MNPs

The TEM results clearly indicated nanoparticles in the size range of 20–35 nm (Fig. 1a). The EDS data (Fig. 1b) confirms the presence of gadolinium in the sample. Although the particle size range for the gadolinium doped nickel ferrite nanoparticles are larger than gadolinium oxide based contrast agents [5], their size is in coherence with magnetic nanoparticles for use in hyperthermia and drug delivery systems [1].

XRD analysis provided useful information regarding the crystal structure of the as-synthesized MNPs. The analysis conducted between 2θ values 20° and 80° results indicated the formation of single phase face centered cubic spinel ferrites with strong reflections for (220), (311), (222), (400), (422), (511) and (440) planes corresponding to JCPDS# 044-1485 (Fig. 1c). The crystallite size was estimated at 32 nm from Scherrer's formula which corresponds well with the particle size estimated using TEM analysis. Further Rietveld refinement on the XRD pattern showed an increase in the lattice parameters along with 2% increase in the lattice strain which suggests the successful substitution of iron with gadolinium at the octahedral site. It is well known that gadolinium prefers octahedral site owing to its large ionic radius and low crystal field stabilization energy. These results correspond well with structural properties of nickel ferrites prepared by similar hydrothermal method process [8].

Magnetic properties of the MNPs were evaluated at 300 K under a constant 7 T magnetic field in a MPMS. The results showed the presence of superparamagnetic particles with a magnetic saturation of 40 emu/g (Fig. 1d). The nanoparticles saturate at a relatively small field of 0.25 T indicating the ease of magnetization of the sample. The presence of high saturation magnetization and susceptibility value coupled with superparamagnetic property makes gadolinium doped nickel ferrites ideal candidates for magnetic hyperthermia and drug delivery application.

3.2. FTIR analysis

The FTIR analysis on the polymer complex was conducted to verify the formation of NH-PNIPAM–chitosan–folic acid–fluorescein complex. Dry lyophilized samples were mixed with KBr for FTIR analysis. The presence of absorption peaks at 1705, 1660, 1569, 1401, 1244, 1065, 1003, 724, 666, 466, 456, and 441 cm^{-1} can be seen. The peak at 1660 cm^{-1} indicated the formation of amide bonds between carboxylic acid groups of folic acid and fluorescein with the amine group of chitosan.

Appearance of peaks at 1569 cm^{-1} and 1705 cm^{-1} in the FTIR spectrum corresponds to asymmetric bending vibrations of –CO and stretching of 1° amines –NH signified the adherence of folic acid to the amine groups of chitosan [10]. The other frequencies represent the characteristic absorption peaks for NH-PNIPAM and chitosan. A comparative FTIR graph between the untreated polymers and the fabricated polymer complex in Fig. 2 showed the change in the absorption spectra with the addition folic acid and fluorescein.

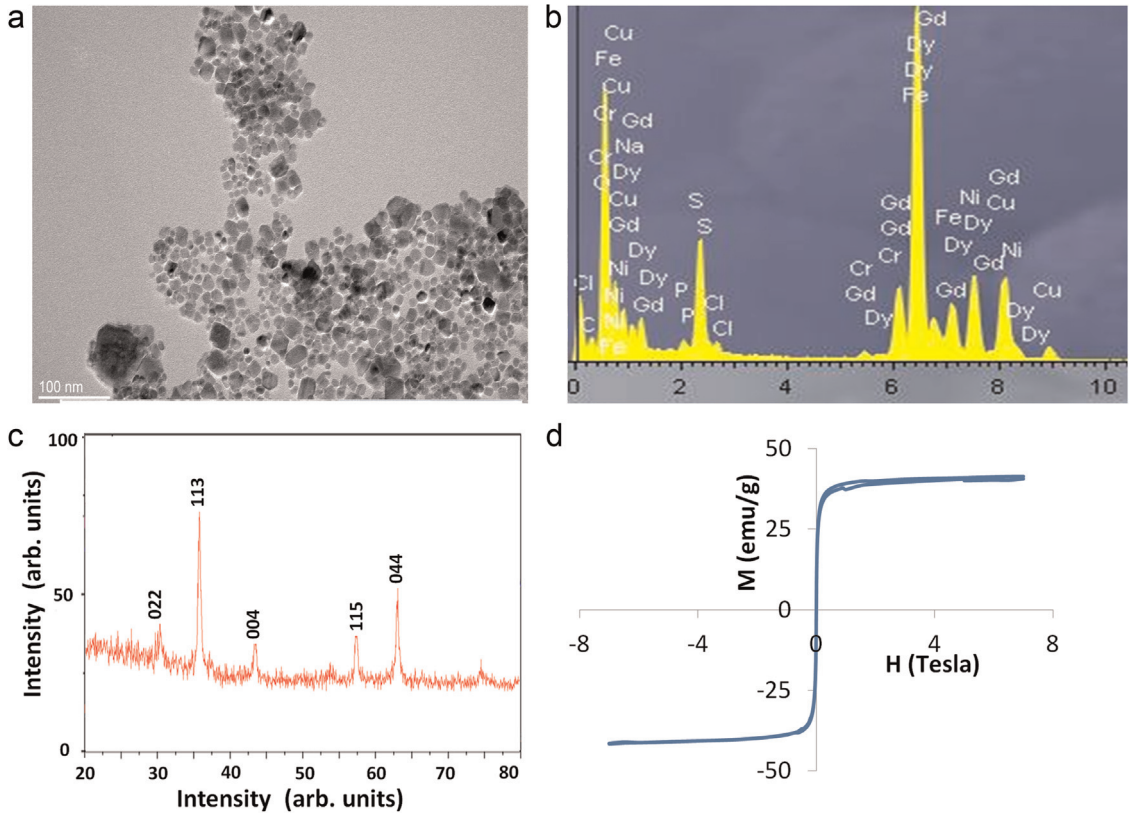

Fig. 1. (a) TEM micrograph of the MNPs. (b) EDS of the particles showing presence of gadolinium in the MNPs. (c) XRD analysis showing FCC spinel structure of gadolinium doped nickel ferrite. (d) VSM graph showing a superparamagnetic loop for the MNPs.

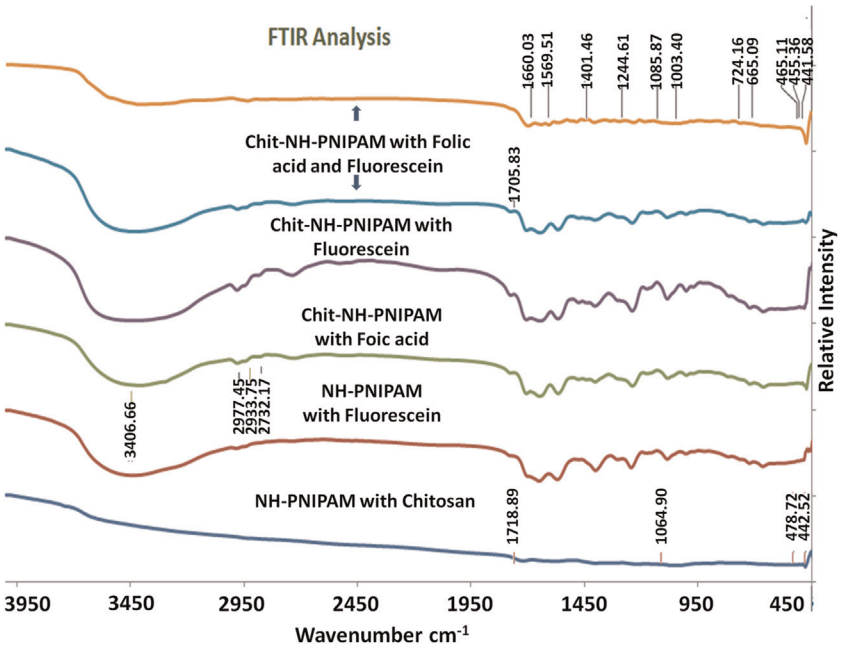

Fig. 2. FTIR graphs representing the formation of folic acid and fluorescein tagged NH-PNIPAM–chitosan complex.

3.3. Characterization of MPCs and DDS

SEM analysis of the MPC (Fig. 3a) showed the formation of spherical nanostructures in the size range of 150 ± 10 nm. The nanopolymers were found to be spherical in shape with high level of consistency in size across the sample. The consistent shape of MPCs in the 150 nm size range is similar to nanopolymer systems previously used in drug delivery systems [1,6] as effective drug carriers to desired sites.

Magnetic hyperthermia studies were conducted on 3 mL of 1 mg/mL MPC solution under an AC magnetic field of 250 kHz and 35 mT with the help of a 3 in. diameter coil at ambient room temperatures for a period of 600 s (Fig. 3b). The results indicated a sharp increase in the temperature of the solution from 27 °C to 68 °C in 600 s. It can be observed that the heating rate of the MPCs is lower compared to conventional polymer coated magnetite nanoparticles used in hyperthermia applications [7]. However, the temperature increase was recorded to be higher than the LCST of the polymer complex being employed, which would serve the purpose of drug release as a result of thermal stimulation. It was observed that the increase in the temperature of the MPC was followed by the decrease in the transparency of the solution. This can be attributed to the presence of a thermosensitive polymer (NH-PNIPAM) in the MPC which clumped to form larger white precipitates when the temperature reached the LCST.

High longitudinal (T1) relaxation rate of 4.6 s^{-1} was observed in aqueous samples containing 0.1 mg/mL of DDS compared to control sample. These studies revealed an increase of 200% in the relaxation rate (Fig. 3c) of protons in the presence of 0.1 mg/mL of the DDS in the sample. The nanoparticulate system increased the relaxation rate of the protons extensively due to the presence of large paramagnetic gadolinium nuclei in the sample. The relaxation rate of the MPC is comparable with previous studies conducted on gadolinium chelates [4], gadolinium oxide nanoparticles [5], and gadoteric acid [11] confirming the effective proton relaxation ability of gadolinium doped nickel ferrite nanoparticles.

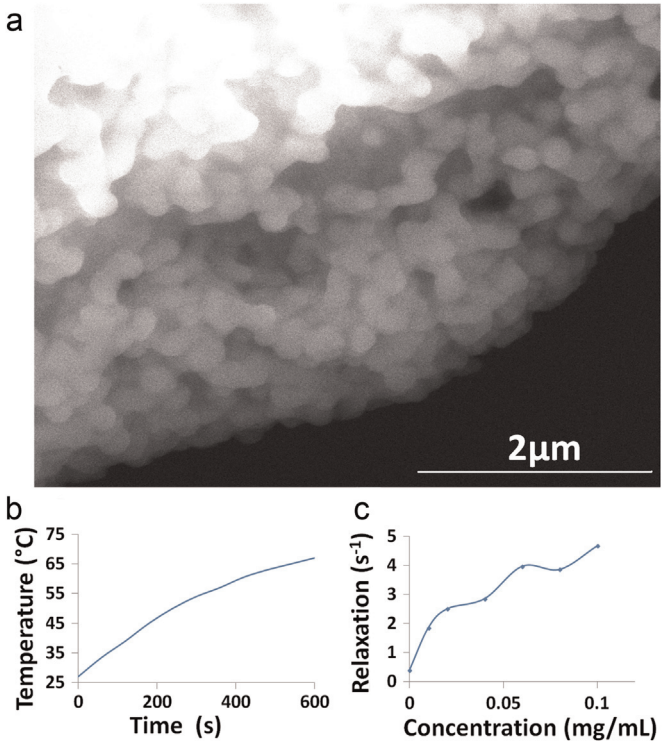

Fig. 3. (a) The SEM micrograph of the drug loaded polymer nanoparticles with a size range of 150 ± 10 nm. (b) Magnetic hyperthermia studies showing the increase in temperature of the sample with time. (c) Proton relaxation studies showing an increase in the T1 relaxation rate by 200% with the addition of MNPs.

3.4. Drug loading and drug release

The loaded drug concentration was found to be 25 mg of curcumin in 60 mg of DDS against 30 mg of total curcumin and drug loading capacity of 0.416 (mg curcumin per mg DDS). The effective loading concentration was found to be 86% which was higher than previously reported starch microspheres (83.9%) [12], poly(D,L-lactic-co-glycolic acid) (PLGA) nanospheres [13] and solid

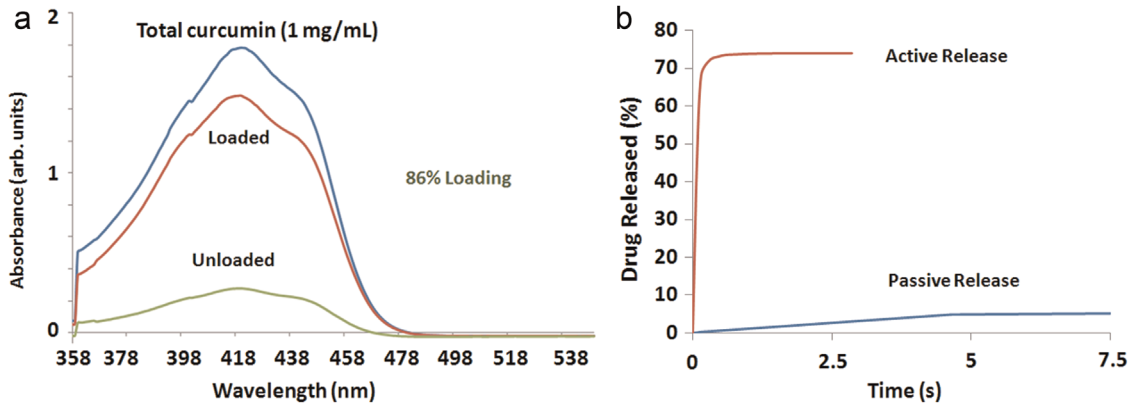

Fig. 4. The figure shows the (a) UV absorption data suggesting the effective drug loaded and not loaded into the DDS and (b) comparative release study between active and passive drug release models conducted on the DDS.

lipid nanoparticles (81.3%) [14,15] loading efficiency for curcumin. The UV absorbance graphs for the loaded and unloaded system can be seen in Fig. 4a.

Passive drug release model of the DDS showed a release of 10% of the total curcumin released over a period of 24 h (data shown only for 8.3 h), while the active release model of DDS at a temperature of 45 °C showed the release of 70% of the curcumin within 600 s of application of magnetic hyperthermia. The burst release of the drug in the case of active drug release can be attributed to the shrinking property of the thermosensitive polymer complex in the DDS which releases the entire drug when the temperature is raised over its LCST. Similar results have been observed on PLGA nanopolymers (70%) [13] using an active magnetic hyperthermia based release. The active and passive drug release models for DDS can be seen in Fig. 4b.

3.5. *In vitro cytotoxicity assay*

Anti-cancer properties of the curcumin loaded DDS and cytotoxicity of MPCs and MNPs were evaluated using MTT assay [16]. In this assay, the MTT is taken up by the cells and reduced in a mitochondria-dependent reaction to yield a formazan product. This product dyes the cell violet in color which can be detected using a simple colorimeter and is an indication of mitochondrial activity or a direct measure of cell viability.

The results in all the cell lines indicated that the DDS (pre-treated with magnetic hyperthermia) alone affected the cancer cell lines with no coloration of the wells. The wells injected with MPC and MNPs showed violet coloration signifying their non-toxic nature. An ELISA plate reader was used for evaluating the amount of formazan in the wells and the results are shown in Fig. 5. The DDS in all concentrations including the lowest concentration (0.01 mg/mL) resulted in decreasing the cell viability to 40% within the first three hours of incubation, while the highest concentration of 0.5 mg/mL decreased the cell viability to fewer than 15%. These results are in accordance with reported anticancer properties of curcumin [16–18] released by the DDS into the well plate. The drug release model discussed in this study is comparable to previously reported studies where cell viability of 30% [13] and 40% [14,17] within three hours of incubation with cancer cell lines. DDS developed in this paper are observed to have higher (if not equal) efficiency of drug release and anticancer properties when

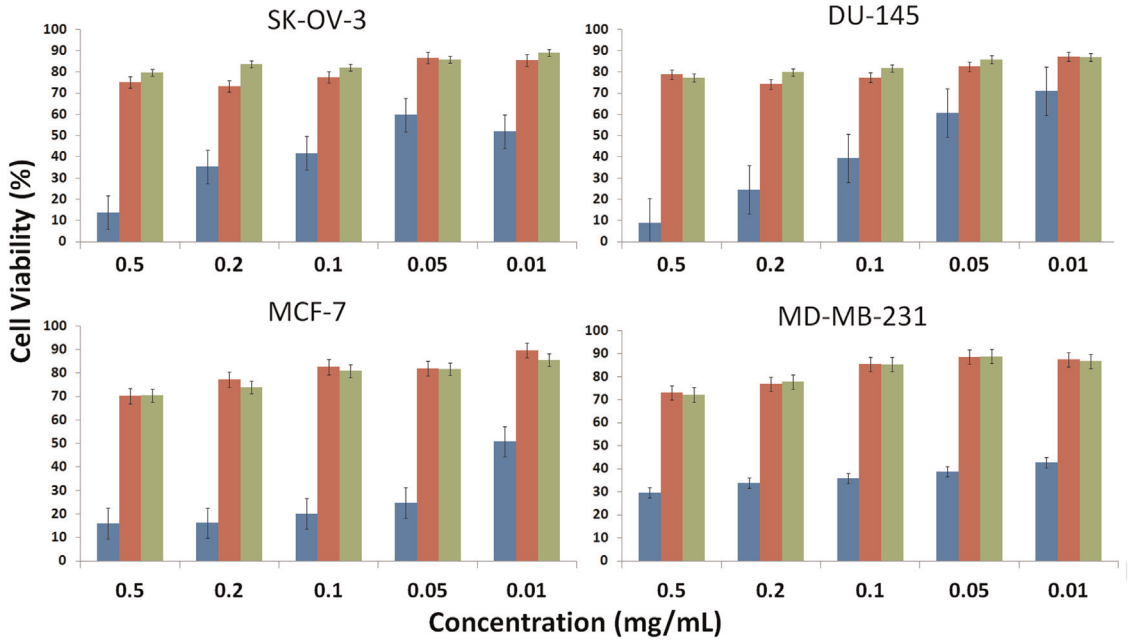

Fig. 5. The bar diagram represent the percentage cell viability of four cancer cell lines namely SK-OV-3, MCF-7, DU-145 and MD-MB-231 when exposed to various concentrations of DDS (blue), MPC (red) and MNPs (green) through MTT assay. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

compared to earlier reported studies. The MPC and MNPs seem to have very minimal effect over the cell lines indicating their non-toxic and bio-compatible nature due to the formation of formazan in the cells.

4. Conclusions

An effective targeted drug delivery system with multimodal imaging capabilities such as T1 MRI contrasting has been prepared in this paper. The particles have high magnetic saturation and superparamagnetic nature which are desired characteristics for a nanoparticle drug delivery system. The contrasting ability of the particles have been evaluated which show a 200% increase in the proton relaxation rate of water molecules. The hyperthermia heating measurements show that the particles rapidly heat to 60 °C within 600 s of application of AC magnetic field. The nanoparticles and the magnetic polymer complex developed in this work were proven to be bio-compatible in nature with no toxic side effects. A drug loading of 86% and a 70% drug release profile was recorded for the magnetic DDS. MTT assay of the DDS recorded cell death within 3 h of incubation with four cancer cell lines. It can be concluded that this drug delivery system is an effective multimodal dual responsive targeted drug delivery system which uses curcumin (an Indian spice) as a therapeutic agent with low toxic side effects. Nuclear magnetic resonance studies to understand the molecular structure of the MPC along with in-vivo investigations of DDS are underway and will be able to suggest the effective imaging and therapeutic capabilities of the developed DDS in rat models. While the current model does not emulate information on the synergistic effect of hyperthermia along with the curcumin, the authors hope to further study the combinatorial effects of the same.

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