Fabrication and biological evaluation of chitosan coated hyaluronic acid-docetaxel conjugate nanoparticles in CD44+ cancer cells

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Abstract

Background: Hyaluronic acid (HA) has been used for target-specific drug delivery because of strong affinity to CD44, a marker in which overexpressed in cancer cells and cancer stem cells. Conjugation of HA to the cytotoxic agents via active targeting can improve efficacy, biodistribution, and water solubility. To be able to benefit from passive targeting as well, a nanoparticulate system by counter ion using a polycation like chitosan may lead to a perfect delivery system.

Methods: Water soluble Hyaluronic acid-Docetaxel (HA-DTX) conjugate was prepared and used to formulate chitosan-coated HA-DTX nanoparticles by polyelectrolyte complex (PEC) method and optimized using Box-Behnken design. Biological evaluation of nanoparticles was done in CD44+ cancer cells.

Results and discussion: Biological evaluation of optimized formula showed IC50 of nanoparticles for 4 T1 and MCF-7 cell lines were 45.34 μM and 354.25 μM against 233.8 μM and 625.9 μM for DTX, respectively with increased cellular uptake showed by inverted confocal microscope.

Conclusion: Chitosan-coated HA-DTX nanoparticles were more effective against CD44+ cells than free DTX.

Keywords: Glyconanoparticles, Nanomedicine, Polyelectrolye Complex, Macromolecular Drug Delivery, Polysaccharides

Background

Most of the anticancer drug products have systemic toxicity because of the wide uncontrolled distribution in the body. Besides, their lack of tumor localization and short half-lives are considerable obstacle facing effective cancer chemotherapy. Development of nanoparticulate drug delivery systems and polymer-drug conjugates of low molecular weight cytotoxic drug molecules to macromolecular carriers are effective ways to address these problems by enhanced permeation and retention (EPR) effect [1, 2]. In addition, conjugation of cytotoxic drugs to hydrophilic macromolecules can increase the water solubility of insoluble drugs such as docetaxel (DTX) and will enhance their biodistribution and therapeutic efficacy [3, 4].

DTX is an anticancer agent belongs to the Taxanes family and is a semi-synthetic derivative from the Taxus brevifolia [5]. DTX shows its cytotoxic effect by inhibiting the depolymerization of microtubules and M-phase cell arrest [6]. The conventional formulations of DTX in drug market suffering from low solubility of the active pharmaceutical ingredient which has been resolved using tween 80 as surfactant. This issue led to some complications in the clinic to control formulation-related adverse drug reactions [7]. Due to the importance of this drug molecule in chemotherapy protocols, a lot of efforts dedicated to address novel formulations of DTX. In one of these approaches water-soluble macromolecular drug conjugates has been proposed to prepare tween-free
formulations along with targeted drug delivery such as hyaluronic acid-docetaxel (HA-DTX) conjugates. In our previous report [4], although prepared conjugate had better solubility profile but the efficacy did not benefit from its polysaccharide CD44-targeting properties and heparine like effects of hyaluronic acid in blood circulation. Therefore this report is an attempt to add passive targeting enhanced permeation and retention of nanoparticulate system while keeping hyaluronate moiety. For this purpose, the water soluble conjugate became coated with chitosan by polyelectrolyte complex (PEC) method to prepare a nanoparticulate drug delivery system to improve pharmacokinetic features and efficacy.

For this purpose, hyaluronic acid (HA) as one of the common polysaccharides carriers has been used for conjugation of low molecular weight cytotoxic drugs such as DTX. HA is biocompatible, biodegradable and non-immunogenic [8]. The most considerable advantage of HA is its strong affinity for CD44, a cell surface protein which is overexpressed in many cancer cells and cancer stem cells [9, 10]. CD44 is a specific biological receptor for HA [11, 12]. HA could have an enhanced attachment and uptake into malignant cells with metastatic activities [13] and has been used for target-specific drug delivery [14, 15]. We hypothesized using HA as carrier and targeting moiety simultaneously may reverse the multiple drug resistance of cancer stem cells via affecting CD44 and regarding physical correlation of P-gp and these markers [16]. In this regard, HA-DTX conjugates has been prepared and evaluated which showed suitable efficacy and safety profile. This research reports optimized polyelectrolyte complex nanoparticles using HA-DTX as cationic part.

Polyelectrolyte complexes which were prepared by electrostatic interaction between unlike charged polymers have received substantial attention in drug delivery systems. The synthesis of PEC nanoparticles is simple and can be easily carried out under mild conditions without using toxic organic solvents or chemical crosslinkers [17]. In the present study, chitosan as a N-deacetylated derivative of chitin has been used extensively as a biocompatible polysaccharide [18] with a cationic nature that can be protonated in weak acidic environment [19], and therefore it can improve the bioavailability of DTX [20].

Achieving optimization of the nanoparticle preparation could be performed by classical method of changing one variable at a time while others have been remained constant. However this method needs lots of series of experiments and time. Moreover in this approach the possible interaction between independent factors will not be observed. Therefore, the fine optimized formulation will not be achieved. Design-of-experiment (DoE) method has been used in pharmaceutical studies to solve this problem. Optimization by response surface methodology including Box-Behnken method is considered as the major application of DoE [21, 22].

In the present study, the water soluble conjugated HA-DTX was synthesized. Then, chitosan coated HA-DTX nanoparticles were prepared by PEC method considering the anionic structure of HA-DTX and cationic chitosan. Box-Behnken statistical design using a response surface methodology has been employed to obtain the optimized condition in terms of particle size, size distribution, drug loading and zeta potential. For determination of efficacy of optimized chitosan coated HA-DTX conjugate nanoparticles, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed on MCF-7, human cancer cell line, and 4 T1 mouse breast cancer cell line. MCF-7 and 4 T1 cell lines were also used for cell uptake study.

Materials and methods

Materials

Sodium hyaluronate (MW 25 kDa) was purchased from GuanLong (Shandong, China). Anhydrous DTX was purchased from Jiangsu Yew (Jiangsu, China). Chitosan (MW 50 kDa, (Primex, Karmoy, Norway), 1-Ethyl-3-[3-(dimethylamino)-propyl] carbodiimide (EDC), N-hydroxy succinimide (NHS), 4,6-diamidino-2-phenylindole (DAPI) and triethylamine were purchased from Sigma Aldrich (Seelze, Germany). MTT dye was from Merck (Darmstadt, Germany). Dulbecco’s Modified Eagle Medium (DMEM) with high glucose, RPMI 1640, FBS (Fetal Bovine Serum), trypsin, penicillin and streptomycin were purchased from Biosera (Vienna, Austria). Ultra-purified water was used throughout the analysis and all other chemicals were of analytical grade. 4 T1 and MCF-7 cell lines were obtained from National Cell Bank of Iran (Pasteur Institute of Iran, Tehran, Iran).

Methods

Synthesis of hyaluronic acid-docetaxel (HA-DTX) conjugates

At first for preparing desalted HA, HA (MW 25 kDa) (1 g) was dissolved in 100 mL of deionized water, then the solution was dialyzed (MWCO 12 kDa) in deionized water for 24 h and lyophilized [23]. Desalted-HA (400 mg) was dissolved in 80 mL of deionized water. Then EDC and NHS were added to the solution in 11 and 10 molar ratios of carboxyl groups of HA, respectively. The mixture was stirred at 40 °C for 3 h. Then 80 mL dimethylformamide (DMF) containing 400 mg of DTX and 20 mL of triethylamine were added. After 24 h the mixture was refluxed at 70 °C and after cooling to room temperature was transferred to pretreat...
dialysis tubing (MWCO 3500 kDa). The mixture was purified by dialysis against water-acetone solution (50:50, v/v) for 1 h, water-acetone (75:25, v/v) for 1 h and water for 1 h, respectively. The obtained HA-DTX conjugate is water soluble. To eliminate the remained DTX, the mixture was transferred to a separating funnel and extracted three times with dichloromethane. The aqueous phase was lyophilized. Chemical integrity of the resulted product was checked by ¹H-NMR (Bruker AC 500 Spectrophotometer, Germany) and fourier transform infra-red (FTIR) spectroscopy (Nicolet Magna-FTIR 550 Spectrometer, WI, USA). The concentration of DTX was measured by UV Spectrophotometry (UV-visible Spectrophotometer, 160A, SHIMADZU, Japan) at 229 nm versus a suitable blank solution containing the appropriate concentration of HA.

**Preparation of chitosan coated HA-DTX conjugate nanoparticle**

Chitosan coated HA-DTX conjugate nanoparticles were prepared by PEC method, considering the anionic structure of HA-DTX conjugates and cationic chitosan [24]. As a representative example, 1 mL of aqueous solution of HA-DTX (4.25 mg/mL) was added slowly to 1 mL of dilute chitosan solution (0.250 mg/mL of 1 % acetic acid) in 1 min while stirring at 370 rpm at room temperature. It should be mentioned that during the screening and optimization procedure, various factors including ratios of HA-DTX conjugate to chitosan, stirring rate and temperature were evaluated.

**DTX determination in the conjugates and nanoparticles**

The concentration of DTX in conjugate was measured by UV spectrophotometry. Determination of DTX in prepared nanoparticles was performed by UV absorbance at 229 nm to determine drug loading and entrapment efficiency. The final solution of chitosan coated HA-DTX nanoparticles were transferred to microtubes and centrifuged by an ultracentrifuge (Optima MAX-XP Ultracentrifuge, Beckman Coulter, USA) at 22000 rpm (150700 g) for 20 min at 10 °C. After collecting the supernatant, the free remaining non-conjugated DTX in the reaction medium was measured by UV spectrophotometry. The encapsulated efficiency and DTX loading in nanoparticles were calculated, applying the following equations:

\[
\text{Entrapment efficiency (\%) = } \frac{\text{Total amount of DTX} - \text{Amount of DTX in supernatant}}{\text{Total amount of DTX}} \times 100
\]

\[
\text{Drug loading (\%) = } \frac{\text{Weight of drug found loaded}}{\text{Weight of nanoparticle}} \times 100
\]

**Fourier transform infra-red spectroscopy of conjugates**

Freeze-dried conjugated HA-DTX, HA, DTX were analyzed by FTIR Spectrometer. The data was achieved in the range of 400–4000 cm⁻¹ for each sample. The FTIR spectra of conjugated HA-DTX were compared with pure substances.

**Experimental design studies**

Box-Behnken statistical design which is a response surface methodology has been employed in the present study. In this study the effect of three quantitative independent variables consisting of stirring rate (rpm), ratio of HA-DTX conjugate to chitosan and temperature (°C) were investigated on dependent variables and responses, including particle size (nm), zeta potential (mv), polydispersity index (PdI) and DTX loading in nanoparticles with Design Expert software (V. 7.0.0, Stat-Ease Inc., Minneapolis, USA). Dependent and independent variables were elucidated based on the preliminary studies which are shown in Table 1.

The aim of the design was to achieve to the optimum formulation, with both minimum size and maximum loading. The PdI factor should be at possible lowest level and zeta potential should be appropriate to have stable nanoparticles. Obtained responses from three optimized formulations were compared with the suggested experimental responses to evaluate the precision of model.

**Characterization of the nanoparticles**

The size and zeta potential of the nanoparticles were determined using a Zetasizer Nano ZS Analyzer (Malvern Instruments, UK) with a He-Ne laser beam at wavelength of 633 nm at 25 °C.

Surface morphology of the nanoparticles observed using Scanning Electron Microscopy (SEM) (Philips XL30, The Netherlands) and Atomic Force Microscopy (AFM) (dualscope® DS 95-200/50, Denmark) microscopy. For AFM evaluation, one drop of nanoparticle suspension was dried on the surface of clean silicon wafer at room temperature. AFM study was performed with 20 μm scanner in tapping mode. For SEM imaging, dried nanoparticles were gently coated by gold layer with a sputter coater and evaluated at 30 kV using a 6300 field emission scanning electron microscope.

Differential scanning calorimetry (DSC) was performed using Mettler-Toledo DSC822e (Greifensec, Switzerland) and data acquisition and analysis was carried out by a software package of STAR® 9.01. The

\[
\text{Entrapment efficiency (\%) = } \frac{\text{Total amount of DTX} - \text{Amount of DTX in supernatant}}{\text{Total amount of DTX}} \times 100
\]

\[
\text{Drug loading (\%) = } \frac{\text{Weight of drug found loaded}}{\text{Weight of nanoparticle}} \times 100
\]
system was conducted by using 8 mg of sample, deposited in 40 μL aluminum pans and hermetically sealed, under a nitrogen gas dynamic flow at a scanning heating rate of 10 °C/min over a range of 20 °C to 300 °C. Empty hermetically sealed aluminum pan was used as a control.

Cytotoxicity evaluation of nanoparticles

For cytotoxicity study of nanoparticles, MTT assay was performed on MCF-7, human breast cancer cell line, and 4 T1 mouse breast cancer cell line [25]. Cell culture medium was DMEM with 10 % FBS and 5 % penicillin-streptomycin. Cells maintained at 37 °C and humidified environment with 5 % CO₂. Cells were seeded into 96-well plate separately at a seeding density of 5000 cell/well. After 24 h incubation, various concentrations of free DTX and nanoparticles (0.1, 10, 100, 500, 1000, and 1500 μM) (based on DTX equivalent concentration) were used as treatments and incubated for 48 h. Then 50 μL MTT (1 mg/mL) solution in PBS was added to each well and incubated for 4 h. Formazan precipitates dissolved by 150 μM dimethyl sulfoxide (DMSO). The absorption was measured at 570 nm and reference well at 620 nm by ELISA reader [26]. Cell viability was calculated by the following equation where OD is optical density:

\[
\text{Cell viability} \% = \left( \frac{\text{OD}_{\text{test well}}}{\text{OD}_{\text{reference well}}} \right) \times 100
\]

Preparation of fluorescent-labeled HA

To prepare fluorescent-labeled HA conjugate, HA was labeled with fluorescamine. One hundred mg of HA was dissolved in 10 mL water and 61.5 mg of EDC and 45.5 mg of NHS were added. The mixture was stirred for 3 h and then 45.84 mg fluorescamine was added and stirred for 24 h. The reaction flask was protected from light by covering with an aluminum foil. After 24 h the mixture was purified by dialysis (MWCO 3500) against deionized water and finally the product was freeze-dried. Fluorescent-labeled nanoparticles were also prepared by fluorescent-labeled HA conjugate instead of HA-DTX conjugate and the nanoparticle preparation method was the same as the optimized method used for normal nanoparticles.

Cell uptake studies

In order to study the cellular uptake of the nanoparticles, 4 T1 and MCF-7 cell lines were seeded at 1 x 10⁵ cell/well in a cover glass and incubated at 37 °C with 5 % CO₂ atmosphere for 24 h. After complete adhesion, the medium was carefully removed and replaced with fresh medium containing fluorescent-labeled nanoparticles and incubated for 2 h. In this stage, the medium containing drug was removed and cells were washed 4 times with PBS and fixed with formaldehyde 4 % for 4 min. Nuclear coloring was performed with DAPI (0.5 mg/mL) in 5 min and then cells were washed 4 times with PBS using inverted confocal microscope (Nikon ECLIPSE Ti, Tokyo, Japan) cell images were taken [27].

Statistical analysis

SPSS 20.0 statistical software and one-way analysis of variance (ANOVA) were used to assess the data groups. All the results were evaluated as mean ± standard deviation (SD). Significance difference of \( p < 0.05 \) was accepted.

Results and discussion

Synthesis and characterization of water-soluble HA-DTX direct conjugate

Indirect HA-DTX conjugate was synthetized previously in our group. Conjugation of DTX in these studies needs preparation of succinyl DTX [4, 28]. The direct formation of conjugated HA-DTX has been reported for the first time with fewer procedure steps (Fig. 1). An esteric bound formed between 2´-OH of DTX and COOH of HA [29]. Formation of conjugated HA-DTX was confirmed by the presence of aromatic protons in ¹H-NMR spectra (Fig. 2). FTIR of freeze-dried HA-DTX conjugate, pure HA, pure DTX were also obtained (Fig. 3). DTX had a specific peak in 1242 cm⁻¹ which had no interaction with HA specific peak. This peak has been repeated in HA-DTX conjugate spectrum with a little shift to 1249 cm⁻¹. Presence of this peak and HA related peaks in conjugate spectrum confirms the formation of HA-DTX conjugate.

The absorption of DTX in conjugate was measured by UV spectroscopy. According to UV absorption, it was shown that 1 mg of HA-DTX conjugate contains 69 μg of DTX.

Preparation of chitosan coated HA-DTX conjugate nanoparticle

Publications reported several nanoparticular based methods to improve DTX drug delivery to cancer cells (DTX loaded chitosan nanoparticle [30], targeted DTX nanoparticles with different targeting agents like folic acid [2] and albumin nanoparticles of DTX [28]). HA
Fig. 1 Schematic representation of hyaluronic acid-docetaxel (HA-DTX) conjugate and chitosan coated HA-DTX nanoparticles preparation.

Fig. 2 Formation of hyaluronic acid-docetaxel (HA-DTX) conjugate was confirmed by the presence of aromatic protons in $^1$H-NMR spectra.
could cause heparin-induced thrombocytopenia because of its nature as a polysaccharide [31]. This event should be considered if the HA-DTX used alone. Coating the HA-DTX conjugate with chitosan may limit the exposure of HA with platelet in blood circulation and reduce thrombocytopenia. Chitosan-coated HA-DTX conjugate nanoparticles were prepared based on these findings.

Optimization studies
To obtain an optimized formulation of HA-DTX nanoparticles, Box-Behnken statistical design was used. Table 2 shows 17 runs based on Box-Behnken design to analyze the effects of independent variables on dependent variables and the data achieved to find out optimized formulation.

As a result, 17 runs were needed to achieve the optimized formulation and the second-order polynomial functions explained the relationship between the dependent and the independent variable as following equation:

\[
Y_{1, 2, 3} = b_0 + b_1A + b_2B + b_3C + b_{11}A^2 + b_{22}B^2 + b_{33}C^2 + b_{12}AB + b_{13}AC + b_{23}BC
\]
which A, B and C are independent variables, and Y is the predicted dependent factor, $b_0$ is the intercept, $b_1$, $b_2$, and $b_3$ are linear coefficients, $b_{11}$, $b_{22}$, and $b_{33}$ are squared coefficients, and $b_{12}$, $b_{13}$, and $b_{23}$ are the interaction coefficients of equation.

**Size of nanoparticles**

Size is the most important parameter in determining the nanoparticles cellular uptake. Nanoparticles were optimized to achieve minimum size while the PdI kept at minimum and loading maximum. As seen in Fig. 4a in the middle range of stirring rate, size of nanoparticles would be reduced. Higher stirring rate could generate bubble and solution splashing so it could not prepare suitable particles. It seems stirring rate at lower limit do not supply enough energy to product small particles, so enlargement has seen in this rate. Figure 4b represents that by increasing the ratio of HA-DTX to chitosan, the size of nanoparticles would be increased. Temperature had minor effect on the size of nanoparticles.

\[
\text{Particle size} = 191 + 64A - 27.63B - 3.87C + 48.62A^2 + 31.38B^2 - 19.13C^2
\]

**Polydispersity index**

Polydispersity index represents the homogeneity of nanoparticles and changes between of 0 to 1 with a desire to be near 0. The less PdI value indicates the more size uniformity in nanoparticle. As shown in Fig. 4c by increasing the temperature, PdI reduced and it would be at minimum of 50 °C. On the other hand, lower PdI is observed in the middle range of stirrer rate. Figure 4d represents that by increasing the ratio of HA-DTX to chitosan, PdI would be decreased.

\[
PdI = 0.055 - 0.041A - 0.00563B - 0.00613C + 0.022BC + 0.02A^2 + 0.013B^2 - 0.014C^2
\]

**Drug content**

Higher amount of drug content is desired and represented an acceptable formulation strategy. HA-DTX to chitosan ratio was the most important factor in drug content. By increasing the ratio of HA-DTX to chitosan, higher drug content would be obtained.

\[
\text{Drug content} = 3.16 + 0.099A + 0.00125C - 0.018A^2 - 0.00138C^2
\]

**Confirmation of designed optimized experiments**

After analyzing data and 3D diagrams by utilizing Box-Behnken method, an optimized formulation achieved which the independent variables were 18.5 for ratio of HA-DTX to chitosan, 723 rpm for stir rate and 50 °C for temperature. It also predicted the amount of

| Run | Stirring rate (rpm) | Ratio of HA-DTX conjugate to chitosan | Temperature (°C) | Particle size (nm) | Zeta potential (mV) | Polydispersity index (PdI) | DTX loading (%) |
|-----|---------------------|---------------------------------------|-----------------|-------------------|--------------------|--------------------------|-----------------|
| 1   | 300.00              | 13.00                                 | 25.00           | 195               | 21.7               | 0.128                    | 3.043           |
| 2   | 300.00              | 21.00                                 | 25.00           | 444               | 18.6               | 0.065                    | 3.257           |
| 3   | 1300.00             | 13.00                                 | 25.00           | 176               | 22.3               | 0.129                    | 3.034           |
| 4   | 1300.00             | 21.00                                 | 25.00           | 269               | 17.4               | 0.030                    | 3.235           |
| 5   | 800.00              | 13.00                                 | 0.00            | 174               | 22.3               | 0.120                    | 3.042           |
| 6   | 800.00              | 21.00                                 | 0.00            | 280               | 18.0               | 0.019                    | 3.235           |
| 7   | 800.00              | 13.00                                 | 50.00           | 182               | 21.9               | 0.086                    | 3.048           |
| 8   | 800.00              | 21.00                                 | 50.00           | 246               | 19.2               | 0.019                    | 3.233           |
| 9   | 300.00              | 17.00                                 | 0.00            | 210               | 21.3               | 0.083                    | 3.159           |
| 10  | 1300.00             | 17.00                                 | 0.00            | 199               | 19.6               | 0.033                    | 3.161           |
| 11  | 300.00              | 17.00                                 | 50.00           | 210               | 21.3               | 0.031                    | 3.157           |
| 12  | 1300.00             | 17.00                                 | 50.00           | 194               | 24.1               | 0.070                    | 3.160           |
| 13  | 800.00              | 17.00                                 | 25.00           | 195               | 21.3               | 0.061                    | 3.160           |
| 14  | 800.00              | 17.00                                 | 25.00           | 191               | 21.6               | 0.039                    | 3.160           |
| 15  | 800.00              | 17.00                                 | 25.00           | 179               | 22.7               | 0.064                    | 3.159           |
| 16  | 800.00              | 17.00                                 | 25.00           | 193               | 21.1               | 0.045                    | 3.159           |
| 17  | 800.00              | 17.00                                 | 25.00           | 197               | 21.3               | 0.067                    | 3.159           |
dependent factors would be 205 nm for size of nanoparticles, 0.02 for PdI and 3.19 % for DTX content and +21.5 mV for zeta potential. Analysis of variance (ANOVA) and lack of fit parameters for the responses according to quadratic model is provided in Table 3.

Three experiments were performed in the lab according to the optimized formulation and there was no significant difference between the obtained and predicted results. The mean amount obtained in these experiments was 234 nm for size of nanoparticles, 0.088 for PdI and 3.18 % for DTX content and +20.03 mV for zeta potential. The mean entrapment efficiency for these experiments was 62.78 %. The entrapment efficiency showed that nanoparticles could be an effective carrier for DTX.

**Characterization of nanoparticles**

**Particle size, distribution and zeta potential**

Particle size and size distribution of nanoparticles were measured by dynamic light scattering (DLS). The mean obtained size and PdI of the optimized chitosan coated HA-DTX conjugate nanoparticles were 234 nm and 0.088 respectively (Fig. 5a). Zeta potential of optimized nanoparticles was +20.03 mV which showed complete chitosan coating (Fig. 5b). This amount of zeta potential could provide the appropriate repulsive force to prevent

### Table 3

| Parameters        | Source       | Sum of squares | Degrees of freedom (df) | Mean squares | F value | P-value |
|-------------------|--------------|----------------|-------------------------|--------------|---------|---------|
| Particle Size     | Quadratic vs 2Fi | 15730.13       | 3                       | 5243.38      | 4.93    | 0.0378  |
| Zeta Potential    | Quadratic vs 2Fi | 8.71           | 3                       | 2.9          | 5.3     | 0.0321  |
| PdI               | Quadratic vs 2Fi | 3.11E-03       | 3                       | 1.04E-03     | 9.9     | 0.0065  |
| Drug Loading      | Quadratic vs 2Fi | 1.46E-03       | 3                       | 4.86E-04     | 9.99    | 0.0063  |
| Lack of Fit       | Quadratic     | 7239.25        | 3                       | 2413.08      | 48.26   | 0.0013  |
| Particle Size     | Quadratic     | 2.2            | 3                       | 0.73         | 1.79    | 0.2889  |
| Zeta Potential    | Quadratic     | 1.16E-04       | 3                       | 3.86E-05     | 0.25    | 0.8579  |
| PdI               | Quadratic     | 3.39E-04       | 3                       | 1.13E-04     | 376.94  | 0.0001  |
Fig. 5 Nanoparticles characterization: a particle size and distribution; b zeta potential; c SEM picture; d AFM micrographs.

Fig. 6 DSC thermogram of freeze-dried nanoparticles, hyaluronic acid-docetaxel (HA-DTX) conjugate, pure chitosan, pure hyaluronic acid (HA), pure docetaxel (DTX) and physical mixture of HA-DTX conjugate and chitosan.
nanoparticle aggregation and improve the stability of formulation.

**Differential scanning calorimetry**
Freeze-dried nanoparticles, conjugated HA-DTX, chitosan, HA, DTX and physical mixture of HA-DTX conjugate and chitosan was analyzed during predetermined increasing temperature rate to obtain DSC thermogram. The DSC thermograms of DTX exhibit an endothermic peak showed melting around 170 °C. HA exhibited one exothermic peak presenting crystallization around 230 °C. Characteristic peaks of DTX and HA were not exist in the thermograms of conjugated HA-DTX. These findings confirm the development of HA-DTX conjugate (Fig. 6) [32].

DSC thermogram specificities of HA has an influence on DSC thermogram of physical mixture of HA-DTX conjugate and chitosan. Because of the ionic charge of the chitosan (cationic) and HA (anionic), these two compounds can form a charge-transfer bond (the same as PEC formation) which may attenuate the difference in comparing these physical mixture and polyelectrolyte complex. So, DSC thermogram of nanoparticles may not express this influence. These results revealed that chitosan coated nanoparticles protected conjugated HA-DTX and weakened the effect of temperature, which showed the formation of chitosan coated HA-DTX nanoparticles.

**Nanoparticle morphology**
Chitosan coated HA-DTX nanoparticles were morphologically studied by SEM and AFM. SEM showed (Fig. 5c) that nanoparticles are uniform spheres and

![Fig. 7](image-url)

**Fig. 7** In vitro cell studies: a MTT assay of DTX and nanoparticles on 4T1 cells (blue bar for nanoparticle and red bar for DTX); b MTT assay of DTX and nanoparticles on MCF-7 cells (blue bar for nanoparticle and red bar for DTX); c uptake image of 4T1 cell line (nuclear coloring with DAPI, FITC labeled chitosan coated HA-DTX conjugate nanoparticles, merge image of DAPI and FITC labeled chitosan coated HA-DTX conjugate nanoparticles and control cell) and uptake image of MCF7 cell line (nuclear coloring with DAPI, FITC labeled chitosan coated HA-DTX conjugate nanoparticles, merge image of DAPI and FITC labeled chitosan coated HA-DTX conjugate nanoparticles and control cell); *p < 0.05; **p < 0.01; ***p < 0.001
non-aggregated. AFM micrographs (Fig. 5d) confirmed the spherical shape of nanoparticles too.

In vitro cytotoxicity
The cytotoxicity of chitosan coated HA-DTX nanoparticles and DTX were assessed by MTT assay on 4 T1 and MCF-7 cell lines. By increasing the amount of nanoparticles or free DTX the cytotoxicity increased. The calculated IC50 of chitosan coated HA-DTX conjugate nanoparticles for 4 T1 and MCF-7 cell lines were 45.34 µM and 354.25 µM respectively while 233.8 µM and 625.9 µM for DTX on 4 T1 and MCF-7 cell lines after 48 h incubation. DTX loaded chitosan nanoparticles were more effective against cancer cells than free DTX drug [30]. Being agree to this result in our study cell viability (%) and IC50 of optimized nanoparticles were less than free drug. It may be because of higher DTX concentration which was available in intracellular space. Small size of nanoparticle as a passive targeting, effect of HA and the adhesive effect of chitosan coat may cause this availability. Therefore, nanoparticles were more potent than free DTX in cytotoxic effect on 4 T1 and MCF-7 cells (Figures 7a, b).

Cell uptake studies
Entrance of nanoparticles in cancer cells had a direct relation with their observed cytotoxic effect. Free DTX molecules could be transported out by P-glycoprotein (P-gp) pumps, but drug loaded nanoparticles were taken up by cells through an endocytosis pathway. The result represented higher cellular uptake of nanoparticles because of their ability to escape from the effect of P-gp pumps [33]. The uptake of optimized FITC-labeled chitosan-coated HA-DTX conjugate nanoparticles by 4 T1 and MCF-7 after 24 h incubation is shown in Fig. 7c. No treatment cells of each cell line are presented in Fig. 7c as a control. Based on fluorescence intensity, FITC-labeled chitosan-coated HA-DTX nanoparticles showed appropriate entrance into 4 T1 and MCF-7 cells. As a result it can be proposed that developed nanoparticles could bring loaded drug molecules effectively to cell cytoplasm as a novel drug delivery system.

Conclusion
Water soluble HA-DTX conjugate was prepared according to HA strong affinity for CD44, a cell surface protein which is overexpressed in many cancer cells and cancer stem cells. Chitosan-coated HA-DTX nanoparticles by polyelectrolyte complex method improved DTX availability. The fine optimized formulation was achieved with proper particle size, PdI, zeta potential and drug loading. The biological evaluation of nanoparticles showed they were more potent than free DTX in cytotoxic effect on MCF-7 and 4 T1 cells beside of their appropriate entrance in to cells. These findings need further evaluation to take into account potential improved pharmacokinetic of nanoparticulate drug delivery system.

Authors’ contributions
NSR carried out all experiments and drafted the manuscript. NG supervised and finalized the paper. FA participated in nanoparticles fabrication. MA conducted structure elucidation. EF revised the manuscript and helped in analysis. MRK lead statistical analysis and DOE. ZHM helped in cellular studies. FA supervised all pharmaceutical and cellular studies. RD supervised all experiments and approved the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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References
1. Goodarzi N, Varshochian R, Kamalinia G, Atiyabi F, Dinavand R. A review of polysaccharide cytotoxic drug conjugates for cancer therapy. Carbohydr Polym. 2013;92:1280–93.
2. Tavassolian F, Kamalinia G, Rouhani H, Armin M, Ostad SN, Khoshayand MR, Atiyabi F, Tehrani MR, Dinavand R. Targeted poly (I-y-glutamyl glutamine) nanoparticles of docetaxel against folate over-expressed breast cancer cells. Int J Pharm. 2014;467:123–38.
3. Dosio F, Stella B, Arpicco S, Cattel L. Macromolecules as taxane delivery systems. Expert Opin Drug Deliv. 2011;8:33–55.
4. Goodarzi N, Ghahremani MH, Armin M, Atiyabi F, Ostad SN, Shabani Ravari N, Nategian N, Dinavand R. CD44-targeted docetaxel conjugate for cancer cells and cancer stem-like cells: A novel hyaluronic acid-based drug delivery system. Chem Bio Drug Des. 2014;83:741–52.
5. Mugabe C, Liggins RT, Guan D, Mansi J, Chafeeva I, Brooks DE, Heller M, Jackson JK, Bun HM. Development and in vitro characterization of paclitaxel and docetaxel loaded into hydrophobically derivatized hyperbranched polyglycerols. Int J Pharm. 2011;404:238–49.
6. Akhlaghi SP, Saremii S, Ostad SN, Dinavand R, Atiyabi F. Discriminated effects of thiolated chitosan-coated pMMA paclitaxel-loaded nanoparticles on different normal and cancer cell lines. Nanomedicine. 2010;6:689–97.
7. Cho HJ, Moon HY, Koo H, Ko SH, Shim JS, Lee JH, Kim K, Chan Kwan I, Kim DD. Self-assembled nanoparticles based on hyaluronic acid-ceramide (HA-CE) and Pluronic * for tumor-targeted delivery of docetaxel. Biomaterials. 2011;32:7181–90.
8. Menzel EJ, Farr C. Hyaluronidase and its substrate hyaluronan: Biochemistry, biological activities and therapeutic uses. Cancer Lett. 1998;131:3–11.
9. Dalerba P, Cho RW, Clarke MF. Cancer stem cells: models and concepts. Annu Rev Med. 2007;58:267–84.
10. Aruffo A, Stamenkovic I, Melnick M, Underhill CB, Seed B. CD44 is the principal cell surface receptor for hyaluronate. Cell. 1990;61:1303–13.
11. Auzenne E, Ghosh SC, Khodadadian M, Rivera B, Farquhar D, Price RE, Ravooi M, Kundra V, Freedman RS, Kloosterjaard G. Hyaluronic acid-paclitaxel antitumor efficacy against CD44 (+) human ovarian carcinoma xenografts. Neoplasia. 2007;9:479–86.
12. Stamenkovic I, Aruffo A, Aumont M, Seed B. The hematopoietic and epithelial forms of CD44 are distinct polypeptides with different adhesion potentials for hyaluronate-bearing cells. EMBO J. 1991;10:343.
14. Kurisawa M, Chung JE, Yang YY, Gao SJ, Uyama H. Injectable biodegradable hydrogels composed of hyaluronic acid–tyramine conjugates for drug delivery and tissue engineering. Chem Commun (Camb). 2005;14:4312–4.

15. Lee H, Mok H, Lee S, Oh Y-K, Park TG. Target-specific intracellular delivery of siRNA using degradable hyaluronic acid nanogels. J Control Release. 2007;119:245–52.

16. Misra S, Ghatak S, Toole BP. Regulation of MDR1 expression and drug resistance by a positive feedback loop involving hyaluronan, phosphoinositide 3-kinase, and ErbB2. J Biol Chem. 2005;280:20310–5.

17. Polexe RC, Delair T. Elaboration of stable and antibody functionalized positively charged colloids by polyelectrolyte complexation between chitosan and hyaluronic acid. Molecules. 2013;18:8563–78.

18. Park JH, Kwon S, Lee M, Chung H, Kim J-H, Kim Y-S, Park R-W, Kim I-S, Seo SB, Kwon IC. Self-assembled nanoparticles based on glycol chitosan bearing hydrophobic moieties as carriers for doxorubicin: in vivo biodistribution and anti-tumor activity. Biomaterials. 2006;27:119–26.

19. Kim SJ, Yoon SG, Lee KB, Park YD, Kim SI. Electrical sensitive behavior of a polyelectrolyte complex composed of chitosan/hyaluronic acid. Solid State Ion. 2003;164:199–204.

20. Saremi S, Dinarvand R, Atyabi F. Enhanced oral delivery of docetaxel using thiolated chitosan nanoparticles: preparation, in vitro and in vivo studies. Biomed Res Int. 2013;2013:150478.

21. Jafari Malek S, Khoshchehreh R, Goodarzi N, Khoshayand MR, Amini M, Atyabi F, Esfandyari-maneshi M, Tehrani S, Mohammad Jafari R, Maghazei MS, et al. Cs-Dichlorodiamminoplatinum (II) glyconanoparticles by drug-induced ionic gelation technique targeted to prostate cancer: Preparation, optimization and in vitro characterization. Colloids Surf B Biointerfaces. 2014;122:350–8.

22. Azadi A, Hamidi M, Khoshayand M-R, Amini M, Rouini M-R. Preparation and optimization of surface-treated methotrexate-loaded nanogels intended for brain delivery. Carbohydr Polym. 2012;90:462–71.

23. Lee H, Lee K, Park TG. Hyaluronic acid–paclitaxel conjugate micelles: Synthesis, characterization, and antitumor activity. Bioconjug Chem. 2008;19:1319–25.

24. Denuriere A, Ferrier D, Damour O, Domard A. Chitosan–chondroitin sulfate and chitosan–hyaluronate polyelectrolyte complexes: biological properties. Biomaterials. 1998;19:1275–85.

25. Luo Y, Prestwich GD. Synthesis and selective cytotoxicity of a hyaluronic acid-antitumor bioconjugate. Bioconjug Chem. 1999;10:755–63.

26. Manoochehri S, Dinarvand R, Khorramizadeh MR, Atyabi F, Akhlaghi SP, Ostad SN, Atyabi M, Dinarvand R. Biotin/folate-decorated human serum albumin nanoparticles of docetaxel: comparison of chemically conjugated nanostructures and physically loaded nanoparticles for targeting of breast cancer. Chem Biol Drug Des. 2015;87(1):69–82.

27. Li C, Yu D-F, Newman RA, Cabral F, Stephens LC, Hunter N, Milas L, Wallace S. Complete regression of well-established tumors using a novel water-soluble poly (L-glutamic acid)-paclitaxel conjugate. Cancer Res. 1998;58:2404–9.

28. Jain A, Thakur K, Kush P, Jain UK. Docetaxel loaded chitosan nanoparticles: Formulation, characterization and cytotoxicity studies. Int J Biol Macromol. 2014;69:546–53.

29. Gandhi NS, Mancera RL. The structure of glycosaminoglycans and their interactions with proteins. Chem Biol Drug Des. 2008;72:455–82.

30. Panyam J, Labhasetwar V. Dynamics of endocytosis and exocytosis of poly (D, L-lactide-co-glycolide) nanoparticles in vascular smooth muscle cells. Pharm Res. 2003;20:212–20.