

**Research Article****Hyaluronic acid anchored nanoparticulate carrier for delivery of Methotrexate**

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**Abstract**

**Objective:** The aim of present study was to evaluate the hyaluronic acid anchored chitosan nanoparticles containing polyphosphoric acid for the delivery of methotrexate. **Material and methods:** Chitosan nanoparticles with MTX were prepared by using ionotropic gelation technique. Preparation of hyaluronic acid (HA) anchored chitosan nanoparticles were prepared by ionic interaction method. NPs were exemplified by zeta potential, differential scanning calorimetry (DSC), particle size, field emission scanning electron microscopy, in-vitro release, entrapment efficiency, X-ray diffraction (XRD), polydispersity index, and stability studies at various temperature  $5 \pm 3^\circ\text{C}$ ,  $25 \pm 2^\circ\text{C}$  and  $40 \pm 2^\circ\text{C}$ . **Results:** Studies demonstrated that HACSNPs have slower release (95.08% in 96 hrs) when contrasted with CSNPs (96.43% in 72hrs). **Conclusion:** The HACSNPs loaded with MTX were obtained successfully by ionotropic gelation method with high entrapment efficiency and low particle size. These formulations provide sustained and prolonged release of drug. Thus the NPs also hold a promising potential for treating the single or multiple ailments to improve patient compliance and reduce toxicity.

**Keywords:** Hyaluronic acid, Methotrexate, Chitosan, Polyphosphoric acid, nanoparticles

**Introduction**

It's a fact that cancer has been considered as one of the most common diseases in all over the world. Every year we come across the new cases that are rising every year. Through new investigation procedures and therapies have been made, the survival rate in this disease could not be raised over the last 30 years (Jamal et al., 2010). Cancer is no doubt is regarded as one of the most important cause of death in the national and worldly scene. If we look into the history of cancer, we found that more than 15.5 million children and youths/adults with cancer survived on 1 January 2016, in the United States. It's guessed that by Jan 1, 2026, the number of cancer survivor will go up to 20.3 million i.e. increase to 20.3 million almost 10 million males and 10.3 million females (Siegel et al., 2012).

Before the past few decades, there was conventional treatment alteration such as chemotherapy and radiation that have seen

many progressive steps in cancer- cure but they had limitation and side effects. Insertion of potent anticancer bioactive by i.v. route and intense side effects when the drug acts on nontarget sites. With such non-specific drug action, the effect of the drug at the tumor site itself goes low. On the other hand, the drug when entered into a healthy tissue takes to produce-toxicity and thus the effective dose becomes an ineffective dose. So as to improve this complexity, the researchers have been giving their attention to the fabrication of tumor-specific drugs or delivery systems that can selectively localize existing agents to the tumor sites. Recent progressive step in nanotechnology assures further developments in target-specific drug delivery systems (Vanvlerken et al., 2006).

Hyaluronic acid is a natural high molecular-weight glycosaminoglycan. It's composed by repeating disaccharides unit of (1- $\beta$ -3) N-acetyl-D- glucosaminy- $\beta$  1, 4 glucuronide (Toole et al., 1990; Goa et al., 1994). Meyer and Palmer (1934) said of a procedure for isolating a novel glycosaminoglycan from the vitreous humor of bovine eyes and named it HA (from the Greek word, hyalos, i.e. glassy or vitreous).

The molecular weight of HA range from 100 to 5000 kDa

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(Bertolami et al., 1992). The HA plays an important key role in the structure and organization of the extracellular matrix (ECM), transport of nutrients, regulation of cell adhesion, morphogenesis and modulation of inflammation (Hardwick et al., 1992; Laurent et al., 1995).

Cell surface HA-binding proteins were discovered during the late 1980s (Aruffo et al., 1990; Turley et al., 1987). Two HA receptors are strongly implicated in the cell signaling cascades associated with cancer beginning and progression: these are CD44 (cluster of differentiation 44) and the receptor for hyaluronic-acid-motility motility (RHAMM) (Yang et al., 2013).

Chitosan is a molecule with a carbohydrate backbone structure similar to cellulose, which consists of two types of repeating units, N-acetyl-d-glucosamine and d-glucosamine, linked by (1-4)- $\beta$ -glycosidic linkage. It is an amino-saccharide cationic polymer.

A molecule named chitosan with a carbohydrate is the backbone structure like cellulose, which is made up of two types of repeating units, N-acetyl-d-glucosamine and d-glucosamine, linked by (1-4)- $\beta$ -glycosidic linkage/chain. It's an aminosaccharide cationic polymer. The mucoadhesive nature of chitosan that grows the time of attachment at the absorption site, the easy availability of free amino group for crosslinking, ease of fabrication of polymeric particles without using hazardous solvents. The cationic nature that allows ionic cross-linking with multivalent anions and finally the ability to control the release of the inserted drug makes chitosan the polymer of choice for developing the particles of polymers.

PPA is an inorganic and contain multi-charged anion, used in detergents, food industry, and biomedical application, Electrostatic complexation allows PPA to bridge chitosan chains through the formation of intermolecular and intramolecular crosslinking forming a nanosized polymeric network and the out-coming materials can be defined as NPs, as hydrophilicity of the ionic groups gets the network to grow larger in size with water. Chitosan/PPA have high water quantity, soft and rubbery firmness when nanomaterials are prepared. The chitosan/PPA ratio regulate particle-size, morphology and surface charge (Gard et al., 1996).

Methotrexate (MTX) is a kind of folate similarity, used in the treatment of cancers (lymphoblastic leukemia, breast cancer and autoimmune diseases etc). It inhibits dihydrofolate reductase (DHFR) and blocks the conversion of dihydrofolic acid (DHFA) to tetrahydrofolic acid (THFA) acting in s-phase of the cell cycle (Mishra et al., 2014; Kye, 1998). MTX is readily absorbed from the gastrointestinal tract at doses of less than 25 mg/m<sup>2</sup>, but larger doses are absorbed incompletely and are routinely administered intravenously. Peak concentration in the

plasma of 1 -10  $\mu$ M are obtained after doses of 25 to 100 mg/m<sup>2</sup>, and concentration of 0.1-1 mM are achieved after high-infusion of 1.5-20 g/m<sup>2</sup>. After intravenous administration, the drug disappears from plasma in a triphasic fashion (Sonneveld et al., 1986). The rapid distribution phase is followed by a second phase, which reflects renal clearance (half-life of about 2-3 hr) and in the third phase has a half-life of approximately 8-10 hrs.

## Materials and Methods

### Materials

Chitosan of low molecular weight (93% degree of deacetylation), Polyphosphoric acid was purchased from chemdyes corporation, India. Hyaluronic acid was purchased from Sigma-Aldrich (USA). Methotrexate sodium (MTX) was obtained as a gift sample from Fresenius Kabi Ltd, Gurgaon, India. Glacial acetic acid and Tween 80 purchased from Merck Limited, Mumbai India and other chemicals which are consumed are of investigative chemical grade and used as brought.

### Preparation of chitosan nanoparticles

A necessary amount of chitosan (10 mg) was dissolved in 0.1 % glacial acetic acid and water in 9:1 ratio and under mechanical stirring at 3000 rpm for 4 hr at room temperature so that it is dissolved completely. CS solution was adjusted to pH 6.2 was performed by adding proper volumes of 0.1 N NaOH. This pH value keeps up CS in its soluble form at all investigated concentration (Hashad et al., 2016 with some modification). Polyphosphoric acid solution of different concentration: 0.5%, 1% and 1.5% in 0.1N sodium hydroxide was put drop by drop in the above-prepared chitosan solution and stirred constantly for 12 hours. 1% solution was made by dissolving and needed quantity of methotrexate in 0.1N sodium hydroxide and was added drop by drop in above-prepared chitosan solution. Afterward, 1.0% tween 80 solution was made and added dropwise in the above solution.

### Preparation of hyaluronic acid (HA) anchored chitosan nanoparticles

The needed amount of HA (10 mg) was dissolved in 10 ml PBS (pH 6.0) and stirred constantly. Its reaction was performed at room temperature for 12 hrs under mechanical stirring at 3000 rpm. This solution was added dropwise in the above solution with continuous stirring. The average of the pH of the HA solution was studied 6.07.

Nanoparticles were made on the solvent interface. Then solvent got evaporated at room temperature. As a result, the suspension of nanoparticles was filtered through a 0.45  $\mu$ m membrane filter (Millipore) and centrifuged for 45 min at

10,000 rpm (Remi, Mumbai, India). The supernatant was removed and HA anchored chitosan NPs were kept for lyophilize (Figure 1).

### **Characterization parameters of chitosan nanoparticles and HACSNPs nanoparticles**

#### ***Surface characteristics by Field Emission-Scanning Electron Microscope***

The topographical study of CS NPs and HACS NPs was carried out using a Field Emission-Scanning Electron Microscope (FESEM; Hitachi, Japan). A spine drop of the diluted lipid nanoparticles suspension was placed onto a carbon-coated copper grid and kept for 15 min for drying. Grids were then coated with gold to a thickness of about 300Å by using a sputter coater. All samples were examined under a FESEM at an accelerated voltage of 10KV and magnification of 2500×.

#### ***Zeta potential and Particle size***

The average particle size, zeta potential, and polydispersity index (PDI) of chitosan nanoparticles and hyaluronic acid anchored chitosan nanoparticles were determined by a Zetasizer (DTS ver. 4.10, Malvern Instruments, England). Briefly, the nanoparticulate dispersion was poured in polystyrene cuvettes diluted with ultra-pure deionized water and analyzed at a 90° fixed angle. The zeta potential of NPs formulation was measured by determining electrophoresis mobility with a laser-based multiple angle particle electrophoresis analyzers, Malvern Zetasizer (DTS Ver. 4.10, Malvern Instruments, England). The nanoparticles were suspended in ultra-pure de-ionized water and kept in an electrophoresis cell with an electric field of 15.24 V/cm and the zeta potential was measured.

#### ***Differential Scanning Calorimetry (DSC)***

Thermal characteristics of MTX, HA, CS and HACS NPs were premeditated by mean of Pyris-6-DSC Thermal analyzer (PerkinElmer, Tokyo, Japan). Both the powdered samples were weighed individually and sealed in the heat-resistant aluminum pans and lid was crimped on its shell by applying pressure using pellet press. The sample and reference pans were placed in the heating compartment, heated from 20°C to 200°C with temperature ascend at a rate of 10°C/min, and DSC spectra were reported.

#### ***Powder X-Ray Diffraction (XRD)***

Powder X-ray diffraction patterns of samples NPs were obtained using power X-ray diffractometer (Bruker, Munich, Germany) a nickel-filtered Cu-Kα radiation (a voltage of 40 KV and a current of 20mA). The scanning rate was 2θ range of 0-40° and with an interval of 0.02.

#### ***Entrapment proficiency***

The drug entrapment efficiency was determined using a dialysis

method for separating unloaded MTX from the NPs. It is a method to estimate indirectly determined the amount of drug bound with the NPs. Taken 5 ml of the MTX-loaded chitosan NPs dispersion was placed into a dialysis bag of MWCO 12000 KDa, (Himedia, Mumbai, India) and dialyzed against 50 ml of 0.1N NaOH for 15 minutes with magnetic stirring (50 rpm). After 15 minute dialysis bag were taken out and drug in reservoir were estimated HPLC (Noquera et al., 2013). All samples (injection volume-20μl) were quantified for MTX content by HPLC at a flow rate of 1ml/min at 300 nm with retention time 2 min. Samples were loaded into the HPLC system to quantify the untrapped amount of MTX (Jain et al., 2014 and Tavano et al., 2013). So as to quantify indirectly the amount of entrapped MTX within the NPs. The same procedure was carried out to measure the entrapped drug in HA anchored chitosan NPs and the quantification was performed using HPLC (Thermo scientific) equipped with a UV detector (PDA detector). The reversed phase C18 column (100 mm×4.6 mm,) was used for chromatographic separation. The mobile phase was a mixture of acetonitrile and water at a ratio of 80:20 v/v.

#### ***In-vitro drug release study***

In-vitro methotrexate release from chitosan nanoparticles and hyaluronic acid anchored nanoparticles was determined using dialysis tube. 5 ml methotrexate loaded chitosan NPs was placed into dialysis bag of MWCO 12000 KDa, (Himedia, Mumbai, India), tied at both the ends are placed in a beaker containing 50 ml of phosphate buffer (pH 7.4). The beaker was placed over a magnetic stirrer at 100 rpm and the temperature maintained at 37 ± 1°C throughout the procedure. At specific time intervals, the whole medium (50 ml) was taken out and replaced with the same volume of fresh 100 ml PBS (pH 7.4) and sink condition was maintained (Garg et al., 2015; Kesharwani et al., 2015).

Then samples were analyzed by HPLC. The HPLC determination was performed using reverse phase column (100 × 4.6), acetonitrile: water as the mobile phase in the ratio of 80:20, v/v with detection at 300 nm with a flow rate of 1 ml/min and retention time was 2.0 min.

#### ***Stability studies***

##### ***Effect of storage on particle size***

Particle size of the formulations stored at 5 ± 3°C, 25 ± 2°C and 40 ± 2°C was determined by using a Zetasizer (DTS ver. 4.10, Malvern Instruments, England) after a definite period of time i.e. 10, 20, 30, and 40 days.

##### ***Effect of storage on residual drug content***

After storage for a specified period of time of 10, 20, 30, and 40 days, the drug content of both formulation was

determined (Fry et al., 1978). Drug entrapment in NPs was determined by using dialysis membrane method.

### *In vitro cytotoxicity study*

In-vitro cytotoxicity was evaluated by tetrazolium dye-based MTT assay on the basis of a method documented earlier. MCF-7 cellline were retained in RPMI-1640 medium supplemented with 10% heat inactivated fetal bovine serum and antibiotics at 37°C in a humidified incubator consisting of 5% CO<sub>2</sub>. The cells were treated with MTX loaded hyaluronic acid (HA) anchored chitosan nanoparticles of different concentrations 10, 20, 30 and 40 µg/ml for 24 hrs. The amount of MTX loaded hyaluronic acid (HA) anchored chitosan nanoparticles needed to prepare molar equivalents of MTX was calculated based on the MTX content in the loaded hyaluronic acid (HA) anchored chitosan NPs. Control was taken without any drug treatment. Afterward, MTT was added and plates were then incubated for further 3 hrs, thenceforth the media was pipetted off and 250 µL DMSO was supplemented. The absorbance of individual wells was perceived at 570 nm via an ELISA plate reader at 25°C. Average values from triplicate were deducted from the average value of control and consequently, the survival percentage of cells was evaluated by the formula.

### **Results and discussion**

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 hyaluronic acid appended chitosan nanoparticles.

### *Morphology*

The shape of chitosan NPs and HA anchored chitosan NPs was determined by the studied of FESEM analysis. The study

revealed that most of the NPs were nearly spherical in shape.

### *Particle size and entrapment efficiency determination*

The particle size of blank chitosan nanoparticles was influenced by polymer concentration. With increasing concentration of polymer from (5 to 10 mg), particle size increased from (137 ± 2.30 to 180 ± 1.50 nm). When concentration of polymer (10 to 15 mg) was increased, the particle size of NPs was increased (168 ± 1.60 to 180 ± 1.60 nm). The particle size of NPs was decreased (176 ± 1.10 to 135 ± 1.50 nm) and entrapment efficiency was increased (59.13% ± 0.50% to 85.38% ± 0.21%), when concentration of drug was increased from (5 to 10 mg). Further increased in concentration (10 to 15 mg), the particle size of NPs was increased from 135 ± 1.50 to 180 ± 2.30 nm and entrapment efficiency of NPs was decreased (85.38% ± 0.21% to 55.40% ± 1.50%). When further increased in concentration of drug (10 to 15 mg) exerts no or little effect on entrapment efficiency.

When concentration of surfactant was increased from 1 to 1.5%, the size of NPs was increased (126 ± 1.60 to 147 ± 1.50 nm) and entrapment efficiency was decreased (91.01 ± 0.50% to 69.03 ± 1.35%). When concentration of surfactant was increased from 1.5 to 2%, the size of NPs was decreased (147 ± 1.50 to 141 ± 2.30 nm) and entrapment efficiency was increased (69.03 ± 1.35% to 74.30 ± 1.45%). Further increased in amount of surfactant (1.5 to 2%) exerts little or no effect on particle size and entrapment efficiency. Results are shown in table 1. When the amount of Tween-80 was increased it was found that the granulometric distribution became narrower. This phenomenon can

**Table 1.** Various optimization parameter of CS and HA anchored CSNPs

S. No.	Drug: Polymer (mg.)	Poly phosphoric acid (ml)	Glacial acetic acid	Tween 80 (ml)	Water (ml)	Particle size (nm)	% Entrapment Efficiency	Polydispersity Index
CHP	0:5	-	9	1%	25	137 ± 2.30	-	-
<b>CHP 1</b>	<b>0:10</b>	-	<b>9</b>	<b>1%</b>	<b>25</b>	<b>168 ± 1.60</b>	-	-
CHP 2	0:15	-	9	1%	25	180 ± 1.50	-	-
CHP 1D1	5:10	-	9	1%	25	176 ± 1.10	72.30 ± 0.50	0.108 ± 0.031
<b>CHP 2D2</b>	<b>10:10</b>	-	<b>9</b>	<b>1%</b>	<b>25</b>	<b>135 ± 1.50</b>	<b>80.38 ± 0.21</b>	<b>0.128 ± 0.012</b>
CHP 1D3	15:10	-	9	1%	25	180 ± 2.30	77.40 ± 1.50	0.112 ± 0.041
CHP1 D1A	10:10	-	9	0.5%	25	126 ± 1.60	86.01 ± 0.50	0.081 ± 0.070
<b>CHP1 D2B</b>	<b>10:10</b>	-	<b>9</b>	<b>1.0%</b>	<b>25</b>	<b>147 ± 1.50</b>	<b>78.03 ± 1.35</b>	<b>0.83 ± 0.022</b>
CHP 1 D3C	10:10	-	9	1.5%	25	141 ± 2.30	83.30 ± 1.45	0.085 ± 0.042
CHP1 D1A1	10:10	0.5%	9	1.0%	25	146 ± 1.60	82.06 ± 1.90	0.113 ± 0.032
<b>CHP1 D2B2</b>	<b>10:10</b>	<b>1.0%</b>	<b>9</b>	<b>1.0%</b>	<b>25</b>	<b>139 ± 1.50</b>	<b>90.09 ± 1.50</b>	<b>0.097 ± 0.024</b>
CHP 1 D3C3	10:10	1.50%	9	1.0%	25	127 ± 2.30	79.13 ± 1.40	0.088 ± 0.041

**Table 2.** Optimum particle size and entrapment efficiency of CSNPs and HACSNPs

S. No.	Formulations	Entrapment efficiency	Particle size (nm)	Polydispersity index	Zeta potential
1	CS NPs	90.09 ± 1.50	134 ± 1.50	0.097 ± 0.026	+5.71
2	HACS NPs	88.13 ± 3.05	140 ± 1.20	0.085 ± 0.079	-3.14

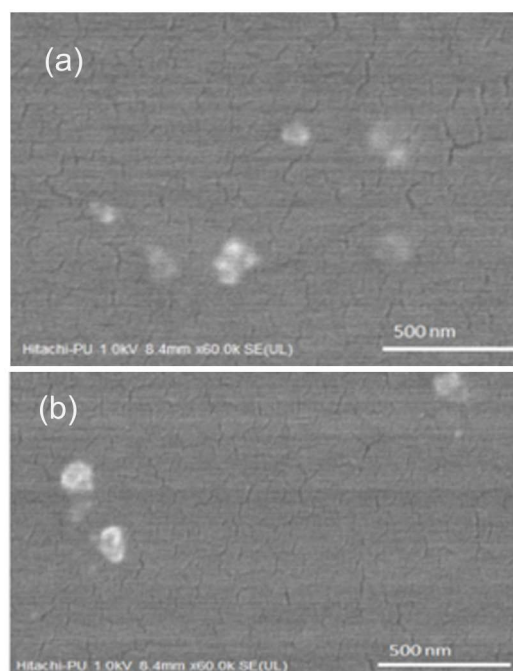
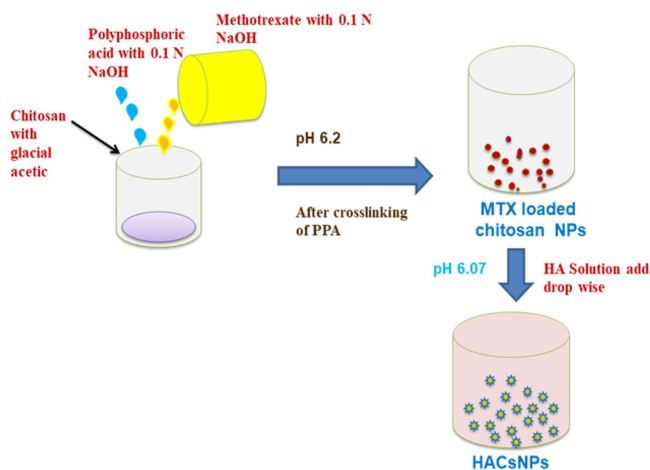
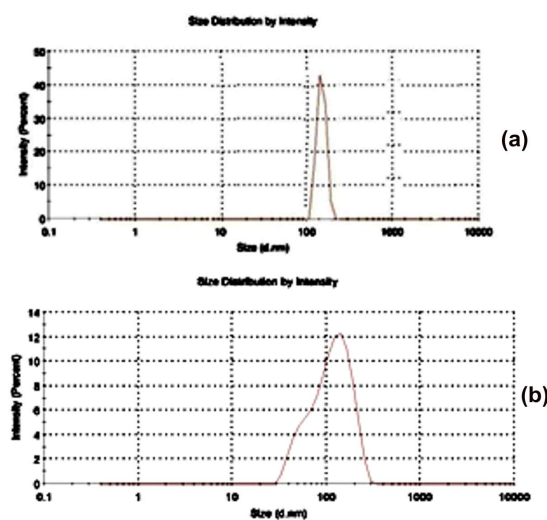
expected from the stabilizing function of a surfactant up to a definite concentration, further increase in concentration of surfactant exerts little or no effect on particle size and entrapment efficiency. It was also observed that when a small concentration of surfactant is used, it causes aggregation of the particles and the size of particles may increase (Garg et al., 2014).

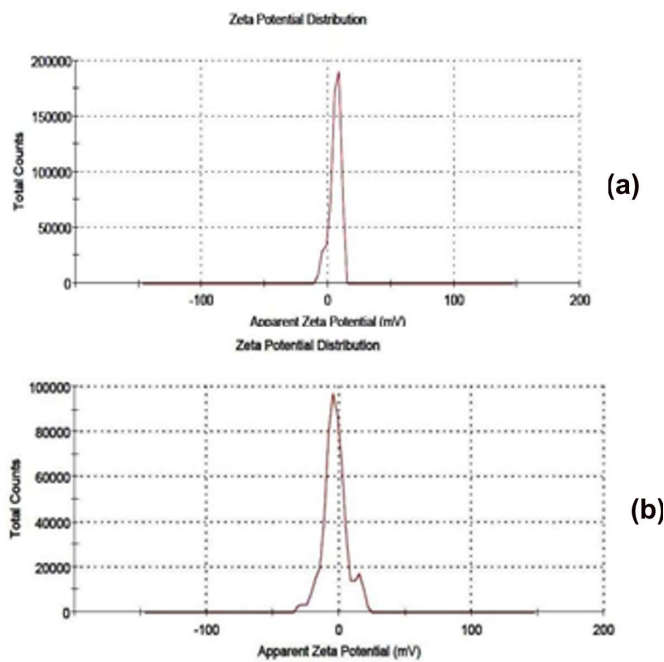
NPs were prepared with 0.5 to 1% crosslinking agent, the particle size of NPs was decreased ( $146 \pm 1.60$  to  $139 \pm 1.5$  nm) and entrapment efficiency was increased ( $70.06 \pm 1.90\%$  to  $80.09 \pm 1.50\%$ ). Further increased in concentration of crosslinking agent 1 to 1.5%, the particle size of NPs was decreased ( $139 \pm 1.5$  to  $127 \pm 2.30$  nm) and entrapment efficiency was increased ( $80.09 \pm 1.90\%$  to  $90.13 \pm 1.40\%$ ), there is no effect or little effect on particle size and entrapment efficiency.

For fabrication of HA anchored chitosan nanoparticles, we selected ionic interaction method. As chitosan has positive charge and HA negative charge for good coating of HA on surface of chitosan nanoparticles. Concentration of HA is need to optimized initially 5 mg of HA solution was added on surface of chitosan.

With increasing concentration of hyaluronic acid (5 to 10 mg) the particle size of HACS NPs was increased ( $136 \pm 1.60$  to  $140 \pm 1.20$  nm) and entrapment efficiency was decreased ( $82.60 \pm 3.51\%$  to  $79.13 \pm 3.05\%$ ). When the concentration of HA (10 to 15 mg) was increased, the particle size of NPs was decreased

( $140 \pm 1.20$  to  $133 \pm 1.40$ ) and entrapment efficiency of HACS NPs was increased ( $79.13 \pm 3.05$  to  $87.50 \pm 2.07$ ). When the concentration of HA was increased (10 to 15 mg) little or no effect on particle size and entrapment efficiency. Thus average particle size and % entrapment efficiency of HACS NPs was found to be  $140 \pm 1.20$  nm and  $87.13 \pm 3.05$ .

**Figure 2.** SEM image of (a) CSNPs (b) Hyaluronic acid anchored Chitosan NPs**Figure 1.** Preparation of MTX loaded HA anchored CSNPs containing polyphosphoric acid (HACS NPs)**Figure 3.** Particle Size of (a) CSNPs (b) Hyaluronic acid anchored Chitosan NPs



**Figure 4.** Zeta potential of (a) chitosan NPs, (b) HA anchored chitosan NPs

Polydispersity index of all chitosan NPs were optimized between  $0.081 \pm 0.070$  to  $0.128 \pm 0.012$  and HA anchored chitosan NPs it was found  $0.069 \pm 0.061$  to  $0.085 \pm 0.079$ . It is a dimensionless number extrapolated from autocorrelation function ranges from a value of 0.01 to 0.02 for monodispersed particles and up to values of 0.5 to 0.7 as broadly distributed and

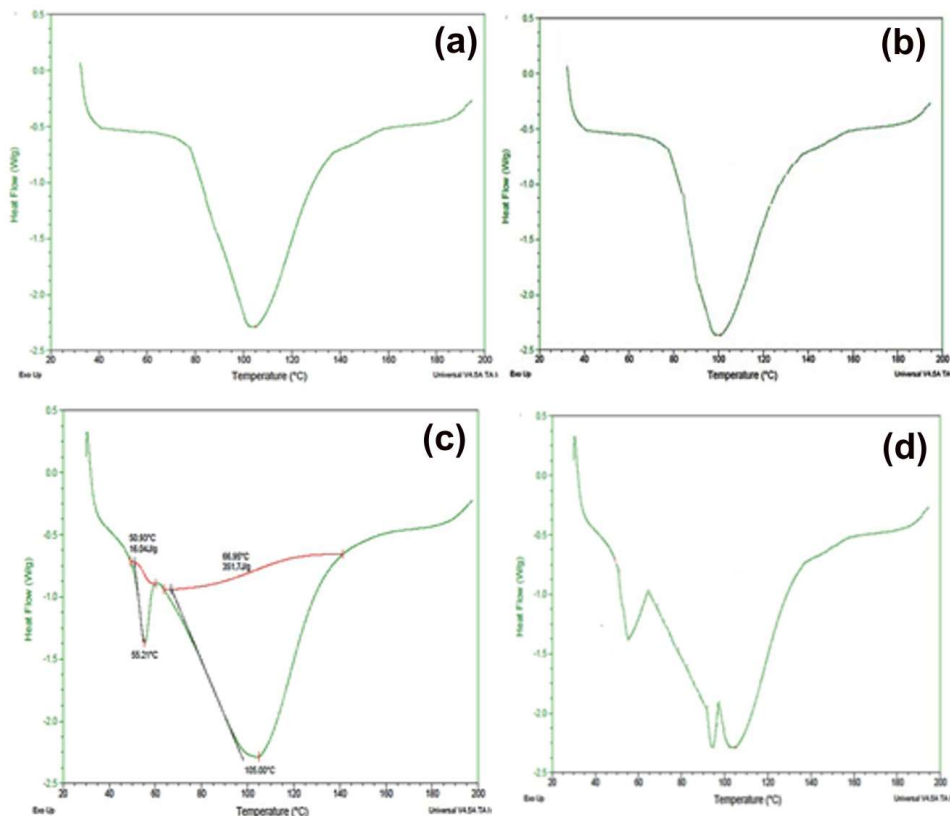
value above  $> 0.7$  indicated extremely broad size distribution that cannot be described by means of PDI (Quintanar et al., 1996).

#### Differential Scanning Calorimetry (DSC)

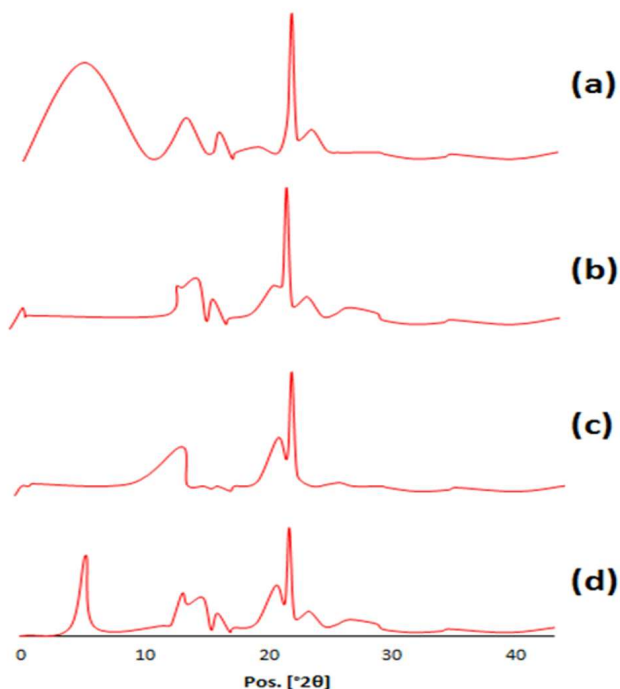
DSC measures the heat loss or gain resulting from physical or chemical changes within a sample as a function of temperature. DSC thermogram of MTX, HA, CS, MTX loaded HACSNPs were performed and thermogram shown in Figure 5. In case of MTX endothermic peak was observed at  $105^{\circ}\text{C}$  (Figure 5a). In case of HA, small endothermic peak was observed at  $80^{\circ}\text{C}$  (Figure 5b). In case of chitosan endothermic peak was observed at  $50.93^{\circ}\text{C}$ ,  $66.95^{\circ}\text{C}$  and  $105^{\circ}\text{C}$  (Figure 5c). In case of HACSNPs, endothermic peak of HA was observed at  $60^{\circ}\text{C}$ , MTX peak at  $95^{\circ}\text{C}$  and chitosan peak was observed at  $105^{\circ}\text{C}$  (Figure 5d). The DSC thermogram of drug loaded NPs, revealing that the drug is present in crystalline form inside the NPs.

#### X-Ray Diffractometer (XRD)

XRD of MTX, HA, CS and MTX loaded HACSNPs are shown in Figure 6 (a), (b), (c) and (d). MTX has shown characteristic intense peaks between  $2\theta$  values of 14, 18, 19 and 30, which confirmed its crystalline nature (Figure 6a). In the XRD of HA, intense peak were found at between  $2\theta$  values of 10-30, which very collates with previous report (Figure 6b) (Morgan et al., 2011). The XRD diffractogram of chitosan has observed peak at 9 and 19  $\theta$  (Figure 6c). In



**Figure 5.** DSC of (a) MTX (b) HA (c) CS (d) MTX loaded HACSNPs

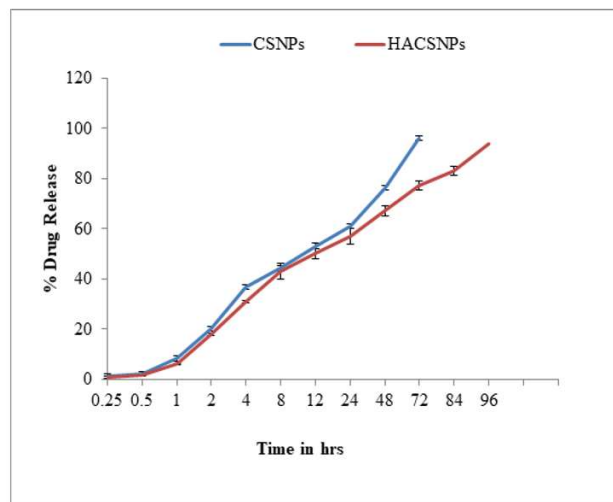


**Figure 6.** XRD thermogram of (a) MTX (b) HA (c) CS (d) MTX loaded HANPs

case of MTX loaded HACS NPs characteristic intense peak were found between 2θ value of 5, 11, 16, 20, 25, 31 and 39 (Figure 6d).

**In-vitro drug release**

The releases of MTX from HACS NPs were found slow release ( $95.08 \pm 2.50$  in 96 hrs) than CS NPs ( $96.43 \pm 1.48$  in 72 hrs).



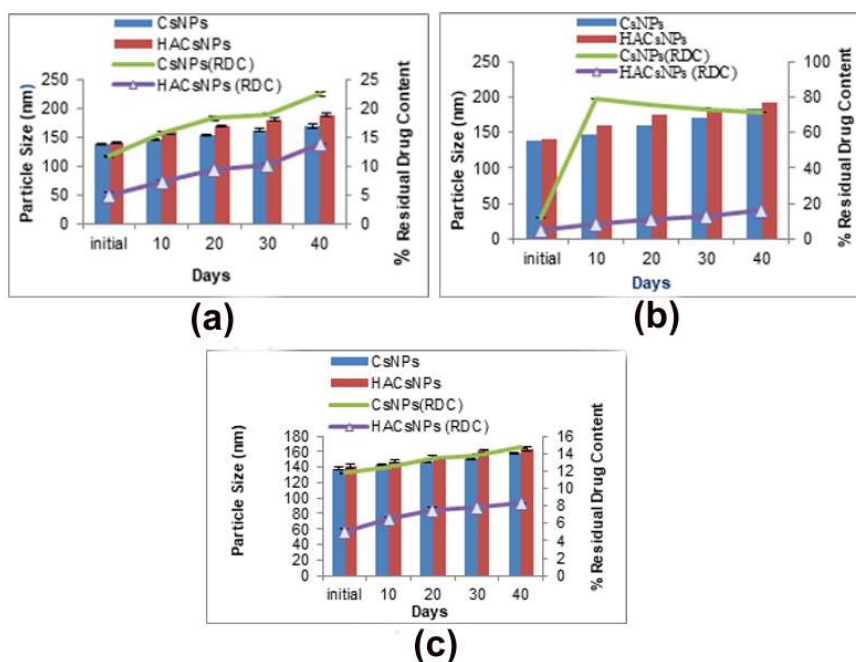
**Figure 7.** In vitro drug release in phosphate buffer (pH 7.4) from NPs formulations

This may be attributed for long circulatory behavior of HACS NPS because of hydrophilic nature of HA which leads to prolonged release of MTX.

**Stability studies**

Both the formulation i.e. Chitosan NPs and Hyaluronic acid anchored chitosan NPs were stored at  $5 \pm 3^\circ\text{C}$ ,  $25 \pm 2^\circ\text{C}$ ,  $40 \pm 2^\circ\text{C}$ . Change in particle size and residual drug content after time interval of 10, 20, 30 and 40 days were determined and results are shown in Figure 8.

When NPs was stored at  $5 \pm 3^\circ\text{C}$  for 40 days, there is little effect or no change in particle size of chitosan NPs and



**Figure 8.** Effect of storage on the particle size and % residual drug content of NPs at (a)  $5 \pm 3^\circ\text{C}$  (b)  $25 \pm 2^\circ\text{C}$  (c)  $40 \pm 2^\circ\text{C}$  with standard deviation mean value (n=3)

HA anchored chitosan NPs. The particle size of chitosan NPs was increased from  $138 \pm 1.84$  to  $153 \pm 1.30$  nm and residual drug content of CSNPs was increased from  $11.77 \pm 0.18\%$  to  $14.70 \pm 0.13\%$ . The particle size of HACSNPs was increased from  $141 \pm 2.30$  to  $164 \pm 3.05$  nm and residual drug content was increased from  $5.08 \pm 0.33\%$  to  $8.28 \pm 0.06\%$ .

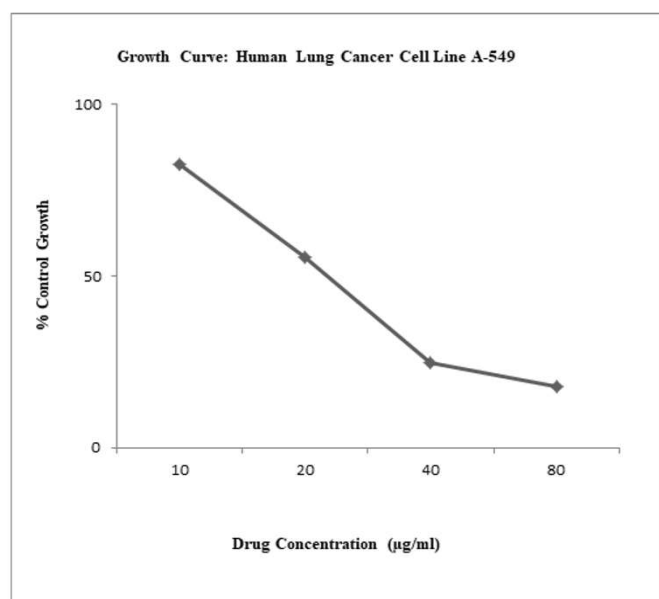
When NPs was stored at  $25 \pm 2^\circ\text{C}$  for 40 days, the particle size of CSNPs was increased from  $138 \pm 1.84$  to  $170 \pm 3.01$  nm and residual drug content was increased  $14.83 \pm 0.15\%$  to  $22.53 \pm 0.39\%$ . The particle size of HACSNPs was increased from  $141 \pm 2.36\%$  to  $181 \pm 0.25$  nm and residual drug content was increased from  $13.60 \pm 0.25$  to  $19.81 \pm 0.16$ .

The particle size of CSNPs was increased from  $138 \pm 1.84$  to  $184 \pm 1.50$  nm and residual drug content of CSNPs was decreased from  $20.22 \pm 0.67\%$  to  $28.15 \pm 0.24\%$  and particle size of HACSNPs was increased from  $141 \pm 2.30$  to  $192 \pm 1.21$  nm and residual drug content was increase from  $19.88 \pm 0.67\%$  to  $30.85 \pm 0.24\%$ . There is broad difference in particle size and residual drug content of NPs, when it stored at  $40 \pm 2^\circ\text{C}$ .

The average particle size of both the formulations was found to be increased on storage, which can be attributed to aggregation of particles. This effect was least in the case of formulations stored at  $5 \pm 3^\circ\text{C}$ . This signifies the low temperature provide stability for nanoparticles, hence ideal storage temperature for NPs is  $5 \pm 3^\circ\text{C}$ . Both the formulation i.e. Chitosan NPs and Hyaluronic acid anchored chitosan NPs were stored at  $5 \pm 3^\circ\text{C}$ ,  $25 \pm 2^\circ\text{C}$  and  $40 \pm 2^\circ\text{C}$ .

#### *In vitro* cytotoxicity study

This may be attributed to the least leaching of drug from formulations stored at  $5 \pm 3^\circ\text{C}$  as compared to that stored at  $25 \pm 2^\circ\text{C}$  and  $40 \pm 2^\circ\text{C}$ .



**Figure 9.** *In Vitro* cytotoxicity of HACSNPs on MCF-7 cell line

The growth inhibition of NPs on MCF-7 cell was assessed by MTT assay. The findings evidently proposed a dose-dependent cytotoxicity that was decreased by cellular sustainability upon raising the concentration of MTX. The survival segment of cells upon incubation of plain MTX and NPs formulation in different concentration is described in (Fig.12). After incubation of MTX loaded hyaluronic acid (HA) anchored chitosan nanoparticles inhibitory effect were noticed on cell growth, the percentage control growth was found to be decreased with the increasing concentration of MTX. Moreover, the cell feasibility reduced when the concentration of MTX either inside the NPs or in free form was up surged. In the concentration range of 10–80 µg/ml, NPs were observed to be cytotoxic to a greater degree in comparison to plain MTX. Cytotoxic effect of loaded hyaluronic acid (HA) anchored chitosan nanoparticles in A549 was found to have a superior inhibitory effect. This may be pretended due to MCF-7 over expressed CD44 receptor (Sun et al., 2009; Arpicco et al., 2013). These outcomes verified that loaded hyaluronic acid (HA) anchored chitosan nanoparticles cytotoxicity caused by MTX to the tumor cells was dose dependent and had a better cytotoxic effect on the tumor cells than did plain MTX. This is approved due to remarkable dispersibility of hyaluronic acid (HA) in aqueous solution and also it provides "stealth" property to the nanoparticles.

#### Conclusion

The HACSNPs loaded with MTX were acquired lucratively by the nanoprecipitation technique with high MTX entrapment efficacy and low particle size. HEC NPs have sustained the release of MTX for a period of more than 96 hrs. The conclusions of cytotoxicity analysis revealed that the cytotoxicity of MTX loaded HACSNPs seems to be less cytotoxic to MCF-7. On the basis of these findings, the preparations formulated in this work could be favorable for *in vivo* MTX drug delivery systems.

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