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In vitro evaluation of folic acid modified carboxymethyl chitosan nanoparticles loaded with doxorubicin for targeted delivery

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Abstract The development of smart targeted nanoparticle that can deliver drugs to direct cancer cells, introduces better efficacy and lower toxicity for treatment. We report the development and characterizations of pH-sensitive carboxymethyl chitosan modified folic acid nanoparticles and manifest their feasibility as an effective targeted drug delivery vehicle. The nanoparticles have been synthesized from carboxymethyl chitosan with covalently bonded bifunctional 2,2′-(ethylenedioxy)-*bis*-(ethylamine) (EDBE) through the conjugation with folic acid. The conjugation has been analyzed by Fourier transform infrared spectroscopy and nuclear magnetic resonance spectroscopy. The resultant nanoparticles with an average size less then 200 nm measured by dynamic light scattering and transmission electron microscopy. Confocal microscopy and flow cytometric analysis have revealed that folate-mediated targeting significantly enhances the cellular uptake of the nanoparticle and thus facilitates apoptosis of cancer cells (HeLa, B16F1). For the application of the nanoparticles as a drug carrier, Doxorubicin a potent anticancer drug has been loaded into the nanoparticles, with the drug loading amount and the drug release pattern observed.

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1 Introduction

The uses of polymeric nanoparticles with functional properties have been widely used in a broad range of bio-applications; like drug and gene delivery, cell and tissue engineering, diagnostic and therapeutic purposes [1–4] etc. Among these applications, the field of drug delivery by nanoparticles with specific and rapid internalization into a target cell has immense promise [5–9]. Nanoparticles of polymer not only protect the bioactive substance but also facilitate the control release of the material in delivering system [10–13]. To improve further for targeting tumor and cancer cell, we have introduced here simple water-soluble biocompatible polymeric carriers which are tagged with folic acid. It is established that the cancer or tumor cells have special affinity to folic acid due it's over expressed folate receptor on its outer membrane.

Chitosan a copolymer of glucosamine and *N*-acetyl-D-glucosamine linked by glycosidic bonds, is the second plentiful biomass produced by deacetylation of chitin. Because of its biodegradability, compatibility, hemostatic, fungistatic, and low cost, chitosan has been used as biomaterial for drug delivery, gene delivery and other biomedical application [14]. The *O*-carboxymethylated chitosan, a derivative of chitosan is a water-soluble polymer where H of hydroxyl group of monomer is replaced by a carboxymethyl group through an ether bond. Its biocompatibility has been proven [15]. The over expressed folate receptor (FR) in many different types of human cancer cell introduces a means for efficient targeting for tumor/cancer for specific drug [16]. When folic acid (FA) is attached to carboxyl site through a pendant group, folate retains its normal receptor-binding affinity and can, therefore, be internalized by receptor mediated endocytosis [17]. This principle has been exploited for the selective delivery

of imaging agents [18], gene [19, 20] therapeutic agents [21], micelle of block copolymer [22], and other complexes of macromolecular [23] to tumor/cancer cells.

Doxorubicin (DOX) is a drug widely used in cancer chemotherapy [24–27]. However doxorubicin is a strong cytotoxic compound to normal tissues and produces extensive biochemical adverse effects on the physiology of the patient. To decrease the toxicity of doxorubicin, targeted delivery of the drug through polymeric nanoparticles is an alternative efficient option for cancer therapy. Cell adhesion and potential cell uptake of nanoparticles of chitosan should be efficient due to its positive charge which is attracted by negatively charged cell membranes in addition to targeted delivery through folic acid.

A significant issue determining the effectiveness of the nanoparticles drug carrier is the ability to control the location and time over which drug release occurs. This challenge has motivated the development of nanoparticle systems that are designed to release their drug load in a controlled manner, upon arrival at the target site. Change in acidity is a particularly useful stimulus to consider in the development of drug carriers because of the numerous pH gradients that exist in both normal and physiological states. It is well known that the extracellular pH of tumors is slightly more acidic than the blood and normal tissue [28–30]. In addition, it is proposed that nanoparticles are taken up by cells via an endocytosis process [31, 32]. The endocytic pathway begins near the physiological pH of 7.4; it drops to a lower pH of 5.5–6.0 in endosomes and approaches pH 4.5–5.0 in lysosomes [33]. Therefore, polymeric nanoparticles that are responsive to pH can be designed to selectively release their payload in tumor tissue or within tumor cells.

In our recent papers [34, 35] we described syntheses, physicochemical characteristics and results in vitro evaluation of selected biological properties of the folic acid modification on magnetic nanoparticles. This paper focuses on the study of the effect on physico-chemical and biological properties of chitosan modification folic acid based nanoparticles and DOX encapsulation and release study at different pH. The conjugates are designed to develop efficient anticancer polymer drugs facilitating tumour-specific drug delivery. This study is aimed to develop a novel, inexpensive nanoparticles containing anti-cancer drug and targeting potential to cancer cells in vitro.

2 Materials and methods

2.1 Materials

Folic acid (FA), chitosan (medium molecular weight), dicyclohexyl carbodiimide (DCC), trifluoroacetic acid, 2,2′-(ethylenedioxy)-*bis*-(ethylamine) (EDBE), di-*tert*-butyldicarbonate (BoC_2O), *N*-hydroxysuccinimide (NHS) and 1-[3-dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC), Fluorescin isothiocyanate (FITC), Rhodamine isothiocyanate (RITC) were obtained from Aldrich Chemicals, USA. Commercially available dimethyl sulfoxide (DMSO) and *N*,*N*-dimethyl formamide (DMF) were purified by vacuum distillation. Pyridine was purified by distillation over KOH. Monochloroacetic acid was obtained from E. Merck, Germany.

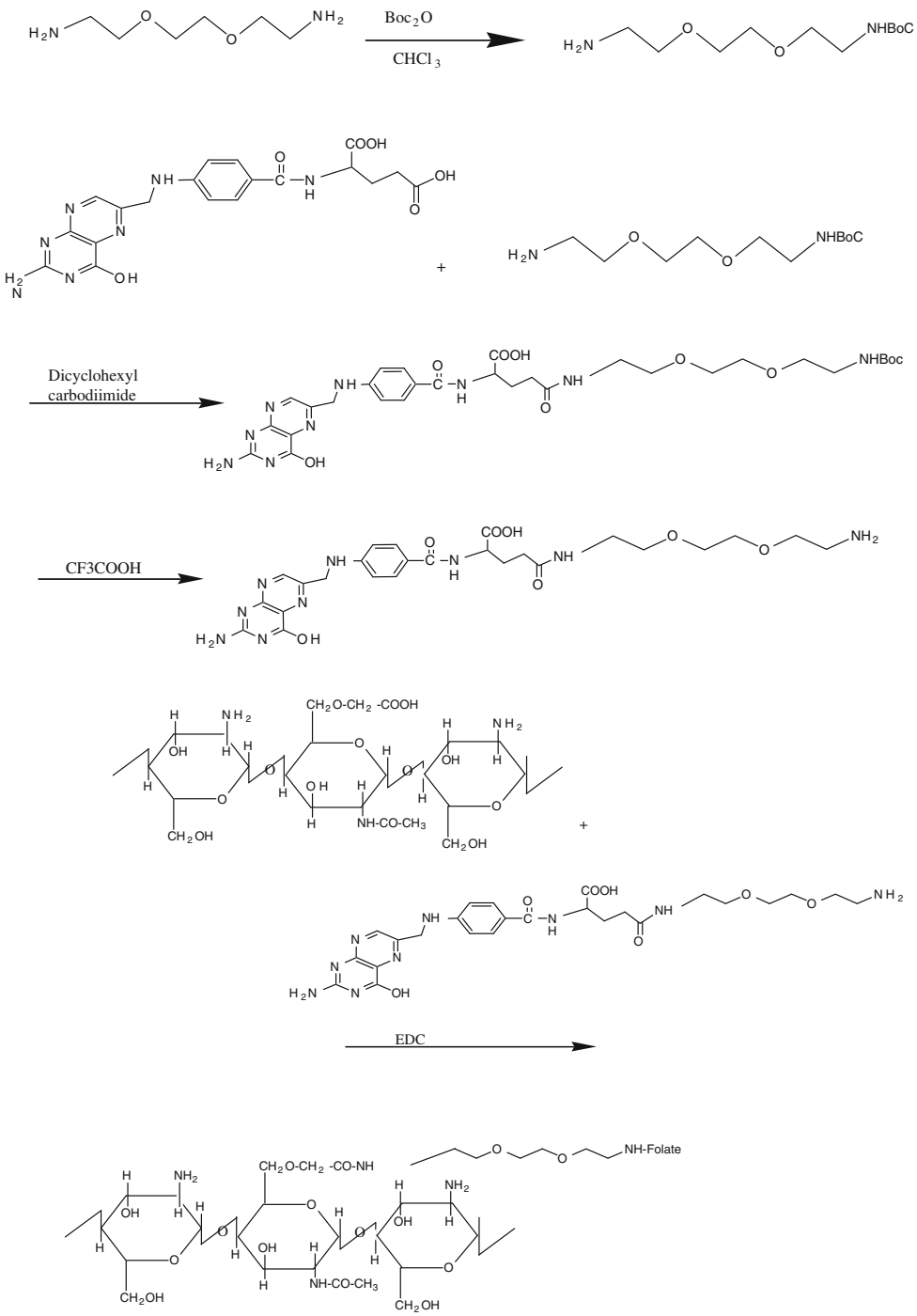
2.2 Preparation of carboxymethyl chitosan (CMC)

Carboxymethyl chitosan (CMC) was prepared as reported previously by Chen and Park [36]. The degree of substitution and the yield are strongly dependent on the reaction time, temperature. Chitosan (2 g) was suspended in 40% (w/w) aqueous NaOH (15 ml) and kept at 0°C overnight. The cold alkaline solution of chitosan was transferred to 2-propanol (60 ml), and monochloroacetic acid (6 g) in isopropanol (2 ml) was slowly added to the solution over 30 min. This mixture was stirred at room temperature for 12 h. Finally HCl was added to the reaction mixture to adjust the pH to 7.0. The CMC was filtered and washed with anhydrous EtOH and the product was vacuum- dried at room temperature. The products were dissolved in dilute ammonia and centrifuged to separate the unreacted chitosan. The CMC was precipitated by ethanol from water-soluble portion and vacuum-dried.

2.3 Synthesis of γ-{*N*-{2-[2-(2-aminoethoxy) ethoxy] ethyl} folic acid (FA-EDBE)

2.3.1 Synthesis of tert-Butyl N-{2-[2-(2-aminoethoxy) ethoxy] ethyl}-carbamate (1)

FA–EDBE was synthesized from *tert*-Butyl *N*-{2-[2-(2-aminoethoxy) ethoxy] ethyl}-carbamate according to our reported methodology [34]. It is presented in Scheme 1. BoC_2O (1 mmol) in 5 ml $CHCl_3$ cooled at 0°C under argon atmosphere was added drop wise to a stirred solution of 2,2′-(ethylenedioxy)-*bis*-(ethylamine) (10 mmol) in 10 ml anhydrous $CHCl_3$. The reaction mixture was kept at room temperature for 24 h with stirring. Then the solvent was removed under reduced pressure and the thick oil so obtained was diluted with CH_2Cl_2. The organic layer was washed with brine solution and dried over anhydrous Na_2SO_4, and concentrated under reduced pressure to give gummy oil which was used for the next step without further purification. Elemental analysis (C 53.01, H 9.03, N 11.25, O 23.67%), IR (KBr) 3358, 2959, 2868, 1715, 1530, 1278, 1258, 1169, 1099 cm^{-1}. ^1H NMR($CDCl_3$): δ 5.15 (br s, 1H, NH), 3.66–3.48 (m, 8H), 3.31(dd,



Scheme 1 The modification of carboxy methyl chitosan with folic acid. Folic acid is conjugated with 2,2′ (ethylenedioxy) *bis*-ethylamine to form $-NH_2$ terminated folic acid, which is subsequently coupled on –COOH terminated carboxymethyl chitosan through the amide bond for synthesis of nanoparticles

$J_1 = 7.0$ Hz, $J_2 = 5.4$ Hz 2H), 2.88 (t, $J = 4.9$ Hz), 1.5–1.3 (br s, 11H). Yield was 90%.

2.3.2 *Synthesis of γ-{tert-butyl N-{2-[2-(2-aminoethoxy) ethoxy] ethyl}-carbamate} folic acid (2)*

The synthesis of γ-{*tert*-Butyl *N*-{2-[2-(2-aminoethoxy) ethoxy] ethyl}-carbamate} folic acid was performed through the following procedure.

A solution of folic acid (0.75 mmol) was made in the mixer of 20 ml anhydrous DMSO and anhydrous pyridine (8 ml). It was added to a mixer of **1** (0.87 mmol) and dicyclohexyl carbodiimide (2 mmol) at room temperature under argon atmosphere with stirring. The stirring was continued for 18 h. The resulting precipitate was filtered and the filtrate was gradually poured into a vigorously stirred Et_2O (50 ml) at 0°C. The yellow precipitate thus obtained was collected and washed with cold Et_2O several

times to remove any trace of DMSO. The adsorbed solvent was removed under reduced pressure to give **2** as a yellow solid. Elemental analysis (C 54.01, H 6.15, N 18.30, O 21.54%), ^{1}H NMR (DMSO-d_6, 400 MHz) spectrum of **2** shows peaks at 1.4–1.5 ppm (br s, –CH_3 9H), 2.5 (br s, β and γ –CH_2– groups, 4H), 2.8 (br s, –CH_2– of CH_2–NHCO, 5 H), 3–3.5 ppm (m, –CH_2– of EDBA, 6H), 4.5 (d, –NH–, 1H), 4 d at 6.5, 6.7, 7.6, 7.7 (benzene ring), 6.9 (s, aromatic H of pteridine), 8.7 (s, OH, 1H). m/z 672.33 ($[M + H]^+$, 70.33%), 572.27 (100), 497 (21), 442 (29), 397 (65), 295 (62). Yield was 70%.

2.3.3 Synthesis of γ-{N-{2-[2-(2-aminoethoxy) ethoxy] ethyl} folic acid (3)

The synthesis of γ-{*N*-{2-[2-(2-aminoethoxy) ethoxy] ethyl} folic acid was performed through the following procedure.

Trifluoroacetic acid (2 ml) was added to **2** (0.669 mmol), and stirred at room temperature. After 2 h, trifluoroacetic acid was removed under reduced pressure and the resulting residue was dissolved in anhydrous *N*,*N* dimethyl formamide. Then pyridine was added until the formation of a yellow precipitate. The yellow precipitate was filtered and washed with Et_2O and dried in vacuum to give **3** (200 mg, 56%). Elemental analysis (C 52.21, H 5.84, N 22.10, O 19.55%). ^{1}NMR (DMSO-d_6, 400 MHz) of product **3** shows peaks at 2.5 (br s, β and γ –CH_2-groups, 4H), 2.9–3.5 ppm (m, –CH_2– of EDBA, 12H),4.3 (br s, NH_2, 2H), 4.5 (d, –NH–, 1H), 4 d at 6.5, 6.7, 7.6, 7.7 (benzene ring), 6.9 (s, aromatic H of pteridine), 8.7 (s, OH, 1H) indicating the formation of FA–NH_2 (product 3). m/z 572 ($[M + H]^+$, 24%), 397(16), 295 (100), 198 (23). Yield was 85%.

2.4 Synthesis of folate-CMC

Final product was prepared through the following process. A reaction was performed between 120 mg of carboxymethyl chitosan and 80 mg EDC dissolved in 35 ml water at 4°C by stirring overnight. Then 75 mg FA-EDBE conjugate was added to the mixture with continuous stirring at room temperature for 12 h. Finally excess acetone was added in the reaction mixture to collect the precipitate. The precipitated product was dried in vacuum at room temperature. The yield was 75%.

^{1}NMR (D_2O 400 MHz) of product shows peaks at 1.9 protons of acetyl groups, 2.5 (br s, β and γ –CH_2-groups, 4H), 2.9–3.3 ppm (m, –CH_2– of EDBA, 12H), 3.6–4.0 ppm (br s ring protons H-3 to H-6), 4.3 (br s, NH_2, 2H), 4.5 (d, –NH–, 1H), 4 d at 6.5, 6.7, 7.6, 7.7 (benzene ring), 6.9 (s, aromatic H of pteridine), 8.7 (s, OH, 1H) indicating the formation FA-EDBE-CMC.

2.5 Preparation of self-assembled nanoparticles

The nanoparticles were prepared by dispersing the fixed amount of CMC-Folate materials followed by sonication using a probe-type sonicator (Sigma Ultrasonic Processor, Sonix-600) having power output 12 W in different pH solutions. The sonication was continued for 2 min.

2.6 Fluorescence-labeled CMC-folate nanoparticles

RITC or FITC labeled CMC-Folate nanoparticles were prepared through the following process.

Two milliliters of DMSO-H_20 (1: 1 v/v) with 1.0 mg RITC or FITC solution was added into 25 ml of prepared CMC-Folate nanoparticles suspension, and the mixture was stirred at room temperature for 24 h in the dark. Then, these fluoro-labeled CMC-Folate nanoparticles were separated by centrifugation at 4°C. The obtained sediment was washed and re-dispersed. This process was repeated three times to remove the un-reacted RITC/FITC. Finally, the obtained RITC or FITC labeled CMC-Folate nanoparticles were dispersed in aqueous solution at pH value of 5.0 for in vitro experiment.

2.7 Preparation of DOX-CMC-folate nanoparticles

CMC-Folate nanoparticles immobilized with doxorubicin (DOX) were prepared through the following process.

The DOX (conc. 0.4 mg/ml) in water solution was introduced in a nanoparticle suspension (1 mg/ml) and stored for 48 h. The suspension was then centrifuged at 15,000 rpm for 20 min to precipitate the nanoparticles and decanted. The process was repeated three times to remove any free DOX in the suspension. The amount of loaded DOX was measured by spectrophotometrically at 481 nm with a UV-1700 spectrophotometer (Olympus). The EE% was calculated from following equation

$$EE\% = \frac{W_{total} - W_{free}}{W_{total}} \times 100\%.$$

2.8 Characterization

2.8.1 Dynamic light scattering (DLS)

DLS analysis was done by Zetasizer Nano ZS (Malvern Instruments). The concentration of the chitosan derivative was 100 μg/ml and was sonicated for 2 min and dynamic particle sizes were measured suspending two drops of aqueous suspension of nanoparticles in 10 ml of Millipore water. The experiments were repeated several times to get average size of nano-particles. Zeta-potential measurements were performed with the zeta option the DLS equipment. Dilutions were the same compared to DLS measurement.

2.8.2 Transmission electron microscope

TEM micrographs were obtained on a Phillips CM 200 transmission electron microscope with an acceleration voltage of 200 kV. For TEM observation, the nanoparticles having concentration was 100 μg/ml.

2.8.3 Analysis of chemical structure of product 1, 2, and 3

The chemical structure of products 1 was analyzed from ^{1}H NMR and product 2 and 3 were analyzed from ^{1}H NMR and mass spectrometry.

2.8.4 Studies on in vitro release

One hundred milligrams of DOX loaded CMC-Folate nanoparticles was re-suspended in 10 ml saline solution and was placed in a dialysis membrane bag (12 kDa cut off) surrounded by PBS buffer at 37°C with continuously stirring. 5 ml of the solution was drawn from outside of the dialysis bag after regular interval of time. The concentration of the released DOX was determined by UV spectrophotometer at 480 nm of the solutions collected at different times.

2.8.5 Cell culture

The mouse melanoma (B16F1) human cervices carcinoma (HeLa), mouse fibroblast cell line NIH 3T3 and L929 cell lines were cultivated for in vitro experiments. Cell lines were obtained from the National Centre for Cell Sciences (NCCS) Pune, India. It was cultured in Dulbecco Modified Eagle's medium (DMEM) and Minimal Essential Medium (MEM) supplemented with 10% fetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin, 4 mM L-glutamine under 5% CO_2 and 95% humidified atmosphere at 37°C.

2.8.6 Cytotoxicity

HeLa, B16 F1, NIH 3T3 and L929 cell lines were seeded into 96 wells of tissue culture plates having 180 μl of complete media and were incubated for 18 h. CMC-Folate nanoparticles were added to the cells at different concentrations (1,10, 50 and 100 μg/ml), were incubated for 72 h at 37°C in a humidified incubator (HERA cell) maintained with 5% CO_2, The cell viability was estimated by 3-(4,5-dimethylthiazol)-2-diphenyltetrazolium bromide (MTT).

2.8.7 Intracellular uptake

Nanoparticle uptake by HeLa cells was studied by confocal microscopy and flow cytometry methods. 5×10^5 B16F1 melanoma or human cervices carcinoma HeLa cells were seeded into 35 mm cell culture plates. It was incubated in a humidified incubator maintained with 5% CO_2 and 37°C. After 8 h the cells were washed with incomplete media and were incubated with 100 μg/ml CMC-Folate/CMC-Folate-RITC for confocal microscopy. CMC-Folate-FITC nanoparticles were used for flow cytometry at 37°C. After 4 h of incubation the cells were washed to remove free nanoparticles and flow cytometry was performed on a FACScalibur (BD Biosciences) with a laser of 488 nm wavelength, and the data were analyzed by CellQuest Pro software. The instrument settings (like PMT voltage and compensation) were adjusted in a manner that the difference in background fluorescence of different cells became insignificant. With the same setting the rest of the experiments were performed, to compare the uptake in different cell lines.

For confocal microscopy the cells were allowed to adhere to a glass cover slip in 35 mm Petriplates, followed by incubation for 4 h at 37°C in a humidified incubator maintained with 5% CO_2. Confocal images were acquired with 488 nm laser for differential interference contrast microscopy and 543 nm lasers for RITC excitation on an Olympus laser scanning microscope.

3 Results and discussion

3.1 Synthesis

FA–EDBE was synthesized according to the method outlined in Scheme 1. Carboxylic group of folic acid and carboxylic group (–COOH) of functionalized carboxymethyl chitosan were connected through the end-amino groups hydrophilic spacer, 2,2′-(ethylenedioxy)-*bis*-ethylamine [EDBE]. EDBE was chosen as a linker in order

(1) To get optimized length of linker to reach accessible receptor sites,
(2) To increase the water solubility and
(3) To make the folate-conjugate system biocompatible.

Folic acid was conjugated to BoC-EDBE (1) following the usual NHS/DCC reaction to get product 2. Though folic acid has two –COOH groups at the end positions and it has already been established that γ-COOH in folic acid is more prone to this reaction due to its higher reactivity [37, 38].

Final product were produced from the reaction between carboxymethyl chitosan (0.1–0.5 mg/ml) and Folate-EDBE (0.5 mg/ml) through the formation of amide bond using EDC between amino group of Folate-EDBE and acid group of carboxymethyl chitosan.

3.2 FTIR study

Figure 1a shows the basic characteristics IR bands of chitosan at 3,455 cm^{-1} (O–H stretch), 2,867 cm^{-1} (C–H

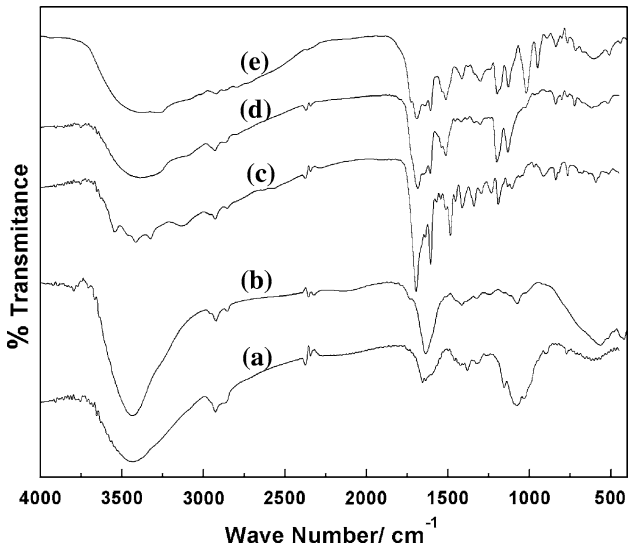


Fig. 1 FTIR spectra of chitosan (*a*), prepared CMC (*b*), folic acid (*c*), FA-EDBE (*d*) and FA-EDBE-CMC (*e*)

stretch), 1,598 cm^{-1} (N–H bend), 1,154 cm^{-1} (bridge-O stretch), and 1,094 cm^{-1} (C–O stretch) and characteristics IR bands of H-form CMC has been shown in Fig. 1b. The peaks at 1,741 cm^{-1} (–COOH), 1,070–1,136 cm^{-1} (–C–O–) and 1,624 and 1,506 cm^{-1} ($–NH_3^+$) were the characteristics of O-CM-chitosan. The IR spectra of folic acid derivative show the various functional groups of the molecule. The important preliminary areas of examination are in the 3,000–1,500 cm^{-1} regions. This region is known as the functional group region and the other important region is the 1,400–700 cm^{-1} regions, which is characteristic of the bending regions of the functional groups. In FA-EDBE (Fig. 1d) shows the characteristic band of folic acid. After conjugation with FA-EDBE with CMC the spectrum of the resultant molecules (Fig. 1e) shows not only the characteristic bands of the original CMC but also the characteristic peaks of the folic acid at 1,635 cm^{-1} (–CONH amide band II) and 1,554 cm^{-1} (–NH amide band II) (Fig. 1c). Furthermore, the absorption of amide band II at 1,635 cm^{-1} increased, which may be due to the formation of the amide linkage between the amino group on the FA-EDBE and the carboxyl group of CMC after being activated by EDC.

^{1}H NMR spectrum was used to conform the binding between CMC and FA-EDBE. The spectrum of FA-EDBE-CMC is shown in Fig. 2. The peaks at about 1.9 ppm in the ^{1}H NMR spectrum of CMC-EDBE-FA were attributed to the methyl hydrogen of acetamido-2-deoxy-β-D-glucopyranosyl unit. The peaks at about 2.9–3.2 ppm attributed to methylene hydrogen atoms of EDBE and 3.5–4 ppm observed the glucopyranosyl hydrogen atoms. It is clear the proton peaks of 8.7, 7.6, 6.9, 6.4 ppm were observed in ^{1}H NMR spectrum of FA-EDBE-CMC. While no such peaks

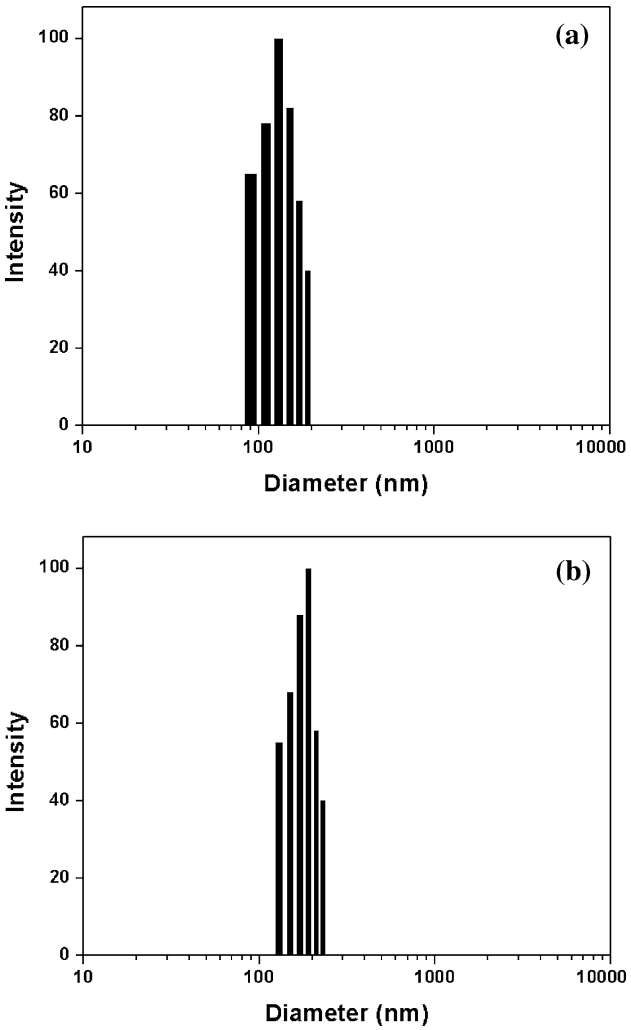


Fig. 2 The size determination of nanoparticles by dynamic light scattering **a** CMC-folate nanoparticles **b** DOX loaded CMC-folate nanoparticles

were observed in the same chemical shifts of ^{1}H NMR spectrum for CMC. The appearance of these peaks confirms the successful conjugation of FA-EDBE with CMC.

3.3 Dynamic light scattering experiment

The size distribution of CMC-Folate nanoparticles and doxorubicin loaded CMC-Folate nanoparticles in aqueous medium was characterized by DLS. The results are represented in Fig. 3. The pH of the samples was adjusted by the addition of citric acid solution in the presence of 0.1 M sodium citrate buffer. The Concentration of the CMC-Folate solution was 0.1 mg/ml a uniform size distribution with the polydispersity index of 0.095 + 0.015 for CMC-Folate nanoparticles was observed, suggesting that the size distribution of the nanoparticles was narrow near physiological pH. The mean size of nanoparticles in aqueous solution was determined to be 150 ± 20 nm as shown in

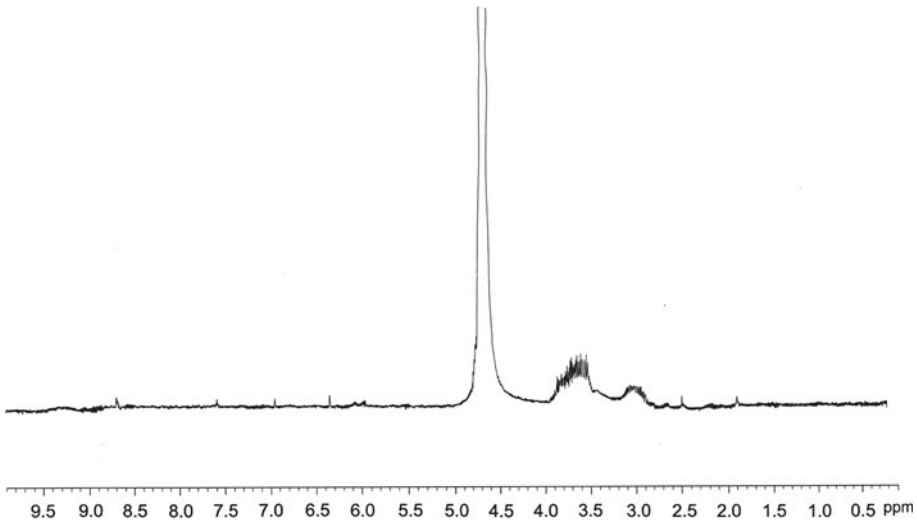


Fig. 3 ^{1}H NMR spectrum of synthesized FA-EDBE-CMC

Fig. 2a, While after DOX encapsulation the particle diameter increases 185 ± 30 shown in Fig. 2b.

In order to investigate the effect of pH values on CMC-EDBE-FA nanoparticles a series of experiments were carried out. The nanoparticles were incubated in buffer solution with different pH values (pH = 1, 2.2, 4.5, 5.2, 6.3, 7.1, 8.2, 9). The nanoparticles were stable in acidic media in the range of pH values from 4.0 to 6.5 but dissolved in acidic medium pH 1 and aggregated quickly at pH values larger than 7. The diameter of the nanoparticles also increases with the increase of pH value from 4.0 to 7.0. The dependence of nanoparticles size on the variation in pH originated from the production of nanoparticles. The degree of protonation of amino groups of chitosan increased when the pH is less than 6.5. Ultimately, deprotonation occurred in pH range 6.0–8.0. So in this range particles size was larger.

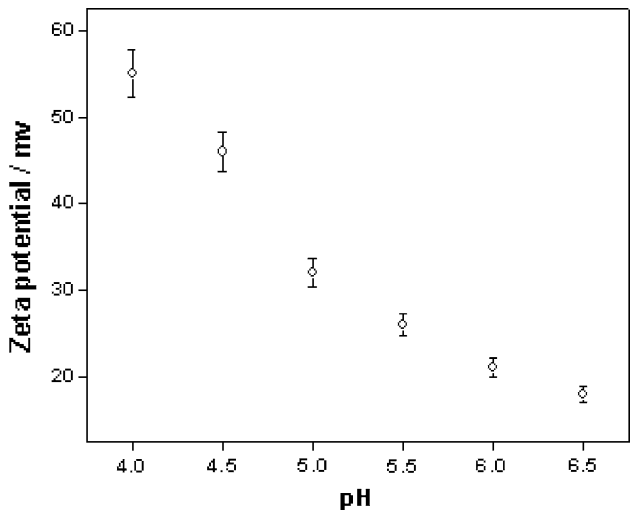


Fig. 4 Zetapotential of nanoparticles determined at different pH

The zeta-potential measurement showed that both CMC-Folate Nanoparticles had a positive zeta potential at different pH indicting that the nanoparticles possess a positively charged surface. It may be due the free amine groups of chitosan. This information is presented in Fig. 4.

3.4 Transmission electron microscopy

Chitosan can be prepared as a film; however, the modified chitosan nanoparticles separated into spherical particles in an aqueous environment and in dried states. TEM micrographs (Fig. 5a) confirmed the nanosize of dried chitosan particles and show the distribution of these derivatives. The size of the dried particles varied in the range 60–90 nm. After DOX encapsulation the particles size increases and also the spherical morphology changes (Fig. 5b). This may be crosslinking of the amines group of the doxorubicin molecules and free acid groups present in the particles. The observed nanoparticles size was approximately larger than the hydrodynamic diameter obtained from the DLS experiment. In TEM described the size in the dried state of the sample, whereas DLS measured the size in the hydrated state of the sample, so that the size measured by DLS was a hydrodynamic diameter and had a larger. However, one has to bear in mind that by TEM we image single particles, while DLS gives an average size estimation, which is biased toward the larger-size end of the population distribution.

3.5 In vitro toxicity studies

The toxicity of the nanoparticles was checked towards different cell lines in vitro. It was previously reported that both carboxymethyl chitosan and folic acid have no significant cytotoxicity. Here carboxymethyl chitosan conjugated with folic acid through (ethylenedioxy)-*bis*-(ethylamine) based

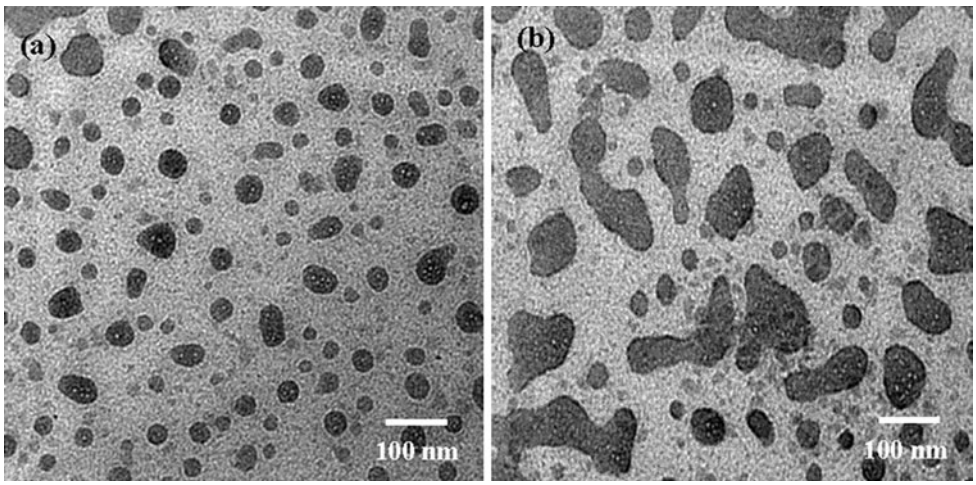


Fig. 5 TEM images of (**a**) CMC-Folate nanoparticles (**b**) DOX loaded CMC-Folate nanoparticles

nanoparticles mediated cytotoxicity to the cells was measured by MTT assay (Fig. 6). It is found that there was no significant difference in cell viability between the cells treated with nanoparticles and cells treated without nanoparticles. It is shown in Fig. 6a. Hence these nanoparticles are expected to be safe for biomedical applications. After encapsulation of Doxorubicin into nanoparticles the cytotoxicity effects were evaluated using two cancer cell lines (HeLa and B16F1) and two non cancerous cell lines (NIH3T3 and L929).Cells were treated with CMC-Folate-DOX at different concentrations. CMC-Folate-DOX nanoparticles showed more cytotoxicity towards cancer cells as compared to normal cells. Data is presented in Fig. 6b.

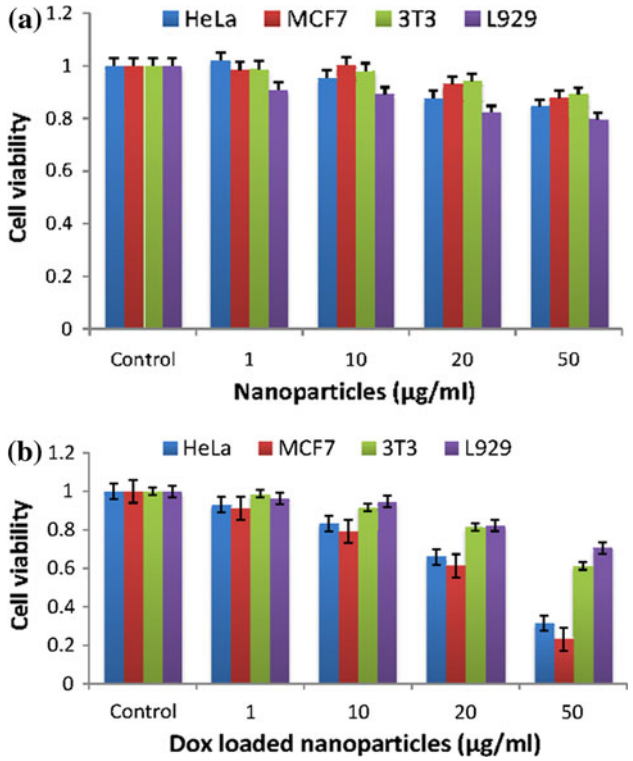


Fig. 6 Cytotoxicity of (**a**) CMC-Folate nanoparticles (**b**) DOX in CMC-Folate nanoparticles at different cell lines

3.6 Intracellular uptake of nanoparticles

Flow cytometry was employed to study the behavior of CMC–Folate-FITC nanoparticles for targeting to B16F1, HeLa cancer cells along with NIH3T3 and L929 non cancerous cells. There was an increase in the fluorescence intensity from cancer cells incubated with nanoparticles in comparison to non cancerous cells shown in Fig. 7. From the figure the mean values of fluorescence intensities for HeLa and B16F1 were 228.62, 103.93 and for NIH3T3, L929 were 37.43, 85.4 respectively. This indicated a significantly higher uptake of nanoparticles by the cancer cells with respect to normal cells. To study the uptake/internalization of the nanoparticles, confocal imaging was performed on HeLa cells. From the confocal images

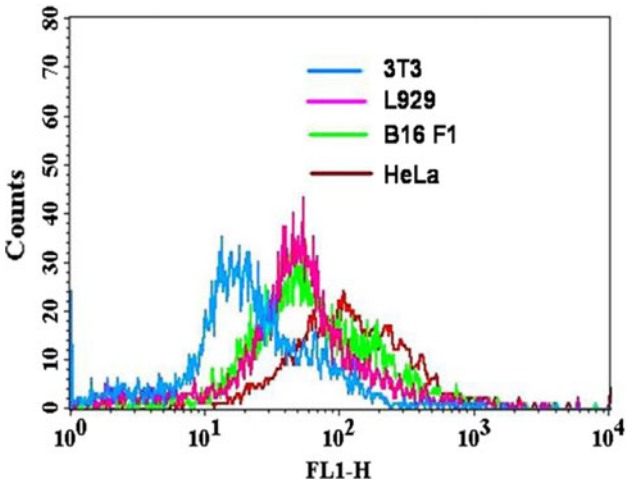


Fig. 7 Cellular uptake of nanoparticles, 10^5 cells treated with nanoparticles for 4 h and the uptake was observed by flow cytometry

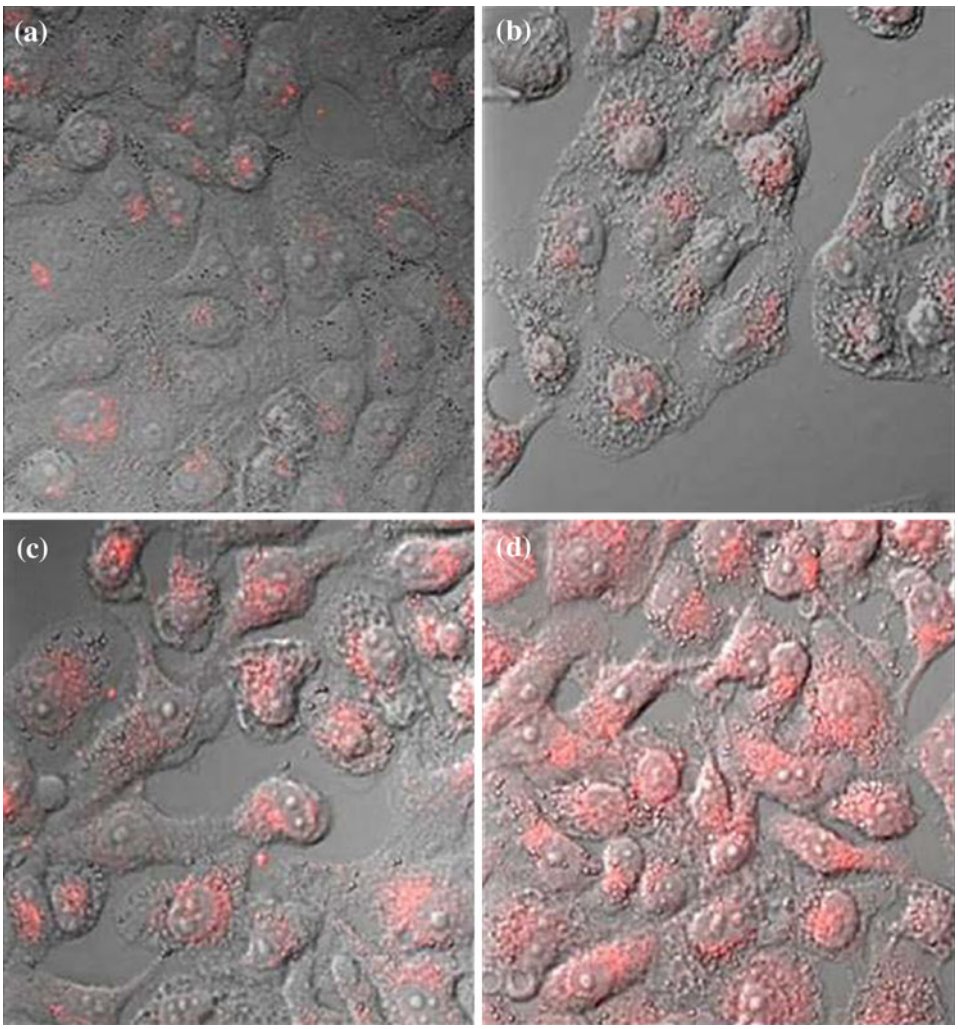


Fig. 8 Folate mediated endocytosis of nanoparticles in HeLa cells incubated with nanoparticles with or without folate for different time interval and observed under confocal microscope (*a*) cells incubated with carboxymethyl chitosan without folate for 4 h and cells incubated with CMC-Folate nanoparticles for 30 min (*b*), 1 h (*c*), 4 h (*d*) respectively

(Fig. 8) the folate conjugated nanoparticles were found to be distributed in the cytoplasm leaving a clear zone of nucleus, indicating cellular uptake instead of adhesion to the surface and the nanoparticles preferentially targeted the cancer cells and were internalized. This internalization might be due to the folate receptor mediated endocytosis. This observation clearly infers that folate conjugated carboxymethyl chitosan is very much effective to use as delivery system for targeted anticancer drug.

3.7 Drug release in vitro

The releases of DOX from CMC-Folate nanoparticles in PBS buffer medium at pH 5 and pH 7.4 were investigated. The percentage of release of DOX from nanoparticles has been presented in Fig. 9. Characteristics of release of DOX in the two different pH media were different. Release of DOX from CMC-Folate nanoparticles in the buffer medium of PBS (pH 5) was 62%, whereas in PBS (pH 7.4) it

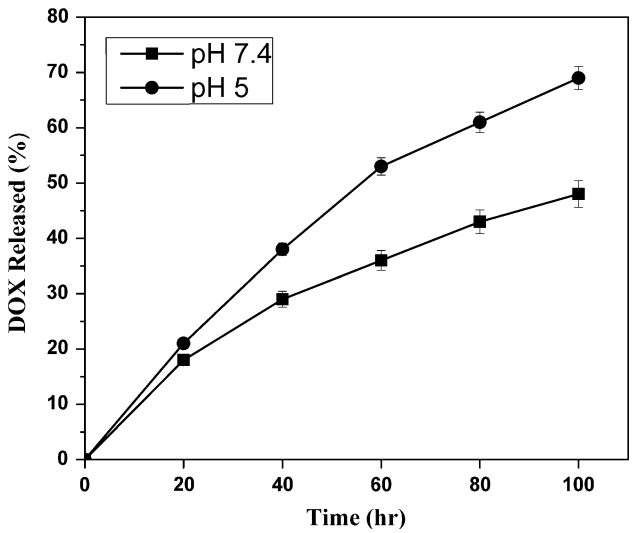


Fig. 9 In vitro release profiles of DOX from nanoparticles at different pH

was 51% after 100 h. The release of DOX continued for 5 days, showing the potential of the nanoparticles for sustained drug delivery. It was found that the pH value of dissolution medium affect the drug release rate of DOX loaded nanoparticles. The drug release rate from nanoparticles reduced with the increasing of the pH value of dissolution medium. The faster drug release rate in lower pH medium could be contributed to two factors: the one is the loose nanoparticles structure, which caused by the stronger protonation of amino groups of carboxymethyl chitosan in lower pH; the other is the higher solubility of DOX in lower pH. The Fickian diffusion and polymer relaxation were the release mechanisms for the present case.

3.8 In vitro anti-tumor activity

It was previously reported that caroboxymethyl chitosan nanoparticles have no significant cytotoxicity. The nanoparticle of CMC-EDBE-Folate-mediated cytotoxicity to the cells was measured by MTT assay. It was found that there was no significant difference in cell viability between cells treated with or without nanoparticles. It was observed that survival in cancer cells treated with DOX loaded nanoparticles was lower than that of normal cell in similar concentrations. It indicated that the folate modified nanoparticles preferentially targeted cancerous cells than normal cells. Release of doxorubicin from the nanoparticles was slow with long duration. These observations help to infer that nanoparticle of CMC-EDBE-Folate will be a very suitable vehicle for treatment of cancer cells.

4 Conclusion

In this study, a simple process has been developed to synthesize carboxymethyl chitosan–folate conjugate nanoparticles using 2,2′-(ethylenedioxy)-*bis*-ethylamine coupling agent. These nano-polymeric systems impart excellent stability in aqueous medium over a wide range of physiological conditions with reasonably good hydrodynamic size. After doxorubicin loading in the nanoparticles, their size increases somewhat. Release behaviors of doxorubicin from nanoparticles showed pH dependence and sustained release pattern. The amount of doxorubicin released from nanoparticles decreased with increasing pH. In vitro cytotoxicity test using an MTT assay method showed that these doxorubicin loaded nanoparticles could reduce cell damage compared unloaded particles. Therefore, we could expect that these polymeric nanoparticles would exhibit pH-responsive behavior and could be useful as a drug carrier for targeted drug delivery. Flow cytometry and confocal microscopy have revealed that nanoparticles are targeting more effectively the cancerous cells than that of normal cells. Due to high specific intracellular uptake, and surface positive charge, this folic acid decorated carboxymethyl chitosan system may further be explored for its applications for delivery of many other anti-cancer drugs. The data presented here suggest that further in vivo studies are warranted to define the therapeutic index of this tumor targeting polymeric carrier and will constitute the basis for the next generation of drug delivery devices. In vivo testing of Drug loaded nanoparticle is in progress.

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