Original Research Article

Usnic acid-loaded bioinspired heparin modified-cellulose acetate phthalate nanoparticle(s) as an efficient carrier for site-specific delivery in lung cancer cells

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Abstract

Introduction: The main goal of the current study was to assess the cytotoxic influence of usnic acid (UA) after enclosement in heparin modified-cellulose acetate phthalate (HEC) nanoparticles (NPs) when targeted to lung cancer A549 cell line.

Materials and Methods: HEC copolymer was manufactured by precipitation method and was substantiated by Fourier-transform infrared spectroscopy and nuclear magnetic resonance spectroscopy. HEC NPs with UA was formulated by employing HEC copolymer and later competed with UA-loaded cellulose acetate phthalate (CAP) NPs. NPs were exemplified by zeta potential, differential scanning calorimetry, particle size, atomic force microscopy, *in vitro* release, entrapment efficiency, X-ray diffraction , and polydispersity index.

Results: Studies revealed that HEC NPs have a slower release (96.21% in 32 h) when contrasted with CAP NPs (97.36% in 8 h). In cytotoxicity analysis of A549, UA-loaded HEC NPs illustrated an immense cytotoxic potential. In addition, HEC NPs were found to be more hemocompatable in comparison to CAP NPs and plain UA.

Conclusion: Decisively, on account of investigational results UA-loaded HEC NPs were percieved to be more cytotoxic against lung cancer cells than UA-loaded CAP NPs and plain UA.

Keywords: Cellular cytotoxicity, cellulose acetate phthalate, heparin, nanoparticles, usnic acid

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INTRODUCTION

Anticancer bioactive(s) are commonly used for eradication of tumors. However, complete eradication of tumor with available cytotoxic drugs is not possible until date. With presently available drug delivery systems, i.e., nanocarriers, anticancer drugs can be delivered specifically to cancer cell only. Most of the anticancer drugs-loaded nanocarriers are given parentally and loaded anticancer drugs can be delivered to tumor cells in a specific manner. Since most anticancer drugs are administered into the blood circulation, regeneration of tumor after post medication is very common. Therefore, oncologists investigated lipid-based and other nanocarriers that can fuse to the cell membrane and enter into the cell. Once inside delivery systems, i.e., liposomes and other lipid carriers, the loaded anticancer drugs can travel into the systemic

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bloodstream and enter cancer cells near blood vessels, they were engulfed by exosomes, which are naturally occurring nanoparticles (NPs) in the body. Given the fact that exosomes can pass through between the cells, the drugs can be delivered efficiently into inner cells of the tumor.[1] Cancer has several biological obstacles,[2] such as distinct blood supply and heterogeneous formations. To obtain a productive medication of cancers, it is very essential to pass through these barriers. Cancer stands for an immense biomedical challenge,[3] for drug release. Cancer therapy is greatly at the mercy of the technique of liberation. At an earlier time, cancer patients use to take various anticancer medicines, but these drugs were found to be less productive and also had considerable side effects. The remedy of cancer by exploiting the targeted drug delivery through NPs is the latest accomplishment in the remedial sector. The scenario of NPs in cancer-based drug delivery is incredible with novel applications frequently being explored. In tumor-based drug delivery the multioperative NPs illustrate a worth mentioning role.[4]

NPs are proficiently valuable for releasing the anticancer agents in the course of delivering the drug into tumor cells. In the modern era, the formulation of drug delivery devices based on nano methodologies is formed to deliver the anticancer drugs the early recognition of cancer cells or distinctive tumor biomarkers and the enhancement of the efficacy of the therapies employed.[5]

Heparin is an enormously sulfated glycosaminoglycan (GAGs) found in the mastocytes of a majority of mammals which are extremely acidic and actively stimulated. Heparin is capable to conjugate with some constituents such as some coagulating and fibrinolysing proteins, a variety of growth elements and immune response proteins, for example, cytokines and chemokines.[6]

The ± usnic acid (UA) enantiomer was aspects to be particular contrary to streptococcus mutans without generating any unpleasant unfavorable property on the oral saprophyte flora.[7] The first time, Kupchan and Kopperman, announced contrary to Lewis lung carcinoma (LLC) for tumor inhibitor action of UA.[8] Takai *et al.* were generated eleven UA derivatives found for action counter to leukemia cells P388 and LLC by, indicating no significant rise in the endurance of animal models.[9] Ding *et al*., researched on six lichens for anticancer properties of components by employing test bioassay of the brine shrimp lethality.[10] UA was found the most dominant bioactive compounds among all other compounds investigated. The prohibiting behavior of UA in the propagation of myelogenous leukemia cell line (K562) and heparin modified-cellulose acetate phthalate (HEC) 50 cells expounded by Cardarelli *et al*., The prohibition of tumor was based on time and dose.[11]

Cellulose acetate phthalate (CAP) is a derivative consists of phthalyl ($C_8H_5O_3$), acetyl (C_2H_3O) groups, calculated on anhydrous free bases having a molecular weight of 60,000. CAP is utilized as a material for enteric film coating as a microencapsulating agent providing sustained and controlled release drug delivery.[12]

In the contemporary investigation, we explored NPs-containing novel anticancer compound UA in heparin-adipic acid-dihydrazide (ADH)-CAP copolymer and assess UA-loaded heparin-ADH-CAP NPs for anticancer cell-lines.

MATERIALS AND METHODS

Materials

UA was procured from Sigma Aldrich, United States of America. CAP (Mol. Wt. 6000 Daltons) was acquired from Central Drug House, Delhi, India. Heparin was charitably supplied by Himedia located in Mumbai, India. N-hydroxysuccinimide, dialysis membranes, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC), dicyclohexyl carbodiimide (DCC), Pluronic F-68 were purchased from Himedia laboratories, Mumbai, India. ADH was purchased from Sigma Aldrich. Acetone, isopropyl alcohol, and acetonitrile purchased from Merck Limited, Mumbai, India and other chemicals which are consumed are of investigative chemical grade and used as brought.

Methodology

Amalgamation of the heparin-adipic acid dihydrazide-cellulose acetate phthalate copolymer

First, 50 mg of heparin was dispersed in 5 ml distilled water, and then 250 mg of EDAC was added in heparin dispersion with constant stirring, EDAC, activated the heparin ends group and provide effective interaction with another group of molecules, then 250 mg of ADH was added on the activated heparin dispersion and the reaction executed up to 12 h in room temperature. CAP was dispersed in solvent system consisting acetone and isopropyl alcohol in the different ratio (8:2) and DCC (50 mg) followed by N-hydroxysuccinimide (25 mg) with constant stirring up to 8 h. After CAP dispersion formation it was added periodically dropwise into Heparin-ADH solution and stirred for 6 h for proper and uniform interaction between amine groups of ADH and carboxyl group of CAP for the formation of amide bonds (carbodiimide conjugation), which shows that Heparin-ADH is binds with the polymer (CAP). The reaction solution was dialyzed expansively (molecular weight cutoffs 12–14 kDa)

to remove untreated heparin (molecular weight 12 kDa) from the heparin-conjugated CAP (Heparin-ADH-CAP). The conjugate was exposed on column for gel filtration in which untreated heparin and CAP molecules, established by the method of titration.[13,14] The copolymer (HEC) acquired was vacuum-dried and authenticated by ^{1}H-nuclear magnetic resonance (^{1}H-NMR) (Bruker DRX, the USA at 400 MHz), and Fourier-transform infrared spectroscopy (FTIR) (IR Tracer-100, Shimadzu) spectroscopic technique.

Formation of heparin modified-cellulose acetate phthalate nanoparticles using heparin-adipic acid dihydrazide-cellulose acetate phthalate copolymer (heparin modified-cellulose acetate phthalate) and plain polymeric nanoparticles (cellulose acetate phthalate nanoparticle)

HEC copolymer (10 mg) was dispersed in 10 ml of a proportionate mixture of acetone: Isopropyl alcohol (9:1). UA (10 mg) was dissipated in acetone and subsequently added dropwise into the prepared HEC solution, then pluronic F-68 (125 mg) was dissolved in distilled water, and various pluronic concentration (0.5%, 1%, and 2%) were prepared. HEC solution was added into pluronic solution with continuous stirring for 2 h. The subsequent suspension of NPs was separated by membrane filter (0.45 μm) and centrifuged at 15,000 rpm for 15 min (C-24, BL, Remi, Mumbai, India). After centrifugation discarded the supernatant and lyophilized the HEC NPs for further use. Plain polymeric (CAP) NPs were prepared according to the method described before. CAP NPs and HEC NPs were further lyophilized and kept for upcoming studies.

Characterization parameters of heparin modified-cellulose acetate phthalate nanoparticles and cellulose acetate phthalate nanoparticles

Surface characteristics by atomic force microscopy

The structure and surface characteristics of prepared NPs were examined with atomic force microscopy (AFM) (SPM-9500, Shimadzu) in contact mode. The above-mentioned AFM of the NPs was done by Si micro-cantilever with sample solution was spotted on mica and allowed to stand for a minute with the substrate and blow off with air and observed for AFM photomicrograph using SPM laboratory software.

Zeta potential and particle size

The particle size and zeta potential of formulated NPs is carried out using Malvern instrument (DTS Ver. 4.10, Malvern Instruments, WR14 1XZ, UK). An appropriately diluted dispersion of CAP NPs and HEC was placed in the compartment of a particle size analyzer, and finally, average particle size and polydispersity index were obtained. The zeta potential possessing the charge over the surface of particle indicates the colloidal system physical stability. The zeta potentials for the HEC NPs and CAP NPs were ascertained by Laser Doppler Anemometry by employing the Zeta Sizer (Malvern Instruments, UK).

Differential scanning calorimetry analysis

The substantial status of NPs was characterized by differential scanning calorimetry (DSC) performed by utilizing DSC 60 instrument (Shimadzu, Kyoto, Japan). The samples about 5 mg (CAP, ADH, UA, Heparin, dried HEC, and UA-loaded HEC NPs) were placed in the aluminum pan further the observation of DSC thermogram was observed at a scanning temperature range up to 300°C with rate of heating 10°C/min under nitrogen atmosphere.

Powder X–ray diffraction

The crystalline characteristics of pure drugs as well as all the materials Gs (Heparin), polymer (CAP), and NP formulations in the manufacturing of NPs was done by X-Ray diffractometry. X-ray diffraction (XRD) thermogram of powder samples (CAP, ADH, UA, Heparin, dried HEC, and UA-loaded HEC NPs) were acquired by utilizing the power X-ray powder diffractometer (Bruker, AXS D8 Advance) having 435, 500, and 600 mm is measuring circle diameter. The source of X-Ray is Cu with wavelength 1.5046 A° consisting of Si (Li) PSD detector. The source of temperature Anton Paar, TTK 450. The scanning rate was 2 θ/min over a 2 θ range of 0–40° and with an interval of 0.02°.

Entrapment proficiency

UA-loaded HEC NPs (10 mg) and CAP NPs (10 mg) were dissipated in the solvent system (acetone). Primarily, the dispersion was centrifuged at 10,000 rpm (cooling centrifuge) for about 10 min, remove the polymeric debris and then the supernatant was collected. The clear supernatant solution was analyzed with HPLC, (Wasters HPLC, Model-515) to calculate the amount of loaded UA in the prepared NP system.[15,16]

The HPLC system (Wasters HPLC, Model 515) having auto-sampler (Model 717 Plus), column oven (Wasters CHM), and PDA detector (Wasters 2998). The collection of data and the analysis was performed using Empower version 2.0 software Milford, MA 01757 USA. This HPLC system generally contains sensor of various wavelength and column (Zorbax C18, 250 mm × 4.60 mm, 5 μ) which was operated for the evaluation of UA. The mobile phases used were methanol and phosphate buffer with a different ratio (70:30 v/v) which was propelled at an optimum flow rate (1 mL/min) at 55°C. The mobile phase is out-gassed under vacuum before use.[17]

In vitro drug release study

Drug (UA)-loaded HEC NPs and CAP NPs were filled in the dialysis bag (Himedia) separately and placed into separate 50 ml of phosphate buffer saline solution at a pH 7.4 with constant stirring at 100 rpm in at 37°C ± 2°C. At a fixed time interval 1 ml of buffer solution was withdrawn and replaced with the similar amount of fresh buffer solution. The amount of drug released from NPs was analyzed using HPLC system (Wasters HPLC, Model 515).[16,18]

Hemolytic toxicity

For hemolytic activity whole human blood was amassed and collected in a collection vial as denoted in Bhadra *et al*.[19] First, human blood was centrifuged at 10,000 rpm for 10 min for complete separation of red blood cell (RBC) and plasma. The plasma was discarded and RBC was taken for further procedure. The RBC was resuspended in saline solution to form 10% hematocrit, then the red blood corpuscles (1 mL) was incubated separately with 10 mL of distilled water, saline solution, and phosphate buffer solution pH 7.4 (taken as 100% hemolytic standard). In case of hematocrit solution with NP (drug solution), the drug-loaded NP formulations was added separately on hematocrit solution (10% hematocrit) of distilled water, saline, and phosphate buffer solution up to 10 mL. The collection tube was allowed to stand for 1–2 h at 37°C, after that the drug-loaded NP in hematocrit mixture was centrifuged at 5000 rpm for 5 min, then the absorbance was taken of supernatant at 540 nm to optimize the effect of NP formulations against RBCs, which was used to predict the percentage hemolysis.[20]

Sulforhodamine B assay

Sulforhodamine B (SRB) assay is an economic, swift, and sensitive process for calculating the cytotoxic potential of test substances, depended on the content of cellular protein for adhered suspension culture in 96 well plates. This process is adequate normal laboratory purposes and for huge scale importance such as the large output of anticancer drug screening (*in vitro*). The desired human lung cancer cell line (A549), was established in a flask-containing tissue culture medium and grown at adequate atmospheric condition temperature (37°C) and relative humidity (5% CO_2 and 90%) to get a large amount of cells. After excavating cells from trypsin-EDTA treatment, cell density was maintained to 10,000 cells/100 µl in a suspension containing cells. The cell suspension (100 µl) was poured to each well of 96 well plate by handy step process, and plates were incubated at 37°C, in an adequate atmosphere relative humidity (5% CO_2 and 90%) for 24 h, then after the sample (NP solution) was added to the wells of 96 well plates at various concentrations (10 µg/ml, 20 µg/ml, 40 µg/ml and 80 µg/ml). After 48 h of addition of sample, the plates were detached from incubator and trichloroacetic acid was added in the concentration 50 µl of chilled 50%, in all the well of the plate to stop the reaction and made up the concentration 10% and plates were incubated again at 4°C for 1 h for fixation of the cell into the underneath of the wells. Plates were washed repeatedly with distilled water followed with air drying. The 100 µl dye solution of 0.4% in 1% acetic acid was poured to each well of the plate and left in room temperature for 30 min at followed by washing with 1% acetic acid and air-dried after that 100 µl (10.5 M) of Tris buffer was added in each well and shaked with mechanical shaker for 20 min. ELISA reader was used for recording optical density of cell at 540 nm wavelength.[21,22]

Pharmacokinetic studies

A group of albino rat consistent body weight (100 ± 20 g) having no prior drug treatment were injected intravenous route with a single dose of free drug UA and NP formulation (30 mg/kg). Blood samples were drained at different time points from the lateral tail vein of rat and placed in centrifuge tubes and immediately centrifuged at 5000 rpm for 15 min. The serum was collected and then deproteinized with the addition of (1 ml acetonitrile/ml of serum) 1 ml acetonitrile then stored at −20°C until analysis and estimated by HPLC.

Statistical analysis

Final research outcome were showed as mean ± standard deviation. This evaluation was achieved by *t*-test. $P < 0.05$ was also important. All processes were performed thrice.

RESULTS

The intention behind the present study was to evaluate the anticancer efficiency of UA which was distinctively delivered to the tumor cells by employing the heparin appended CAP NPs. The schematic representation of formation of HEC NP and their tumor targeting behavior is depicted in Figure 1.

^{1}H-nuclear magnetic resonance and fourier-transform infrared spectroscopy spectroscopic analysis

The ^{1}H-NMR spectra of HEC copolymer is illustrated in Figure 2. The presence of Heparin, ADH and CAP in HEC was confirmed by distinctive peaks presenting in the ^{1}H NMR spectra. The proton assignment of heparin was at 2.8–3.6 ppm and peak of N-acetyl group was notify at 0.9–1.3 ppm, the peak of CAP was obtained between 1.8 and 2.0 ppm and the proton assignment of ADH, shows between 2.4 and 2.6 ppm. All these proton assignment justify the presence of heparin, ADH and CAP in HEC copolymer.

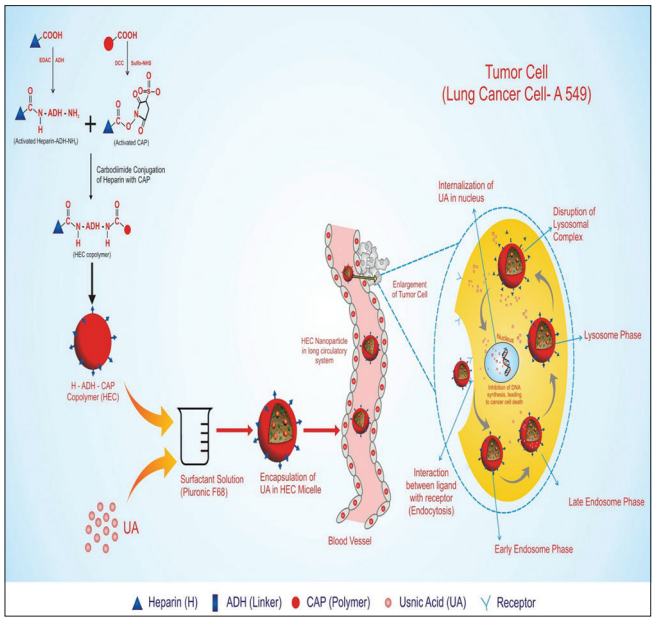

Figure 1: Schematic representation of formation of heparin modified-cellulose nanoparticles and tumor targeting behavior heparin modified-cellulose nanoparticles

The validation of HEC copolymer was accomplished by ^{1}H-NMR and FTIR spectrometer. The spectroscopic graph of ^{1}H-NMRand FTIR is depicted in Figures 2 and 3. The spectra obtained from the FTIR studies are from 3600 to 400 $cm^{-1.}$ The peak obtained by FTIR spectra was, 3440 cm^{-1} due to N-H stretch of amide, 2918 and 1387 cm^{-1} due to presence of C-H alkene bond, 1726 cm^{-1} due to presence of C = O stretch, 1650 cm^{-1} also shows the C = O bond formation, 1599 cm^{-1}, 1588 and 1582 cm^{-1} due to aromatic ring C = C, 1288 cm^{-1} due to C-N stretching of amide bond and 1089 cm^{-1} shows C = O stretching. Characteristic peak of 3440 and 1650 cm^{-1} confirm the presence of amide as well as C = O carboxyl bond, which confirm the construction of amide bond between amine group of ADH with both carboxyl group heparin and CAP.

Surface characteristics

The surface characteristics such as shape, size, and texture were examined using atomic force microscopic technique (SPM-9500, Shimadzu). The prepared HEC NPs were be sphere-shaped which were of nanometric size range (85–230 nm) as monitored by AFM image single and three-dimensional dimensions correspondingly [Figure 4a and b].

Zeta potential determination, particle size analysis and drug entrapment proficiency

The particle size evaluation of HEC NPs was carried out using particle size analyzer and the particle size

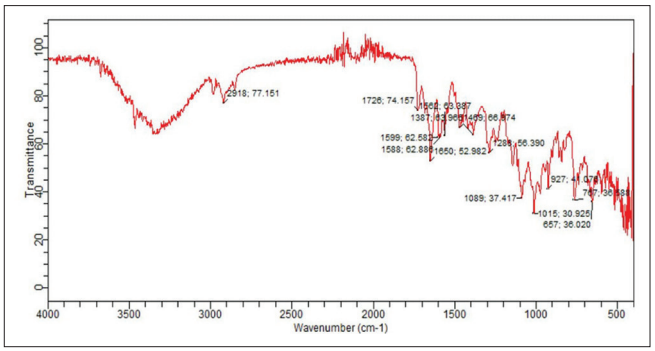

Figure 2: Fourier-transform infrared spectroscopy spectrum of heparin modified-cellulose copolymer

of HEC NPs was observed 85 ± 0.16 nm and the distribution of particle size achieved to be 21.5% of 80.60 nm, 75.8% of 91.4 nm, and 2.7% of 98.9 nm. The distribution of particle size varies between 85 nm and 230 nm because of agglomeration of particles with each other.

According to the result indicated in Table 1, with enhancing the amount of CAP-polymer from 10 mg to 30 mg the size of particles enhance from the range 85 ± 0.16 nm to 230 ± 1.20 nm. In the same way, the particle size range obtained from CAP NPs was from 137 ± 1.6 nm to 280 ± 1.4 nm [Table 2]. The PDI index of HEC NPs was found to be 0.071 ± 0.038 and the PDI value of CAP NPs was observed to be 0.082 ± 0.13. The encapsulation proficiency of prepared HEC NPs and CAP NPs was noticed 91.18 ± 1.30% and 76.82 ± 1.2% and the size of particles notify to be 85 ± 0.16 nm and 137 ± 1.8 nm [Table 3]. Zeta potential of HEC was obtained to be −13.8 mV and for CAP NPs it was observed to be −7.29 mV, respectively [Table 3].

Differential scanning calorimetry analysis

DSC thermograms of ADH, UA, CAP, Heparin, HEC NPs and UAloaded HEC NPs were achieved and displayed [Figure 5]. For heparin, tiny endothermic peak was attained at 80°C, which reveals their distinctive peak of heparin. The endothermic peak of ADH was noticed at 180°C. Whereas in case of CAP, there was no peak was obtained. Endothermic peak was acquired at 202°C of UA. When without drug-loaded HEC NPs were analyzed, endothermic peak at 60°C–70°C of Heparin was achieved, an exothermic peak of ADH at 270°C revealed the crystalline structure of the NP. In the case of drug-loaded NP (HEC NPs), at 60°C, endothermic peak of heparin, at 280°C exothermic peak of ADH and endothermic peak of UA at 202°C signified in the NPs.

Table 1: Ingredients and concentration using in the formulation of heparin modified-cellulose acetate phthalate nanoparticles

Serial number	Drug: Polymer ratio (mg)	Internal phase				External phase		Percentage Entrapment efficiency	Particle size (nm)
		UA (mg)	HEC (mg)	Acetone (ml)	Isopropyl Alcohol (ml)	Pluronic F-68 (mg)	Water (ml)		
F1	10:10	10	10	9	1	250	25	91.18±1.30	85±0.16
F2	10:20	10	20	9	1	250	25	71.23±1.12	172±0.120
F3	10:30	10	30	9	1	250	25	62.58±1.19	230±1.20
F4	10:10	10	10	9	1	125	25	79.98±0.70	125±1.10
F5	10:10	10	10	9	1	250	25	90.4±0.70	86±1.18
F6	10:10	10	10	9	1	500	25	83.45±1.10	113±1.50

F: Formulation, UA: Usnic acid

Table 2: Ingredients and concentration using in the formulation of cellulose acetate phthalate nanoparticles

Serial number	Drug: Polymer ratio (mg)	Internal phase				External phase		Percentage entrapment efficiency	Particle size (nm)
		UA (mg)	CAP (mg)	Acetone (ml)	Isopropyl alcohol (ml)	Pluronic F-68 (mg)	Water (ml)		
F1	10:10	10	10	9	1	250	25	76.82±1.2	137±1.8
F2	10:20	10	20	9	1	250	25	65.71±1.40	203±1.3
F3	10:30	10	30	9	1	250	25	52.31±0.98	280±1.4
F4	10:10	10	10	9	1	125	25	59.63±1.12	265±1.10
F5	10:10	10	10	9	1	250	25	75.32±1.10	139±2.21
F6	10:10	10	10	9	1	500	25	69.8±1.15	198±1.12

F: Formulation, CAP: Cellulose acetate phthalate, UA: Usnic acid

Table 3: Optimum particle size and entrapment efficiency of heparin modified-cellulose acetate phthalate and cellulose acetate phthalate nanoparticles

Formulations	Entrapment efficiency	Particle size (nm)	Polydispersity Index	Zeta potential
HEC NPs	91.18±1.30	85±0.16	0.071±0.038	−13.8 mV
CAP NPs	76.82±1.2	137±1.8	0.082±0.13	−7.29 mV

NPs: Nanoparticles, CAP: Cellulose acetate phthalate, HEC: Heparin modified-cellulose acetate phthalate

Powder X-ray diffraction investigation

The XRD of ADH, UA, CAP, Heparin, HEC, and UA-loaded HEC NPs are explained [Figure 6]. In case of Heparin, X-ray diffractogram displayed the intense peak between 2 θ values of 10 and 30. At 14, 21, 22, 28, 30, 32, and 41 θ, the peak of ADH obtained in XRD diffractogram, signifies the crystalline characteristic of ADH. The diffractogram of CAP has showed small sharp peaks, which revealed its crystalline characteristics. The drug UA has elucidated characteristic sharp peaks at 9, 10, 12, 18, 16, 24, 25, 28, 30, and 33, which validate its crystalline characteristics. Whereas, the diffractogram of HEC has peaks at 16, 19, 23, 27, 28, 36, and 40 θ. In case UA-loaded HEC NPs, characteristic crystalline peaks at 10, 12, 18, 24, 28, and 30 θ were spotted which revealed the increase in crystallinity and UA is mingled with its crystalline form [Figure 6f].

In vitro drug release pattern

The sustained and prolonged release nature of drug from NP system illustrated in graph [Figure 7]. The drug release graph represents the controlled as well as sustains release of UA from the HEC system and from plain polymeric (CAP) nanoparticulate system. The HEC NPs was sustained UA up to 32 h and released 96.21%, however plain CAP NPs were liberated 97.36% UA in 8 h.

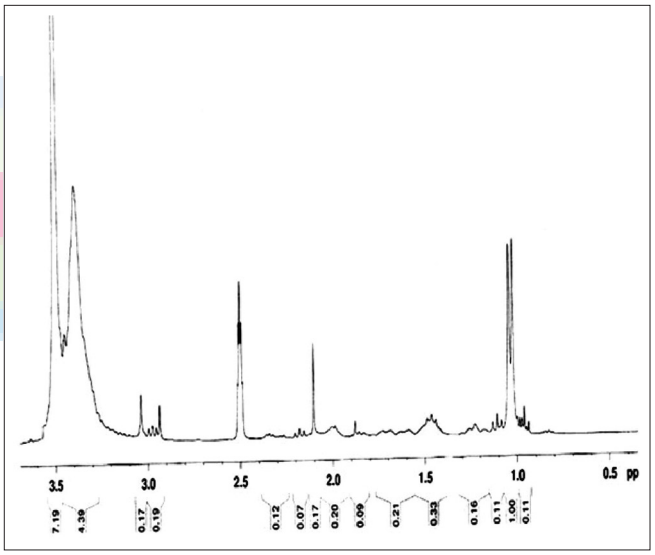

Figure 3: Nuclear magnetic resonance spectra of heparin modified-cellulose copolymer

Hemolytic toxicity study

The hemotoxic effect of the formulated heparin anchored CAP NP and plain CAP NPs was estimated by hemolytic toxicity study. The plain UA, UA-loaded HEC NPs and UA-loaded CAP NPs have exhibited hemolytic toxicity up to 28.31 ± 1.15%, 4.19 ± 1.10% and 8.09 ± 0.90% individually, plain UA and UA-loaded NP formulations consisting of 0.1 μM equivalents of UA. The UA formulation of NPs was evaluated by the

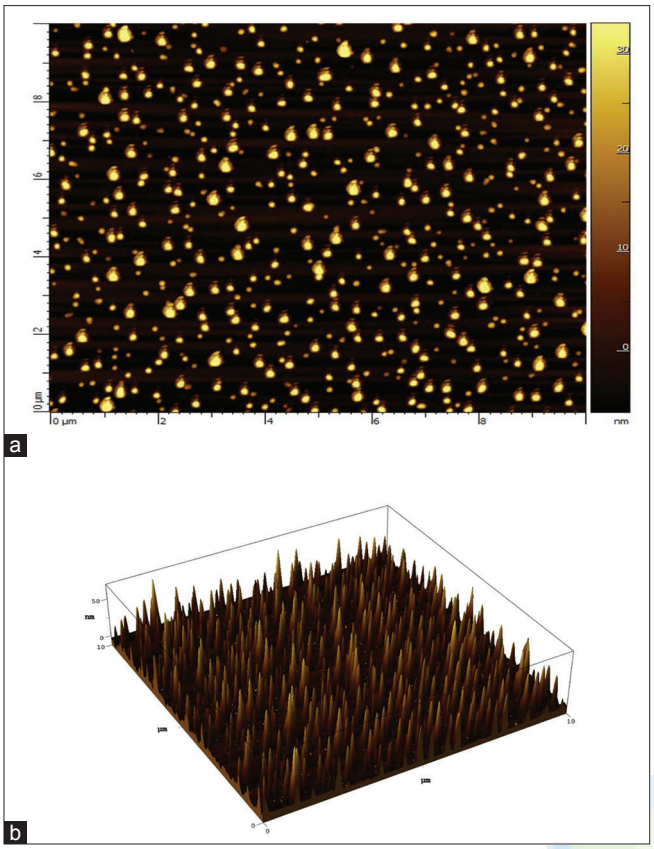

Figure 4: Atomic force photomicrograph of Heparin modified heparin modified-cellulose acetate phthalate Nanoparticles (a) Single dimension image; (b) 3-D image

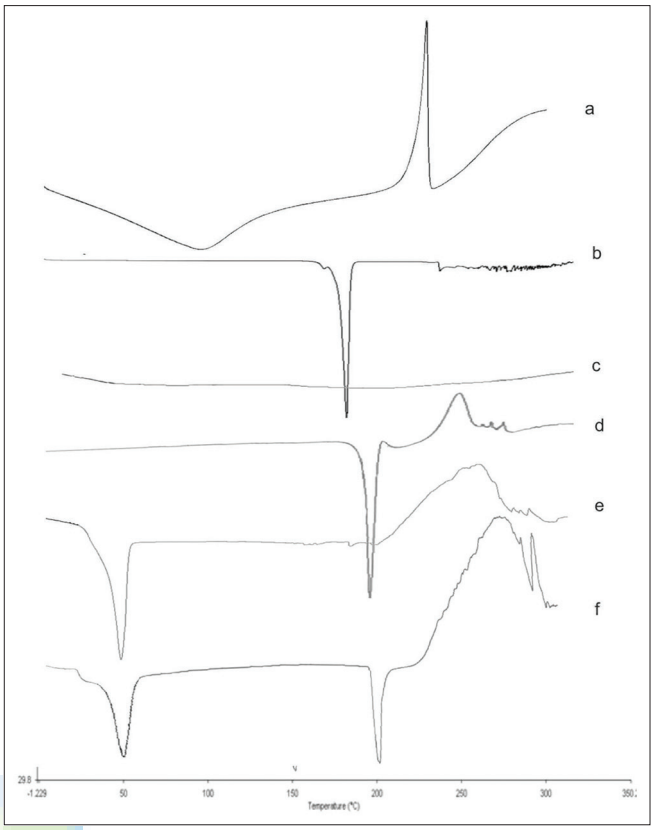

Figure 5: Differential scanning calorimetry thermo gram heparin (a) and adipic acid dihydrazide (b) and cellulose acetate phthalate (c) and usnic acid (d) and heparin modified-cellulose (without drug) (e) and heparin modified-cellulose (drug loaded) (f)

means of content of drugs. There was decline in hemolytic toxicity caused due to delayed release of encapsulated drug molecules in the nanoparticls.

Sulforhodamine B assay

The *in vitro* cytotoxicity screening of NPs in A549, human lung cancer cell line was established by SRB assay. The result obtained by the assay affirm dose dependent assessment of cytotoxicity in which the cellular bioavailability decreased with increasing the concentration of sample. The sample is UA-loaded HEC NPs. The result of percentage growth inhibition of cell is illustrated in Figure 8. Which revealed that higher concentration of UA inhibit the cell growth. Furthermore, the cell viability also gets declined with increase in the UA concentration and is available in free form or in encapsulated form in NPs. NPs formulations were experiential to be cytotoxic to a greater amount with the concentration between 10 and 80 μg/ml, when compared to plain UA.

Pharmacokinetics parameter

The pharmacokinetic analysis of prepared nanoparticulate formulation was accomplished on albino rats to evaluate the viability of delivery of the UA into blood by means of intravenous route. It was well-defined that HEC NPs participated enormously increase bioavailability UA concentration compared to plain UA solution (control). In pharmacokinetic parameters as observed, UA-loaded HEC NPs exhibited higher plasma concentrations (115.45 μg/L) in comparison to the UA plain solution (26.24 μg/L). In the study of pharmacokinetic assay the a scientifically considerable differentiation ($P \leq 05$) was found in values AUC, among the plain solution of UA (595 ± 2.26 μg h/mL) and HEC NPs (9167.7 ± 2.90 μg h/mL). The C_{max} (maximum concentration of drug in plasma), T_{max} (Time to accomplish maximum plasma concentration) and MRT (Mean residence Time) of plain solution of UA as well as HEC NP system was observed to be 339.12 ± 3.295 μg/L, 32 ± 0.5 h, 15.40 ± 0.25 h and 39.70 ± 2.30 μg/L, 1.2 ± 0.4 h, 0.9 ± 0.20 h respectively.

DISCUSSION

^{1}H-NMR and FTIR spectroscopic assay was execute to confirm the conjugation as well as the compatibility between heparin and polymer (CAP). The photomicrograph obtained from AFM analysis shows the uniform

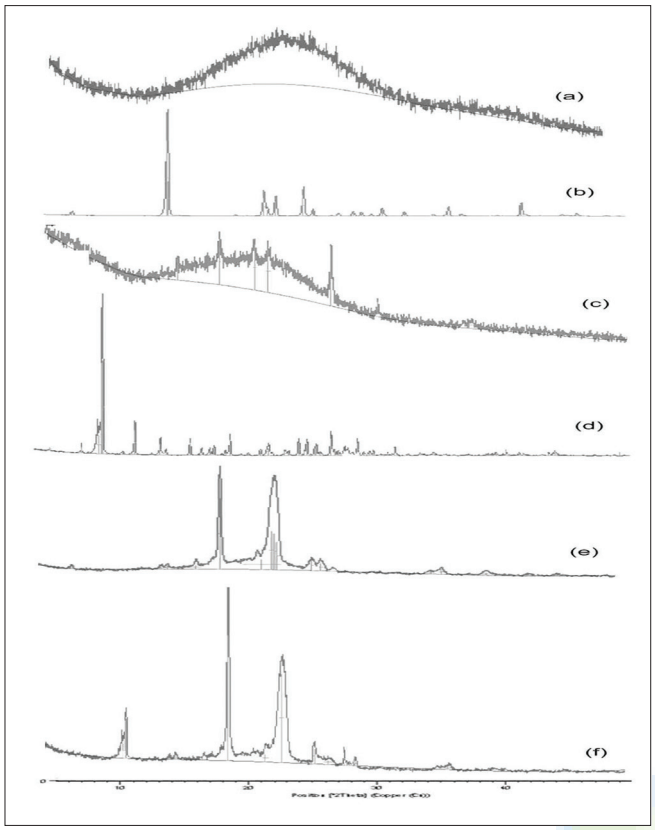

Figure 6: X-ray diffraction thermo gram heparin (a) and adipic acid dihydrazide (b) and cellulose acetate phthalate (c) and usnic acid (d) and heparin modified-cellulose (without drug) (e) and heparin modified-cellulose (drug-loaded) (f)

arrangement as well as similar height of the NPs. The outcomes were anticipated to be in accordance with the previous reports.[20]

According to the result indicated in table the entrapment proficiency of drug the size of particles of NPs was affected due to different concentration of surfactant as well as polymers. On enhancing the amount of CAP (polymer) from 10 to 30 mg the size of particles enhance from the range 85 ± 0.16–230 ± 1.20 nm and reduction in entrapment (drug content) efficiency was observed from range 91.18 ± 1.30% to 62.58 ± 1.19%, due to enhancement of particle size the efficiency of entrapment may decreased [Table 1]. CAP NPs were also affected by polymer concentration similar to as HEC NPs. Enhancement of size of particle from 137 ± 1.6 to 280 ± 1.4 nm with increasing amount of CAP (polymer) from 10 to 30 mg. The encapsulation efficiency may also be affected because of the larger size of particles and higher amount of polymer concentration, the entrapment efficiency of CAP NPs may be reduced from 76.82 ± 1.2% to 52.31 ± 0.98% [Table 2]. The concentration of surfactant (pluronic F68) also acting significant role

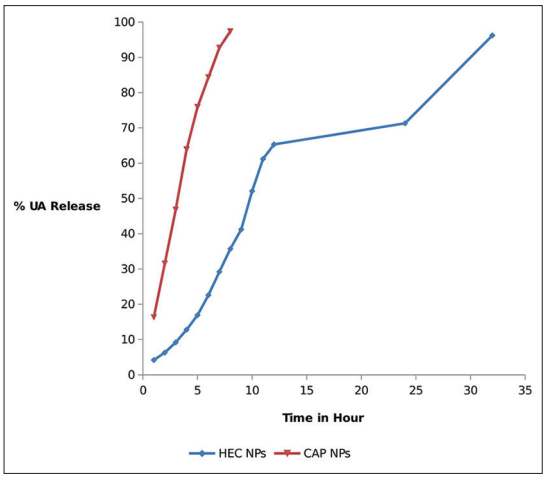

Figure 7: Percentage cumulative usnic acid release of heparin modified-cellulose and cellulose acetate phthalate nanoparticles

in particle size as well as percentage drug entrapment efficiency and possibly will also influence the particle size and entrapment efficiency of HEC NP, with raising the concentration of surfactant from 0.5% to 2%. When increasing the concentration from 0.5% to 1% the particle size may decreased from 125 ± 1.10 nm to 86 ± 1.18 nm and enhancement in drug entrapment was found from 79.98 ± 0.70% to 90.4 ± 0.70%, but when the concentration enhanced from 1% to 2%, the size of NPs (nm) increased (86 ± 1.18–113 ± 1.50), due to gathering of the particles and consequently the % drug entrapped was found to be reduced 90.4 ± 0.70%–83.45 ± 1.10% [Table 1]. As same way in CAP NPs, enhancement of concentration of surfactant from 0.5% to 1%, the particle size of CAP NPs was reduced from the range 265 ± 1.10 nm to 139 ± 2.21 nm and increment in entrapment efficiency may occur from 59.63 ± 1.12% to 75.32 ± 1.10%. However as the concentration increased from 1% to 2% in the CAP NP system the agglomeration of particles may occur which may cause enhancement in particle size and reduction in drug entrapment efficiency which may also affect the stability and quality of NPs depicted in Table 2. The PDI index of HEC NPs and CAP NP was found to be 0.071 ± 0.038 and 0.082 ± 0.13, respectively. PDI is a value available in form of dimensionless number having different ranges, the PDI index of mono dispersed particles is 0.5–0.7 and the sample having extensive range of size distribution depicted the PDI <0.7.[23] The encapsulation proficiency of prepared HEC NPs and CAP NPs was noticed 91.18 ± 1.30% and 76.82 ± 1.2% and the size of particles notify to be 85 ± 0.16 nm and 137 ± 1.8 nm [Table 3]. Zeta potential of HEC and CAP was obtained to be −13.8 mV and −7.29 mV, respectively [Table 3]. The negative value of the zeta potential analysis of NPs may be due to the carboxyl moiety of the polymer as well as the ligand. In the

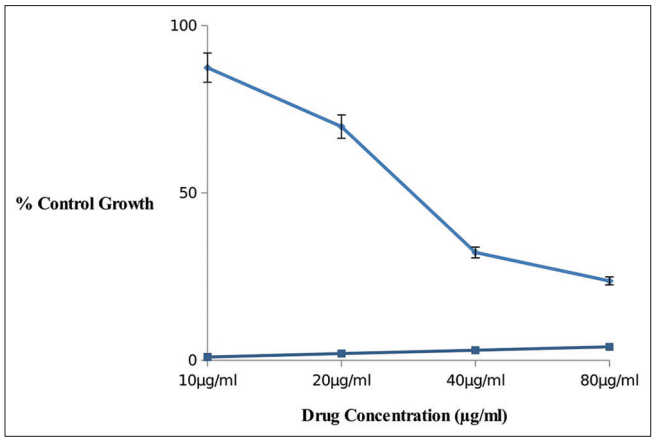

Figure 8: *In vitro* percentage control growth of usnic acid-loaded heparin modified-cellulose nanoparticles in A549 cancer cellline

charged particle having high zeta potential causes formation of more stable particles due to higher repulsive interaction. It was observed that the lower negative zeta potential may increase the stability of NP system.

By the result obtained from DSC analysis, in case of UA-loaded HEC NPs, the exothermic peak of UA was obtained at 202°C in the thermogram, which signifying the presence of UA resides in crystal-like format inside the NP micelle, and also in the diffractogram of XRD, the various difrractogram of UA was spotted in the UA-loaded HEC NPs, it is now recognized that the UA-loaded HEC NPs are suitably applicable for the controlled and prolonged drug release from the NP system.

In the drug release study, the HEC NPs was sustained UA up to 32 h and released 96.21%; however, plain CAP NPs were liberated 97.36% UA in 8 h. This is due to because the solvent system used in the manufacturing of NP carrier, i.e., acetone and isopropyl alcohol may break the hydrogen bond of the heparin and promote the reaction between of the carboxyl group of heparin and polymer with the amine group of ADH (carbodiimide conjugation) and formation of crosslinked core-shell micelle, which having less solubility, may promote sustained release.

The data obtained from hemolytic toxicity study, UA consisting of HEC NPs demonstrated lesser hemotoxicity in comparison with UA-loaded CAP NPs. This might be because of the hydrophilic nature of heparin which brings about the hemocompatible system. The repression of hemotoxicity of drug can be linked among other similar studies of heparin NPs described previously.[24,25]

The result of percentage growth inhibition of cell (A-549), revealed that higher concentration of UA inhibit the cell growth. NPs formulations were experiential to be cytotoxic to a greater amount with the concentration between 10 and 80 µg/ml, when compared to plain UA. Cytotoxic effect of optimized UA-loaded HEC NPs in A549 was discovered to have greater inhibitory effect. This may be recognized due to A549 over expressed high anaplastic lymphoma kinase (ALK) level.[26,27] The ligand receptor binding proficiency provide higher range of cytotoxic effect on cell lines in this way the heparin directly bind to ALK receptor present in the lung cancer cell line (A549) and provide effective receptor mediated endocytosis into the cell and release the drug into the nucleus causes inhibit DNA synthesis which may responsible for cell death. This was due to property of heparin of tremendous dispersing characteristics in aqueous solution and which imparts "stealth" property to the NPs. In this type the heparin anchored CAP NPs is more suitable for inhibiting the cancer cell growth as well as drug delivery carrier than free form of drug.

The pharmacokinetic analysis of prepared nanoparticulate formulation was accomplished on albino rats to evaluate the viability of delivery of the UA into blood by means of intravenous route. The C_{max} (maximum concentration of drug in plasma), T_{max} (Time to accomplish maximum plasma concentration) and MRT (mean residence time) of plain solution of UA as well as HEC NP system was observed to be 339.12 ± 3.295 µg/L, 32 ± 0.5 h, 15.40 ± 0.25 h, and 39.70 ± 2.30 µg/L, 1.2 ± 0.4 h, 0.9 ± 0.20 h, respectively. This may be as a result of hydrophilic Heparin layering on CAP NPs resulting in enhancement of the diffusion of drug.[28,29]

CONCLUSION

The HEC NPs-loaded with UA were acquired lucratively by using nanoprecipitation method by which higher amount of the drug (UA) to be encapsulated in the nanoparticulate system. These HEC nano particle system show sustain and prolong release of UA due to the hydrophilic layer of heparin in the polymer core. The zeta potential studies also revealed the higher stability of NPs because of their negative surface charge. Cytotoxicity analysis revealed that UA-loaded HEC NPs showed potent cytotoxic effect on A549 cancer cell lines. After performing all the analysis it was accomplished that the UA-loaded HEC NPs system possess as potent agent for anticancer drug delivery vehicle.

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Conflicts of interest

There are no conflicts of interest.

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