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pH and magnetic field sensitive folic acid conjugated protein–polyelectrolyte complex for the controlled and targeted delivery of 5-fluorouracil

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ABSTRACT

In the present study, a magnetic protein–polyelectrolyte complex of casein coated iron oxide nanoparticles and folic acid conjugated chitosan-graft-poly(2-dimethylaminoethyl methacrylate) was prepared for the delivery of an anticancer drug, 5-fluorouracil (5-FU) and characterized using FTIR, XRD, VSM, FESEM, TEM, DLS and zeta potential studies. Sustained and controlled release of 5-FU was observed at acidic pH 5.0. Drug release kinetic studies indicated both swelling and diffusion controlled release. *In vitro* cytotoxicity studies revealed the low toxicity of the prepared nano drug carrier towards normal cells and folate receptor targeting efficiency of 5-FU loaded carrier towards cancer cells.

Introduction

The shortcomings of conventional chemotherapy paved the way to the development of innovative Drug Delivery Systems (DDS) with increased therapeutic efficiency. As a result of this controlled drug delivery systems have been emerged as a new area of research. The controlled and targeted delivery of anticancer drugs brings down the side effects and improves its elimination half life and therapeutic index. Nano DDS with particle size less than 200 nm are of particular interest, since they accumulate in the tumor tissue because of Enhanced Permeability and Retention effect (EPR effect). The leaky vasculature around the tumor tissue augments the easy uptake of nanoparticles [1,2]. Stimuli-sensitive polymers can deliver the encapsulated drug according to the internal stimuli such as pH, temperature and external stimuli such as electric field and magnetic field. Among these, pH sensitive polymers are widely studied for cancer treatment since cancer cells are characterized by their acidic environment. pH sensitive protonation and deprotonation of the polymer chain leads to the release of encapsulated drug [3].

Several metal and metal oxide nanoparticles have been incorporated to the controlled delivery system so far with the intention of enhancing their stimuli-responsive properties. Among the metal oxide nanoparticles, Fe_3O_4 nanoparticles possess superparamagnetic nature at room temperature and they can be easily manipulated by applying an external magnetic field. Moreover they have low toxicity. Bare magnetic nanoparticles have to be coated with suitable agents which prevent agglomeration and improve their drug delivery efficiency. The physicochemical and biopharmaceutical properties of the system can be improved by proper modification. Magnetic nanoparticle incorporated DDS can be actively targeted to the neoplastic cell using applied magnetic field [4,5].

Growing interest has been devoted in the field of drug delivery for polyelectrolyte complexes formed between polyanions and polycations *via* electrostatic interaction as drug carriers. They can encapsulate large amount of the drug by physical interactions and deliver it in an external stimulus such as pH [6]. Moreover these polyelectrolyte complexes serve as a biocompatible system. Complexes can also be formed between a protein and a polyelectrolyte at a particular pH. Proteins are positively charged below their isoelectric point (p*I*) and negatively charged above their p*I*. When proteins are mixed with polyelectrolyte at a particular pH which favors the electrostatic attraction between them, protein- polyelectrolyte complexes are formed. A cationic polyelectrolyte can form complex above the p*I* of the protein [7,8].

Casein (CN) is the main constituent of milk protein which can encapsulate bioactive molecules in its micellar structure. 80% of milk protein is made up of casein which has four structural constituents namely, $\alpha_{s1}{}^{-}$, $\alpha_{s2}{}^{-}$, β and κ casein. The pK_a of casein

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varies between 4.2 to 5.8. It is biocompatible and shows pH responsive conformational and functional changes. It is inexpensive, amphiphilic and thermally stable upto 60–70 °C [9,10]. Chitosan (CS) is a natural, biocompatible, non-toxic polymer and widely used in biomedical field. It bears hydroxyl and amine functional groups which can be further modified. Physical properties of CS can be altered through these modifications [11]. Folic acid (FA) is a ligand which can selectively bind with the folate receptor (FR). It is non toxic and act as a precursor in DNA base synthesis. FRs are overexpressed in many neoplastic cells .This can be exploited for targeting these cells by means of FA ligand. FRs are glycoproteins with so many cysteine residues on its surface. FA conjugation to nanoparticles enhance the endocytosis of nanoparticles *via* FRs by utilizing the high affinity of FRs towards FA [12,13]. 2-(Dimethylamino)ethyl methacrylate (DMAEMA) is a monomer which polymerize to form cationic polyelctrolyte. It contains tertiary amino groups which can be protonated at acidic pH owing to its pK_a 8.2 [14]. DMAEMA based cationic polymers are extensively studied for its stimuli responsive properties and its application in DDS [15].

5-FU is a pyrimidine anticancer drug widely used in the treatment of colorectal, breast, stomach and pancreatic cancer. It can be administered orally or intravenously. But one of the main disadvantage of direct administration of 5-FU is that it may affect both normal cells and cancer cells. Since it is a structural analog of pyrimidine uracil, it interferes with DNA or RNA synthesis by mimicking their building blocks. 5-FU is acidic and hydrophilic in nature [16].

In the present study, a novel folic acid conjugated superparamagnetic protein–polyelectrolyte complex (IO-PPEC-FA) was synthesized and well characterized using FTIR, XRD, FESEM, DLS, TEM and VSM. The synthesis procedure includes the preparation of casein coated magnetic nanoparticles. FA was conjugated to CS through EDC/NHS coupling. Then DMAEMA was grafted onto hydroxyl group of CS using ceric ammonium nitrate (CAN). Then a complex of casein coated iron oxide nanoparticles (IO-CN) and folic acid conjugated chitosan-graft-poly(2-dimethylaminoethyl methacrylate) (FA-CS-g-PDMAEMA) was made at alkaline pH. The drug loading was done during the complex formation step. The zeta potential studies were conducted to find out the surface charge. *In vitro* drug release and cytotoxicity studies on L929, MCF-7 and MDA-MB-231 cells were also carried out to find out its applicability in drug delivery.

Materials and methods

Materials

Chitosan was purchased from Himedia laboratories, Mumbai (CS, CAS No. 9012-76-4). 2-(Dimethylamino)ethyl methacrylate (DMAEMA, CAS No.2867-47-2) and folic acid (FA, CAS No. 59-30-3) were obtained from Sigma Aldrich Co, U.S.A. and Merck, Germany respectively. 1-(3-Dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride(EDC, CAS No. 25952-53-8), *N*-hydroxy succinimide (NHS, CAS No. 6066-82-6) and glyoxal (CAS No.107-22-2) were purchased from Tokyo Chemical Industry Co. Ltd. (TCI), Japan. Casein procured from Nice chemicals, India. All the solvents used were obtained from Merck, Mumbai. Double distilled water was used throughout the study.

Preparation of drug carrier

Synthesis of FA-CS-g-P(DMAEMA)

FA-CS was synthesized according to the previous report [17]. CS (0.05 g) was dissolved in 10.0 mL of 1.0% acetic acid solution by stirring overnight on a magnetic stirrer. Then a solution of FA (0.10 mmol), EDC (0.15 mmol) and NHS (0.15 mmol) in DMSO was added dropwise to the CS solution. The whole mixture was stirred overnight on a magnetic stirrer under dark condition. FA-CS was precipitated by adjusting the solution pH to pH 8.0 using NaOH solution. The yellow precipitate obtained was collected by centrifugation, washed with $NaHCO_3$ solution (pH 8.5) and lyophilized.

FA-CS (0.05 g) was dissolved in 30.0 mL of 1.0% acetic acid solution. The FA-CS solution was heated to 60 °C. To this solution 0.50 g CAN initiator and 1.0 mL DMAEMA were added. The mixture was stirred on a magnetic stirrer at 60 °C for 3 h to complete the polymerization. The product, FA-CS-g-P(DMAEMA) was precipitated by changing the pH to alkaline. The solution was centrifuged and the precipitate was washed with hot water and dried in an oven [18]. The percentage of grafting was calculated from the following equation.

$$\text{Grafting percentage} = \frac{W_2}{W_1} \times 100 \quad (1)$$

where W_1 is the total weight of FA-CS and DMAEMA monomer and W_2 is the weight of FA-CS-g-P(DMAEMA) formed.

Synthesis of casein coated iron oxide

For the synthesis of IO-CN, firstly glucose coated iron oxide (IO-Glu) was synthesized as reported elsewhere [19]. IO-Glu (0.50 g) was added to 2.0% (w/v) casein solution in 0.01 M NaOH and stirred for 4 h [10]. Then 5.0 mL of glyoxal was added as crosslinking agent. The mixture was stirred overnight on a magnetic stirrer at room temperature. The precipitate was collected by ultracentrifugation, washed with distilled water and dried in an oven at 40 °C.

Synthesis of magnetic protein–polyelectrolyte complex

IO-CN (0.10 g) was dispersed in 40 mL of distilled water and pH of the solution was adjusted to 8.0 using 0.01 M NaOH. The dispersion was sonicated for 30 min and then placed over a magnetic stirrer and stirred at 1000 rpm. 5.0 mL of 1.0 wt% (w/v) solution of FA-CS-g-P(DMAEMA) containing 5-FU (2 mg/mL) was added drop wise using a microsyringe to the above dispersion. The precipitated 5-FU loaded IO-PPEC-FA was collected using a magnet and dried in oven at 50 °C. IO-PPEC-FA without 5-FU was also prepared in the same way by omitting 5-FU addition. The encapsulation efficiency was calculated using the following formula.

$$\text{Encapsulation efficiency} = \frac{\text{Total amount of 5FU} - \text{Free 5FU}}{\text{Total amount of 5FU}} \times 100 \quad (2)$$

The 5-FU concentration was determined by measuring the absorbance at 266 nm and comparing the value with a standard calibration curve.

Instruments and methods of characterization

The FTIR spectra of iron oxide containing samples were recorded with Shimadzu FT-IR spectrometer in 4000–400 cm^{-1} region by KBr pellet technique and FT-IR spectra of all other samples were recorded with Cary 630 spectrophotometer in 4000–650 cm^{-1} by direct sampling technique. Bruker AXS D8 advance X-ray diffractometer using CuK_α radiation at a wavelength of 1.5406 Å was used for X-ray diffraction (XRD) analysis. Morphology of the samples was obtained from Field Emission Scanning Electron Microscope (Nova nano SEM 450, Czech Republic). Philips CM12 STEM, Netherlands, Transmision Electron Microscope was used to find out the size and morphology of IO-PPEC-FA.