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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