ORIGINAL ARTICLE

Spatial Drug Delivery through Hyaluronic Acid-Paclitaxel Conjugate-loaded Nanoparticles for the Effective Treatment of Solid Tumors

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Abstract

Background: Cancer has become a serious threat to the life of human beings globally. Various strategies are available for the treatment of cancer; however, they are not so effective due to various serious side effects, non-specificity to cancer cells targeting, and noxious effect to healthy cells. **Aim:** To resolve the aforementioned facts related to cancer treatment, we try to exploit inherent characters of cancer cells in the present project, i.e., overexpression of CD44 receptors for the treatment of cancer. Hyaluronic acid (HA) has a high affinity toward the CD44 receptor. Hence, HA was used as a targeting ligand for the delivery of an anticancer drug, paclitaxel (Ptx) through nanoparticles (NPs). **Methods:** The HA-Ptx conjugate was prepared using carbodiimide chemistry and characterized by infrared spectroscopy. The HA-Ptx-loaded NPs (CNPs) were prepared by a modified nanoprecipitation method and optimized using quality by design (QbD), and the formulation was characterized for several parameters. **Results:** The synthesis of the conjugate was confirmed by infrared spectra, and conjugate-loaded formulation was selected on the basis of particle size and entrapment efficiency (EE). The optimized formulation was smooth, spherical in shape and size which was found to be in the nanometer range. The release from the formulation followed the Higuchi model which shows that drug release from the polymeric matrix based on the diffusion process which is directly proportional to the square root of time. The cytotoxicity study was confirmed the lowest IC_{50} (half maximal inhibitory concentration) value for the CNPs. **Conclusion:** The cytotoxicity studies support the targeted drug delivery to tumor cells using the HA molecule as the targeting moiety with the drug. Therefore, CNPs could be considered as a hopeful carrier system for targeted drug delivery to solid tumors.

Key words: Box–Behnken design, design of experiment, hyaluronic acid, kinetic models, paclitaxel, polymeric nanoparticles

INTRODUCTION

Hyaluronic acid (HA) is a linear glycosaminoglycan polymer made up of disaccharide units of glucuronic acid and N-acetylglucosamine in the repeating manner.[1] It is biocompatible and biodegradable in nature, thus acting as an excellent biomaterial for the development of a novel drug delivery system. It has been successfully used as the vehicle for the delivery of anticancer agent. It is reported that CD44 receptors are overexpressed on the various types of cancerous cells such as lung, breast, and skin cancer cells and associated with the tumor progression and metastasis.[2,3] Exogenous HA primarily interacts with these overexpressed CD44 receptors. Thus, the HA has emerged as the targeting moiety when conjugated with drug molecules or carrier system for the effective treatment of various types of cancers.[4]

Paclitaxel (Ptx) is a natural diterpene pseudoalkaloid used as the first-line chemotherapeutic agent for lung, breast, and ovarian cancer. The Ptx is accountable for cell death by disrupting the normal microtubule dynamics required for cell division and also reported to show antiangiogenic property, thus making the

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environment unfavorable for cell growth and proliferation.[5,6] Poly (lactic-co-glycolic) acid (PLGA) was used as a choice of polymer for the preparation of nanoparticles (NPs) and is approved by the US Food and Drug Administration due to low toxicity and biocompatible properties.[7]

The quality and characteristics of NPs depend on the several formulation and process parameters used in different methods of preparation. In the present work, HA-Ptx-loaded NPs were prepared by the modified nanoprecipitation method. Polymer/conjugate ratio, stirring speed, stirring time, the evaporation rate of the solvent, type of polymers, type of organic solvents, rate of addition of organic phase to the aqueous phase, sonication time, and the rate of sonication were found to be main parameters which affect the NP preparation by this method. Therefore, the optimization of these parameters needs to be tuned for the development of effective and high-quality product with the help of suitable optimization techniques. Conventional optimization techniques can determine the effect of only one factor at a time while keeping the other factors constant, so these techniques cannot be much supportive in the prediction of the simultaneous effects of more than one factors on the quality of the product. These conventional techniques required more number of formulations for determining the effect of formulation and process parameters, thus making these techniques very tedious and tiresome. For that reason, there is a need to develop an effective optimization technique for evaluating the simultaneous effects of all the parameters on the quality of the formulation.[8] Quality by design (QbD) method have replaced the conventional techniques, providing a more systematic approach of optimization and vastly utilized method for the determination of the simultaneous effects of various formulation and process parameters. QbD performs the optimization in a systematic and efficient manner, which reduces consumption of time in designing and cost of the formulation. Thus, these QbD-based techniques are widely used in the development and optimization of drug delivery systems in the current scenario.[9] QbD may also be termed as the design of experiment (DoE) which includes various quality-related parameters such as quality target product profile (QTPP), critical quality attributes, critical process parameters (CPPs), risk assessment of factors, and elements associated with the QbD.[8]

The various types of experimental designs are available for the optimization work. Box–Behnken design (BBD) is one of the best methods for response surface methodology for the optimization of formulation because it reduces the time and cost of the formulation during the optimization process. BBD is a specially made design which requires only three levels for each factor, i.e., −1, 0, and +1.[10]

In the current work, the HA was conjugated with the Ptx by a three-step chemistry approach. The prepared conjugate (HA-Ptx) was characterized using Fourier-transform infrared (FTIR) spectroscopy. The conjugate-loaded NPs (CNPs) were prepared and optimized on the basis of EE and particle size using BBD. The optimized CNP formulation was smooth and spherical in shape when characterized with transmission electron microscopy (TEM) and scanning electron microscopy (SEM). The proposed system will offer several advantages such as high drug loading efficiency, reduced dose, lesser side effects, desirable distribution, and increased bioavailability.[11-13] The cytotoxicity study of the optimized formulation (CNPs) was performed and compared with the drug-loaded NPs (PNPs) and pure drug (Ptx) on MCF-7 cell line. The cytotoxicity result showed that the CNPs exhibit the lowest IC_{50} value in comparison to the other formulations.

MATERIALS AND METHODS

Materials

HA was purchased from Spectrochem Pvt., Ltd., India. Ptx was obtained as a gift sample from Alchem International Pvt. Ltd., Haryana, India. PLGA (Resomer® RG-503H), N-hydroxysuccinimide (NHS), and diphenyl phosphoryl chloride (DPP) were purchased from Sigma-Aldrich, Germany. Polyvinyl acetate and dichloromethane (DCM) were obtained from Thermo Fischer Scientific Pvt. Ltd., India. Adipic dihydrazide (ADH), acetone, methanol, and other solvents were purchased from Merck Specialities Pvt. Ltd., India. All the chemicals and solvents were of analytical grade. Milli-Q water was used throughout the experimental process.

Method

Conjugation of HA with Ptx

The conjugation of HA with Ptx was performed in a three-step process. In the first step, the ester form of drug (Ptx-NHS ester) was synthesized using succinic anhydride and 2-hydroxysuccinimide diphenyl phosphate (SDPP). In the second step, activation of HA was completed with the formation of adipic-dihydrazide functionalized HA (HA-ADH). In the third and final steps, both activated moieties were reacted together to produce HA-Ptx conjugate.[14,15]

Synthesis of Taxol–NHS ester (TSE)

The ester moiety was prepared with the formation of taxol-2-hemisuccinate (THS) and SDPP.

Preparation of THS

0.63 mmol of Ptx and 0.76 mmol of succinic anhydride were dissolved in 25 ml of DCM at room temperature (RT). 6.3 mmol of dry pyridine was added in the solution, and the mixture was kept for 3 days under continuous stirring at RT. The mixture was concentrated under rotary evaporator, and the concentrate was dissolved in 5-ml DCM. The product was purified using column chromatography with ethyl acetate:hexane (1:1) as the mobile phase. The percent yield was found to be about 90% and THS was obtained as the white crystalline solid. The schematic representation of the reaction is shown in Figure 1.

Preparation of SDPP

DPP was reacted with N-hydroxysuccinimide (NHS) in triethylamine and DCM, to form SDPP [Figure 2]. The product was filtered and purified using column chromatography. The product was triturated with ether, dissolved in ethyl acetate, washed with H_2O, and dried over $MgSO_4$. The product was concentrated which gave SDPP in the pure form.

Preparation of Taxol–NHS ester

Both the prepared moieties were reacted together to get activated drug ester moiety. First, 300 mg of THS and 164 mg of SDPP were added into 15-ml acetonitrile to make a solution of the mixture. 175 ml of triethylamine was added to the above solution, and the reaction was performed at RT under continuous stirring for about 6 h [Figure 3]. The completion of the reaction was checked with the help of thin-layer chromatography. After the completion of the reaction, the product was concentrated under vacuum at rotary evaporator. The concentrated residue was purified with silica gel column chromatography using ethyl acetate and hexane (1:2) as the mobile phase. About 270 mg of TSE was collected by drying under vacuum at RT for 24 h.

Synthesis of HA-ADH

HA is a water-soluble polymer. First, HA solution (3 mg/ml or 0.25 mmol) was prepared by dissolving 100 mg HA in about 33.3 ml of purified water. About 30 times, molar amount of ADH was added into the prepared HA solution. 0.1 M HCl and 0.1 M NaOH solution were used to adjust the pH of the reaction mixture about 6.8. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was weighed exactly to 192 mg and added in the solution of 1- hydroxybenzotriazole in 20 ml dimethylsulfoxide (DMSO):water (1:1) mixture.

Then, the EDC solution was mixed with the HA-ADH solution at RT. The pH of the solution was adjusted to 6.8 with 0.1 M NaOH, and the reaction was continued overnight under stirring as shown in Figure 4. The confirmation of reaction processing was confirmed by spotting TLC plates. After the completion of reaction, the mixture was dialyzed against different solvent systems, i.e., 100 mM NaCl then 25% ethanol/water mixture and finally purified water. The dialyzed solution was filtered through the 0.2-mm cellulose membrane and then lyophilized.

Synthesis of HA-Ptx

The final step takes place with the reaction between both the prepared products from step 1 and step 2. First of all, HA-ADH solution (1 mg/ml) was prepared in the 0.1 M sodium bicarbonate ($NaHCO_3$, pH 6.8) buffer. Then, the TSE solution was prepared in 2:1 mixture of dimethylformamide

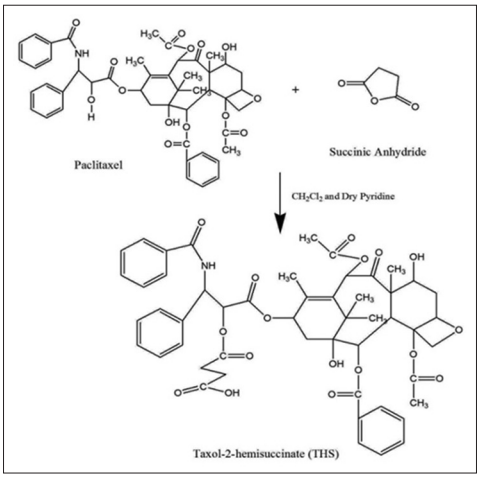

Figure 1: Preparation of Taxol-2-hemisuccinate

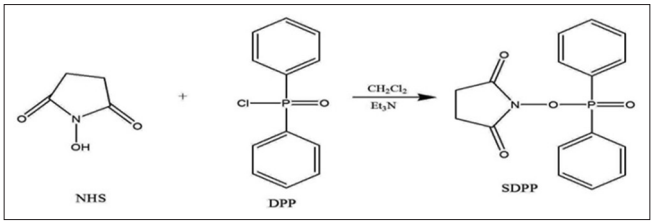

Figure 2: Preparation of 2-hydroxysuccinimide diphenyl phosphate

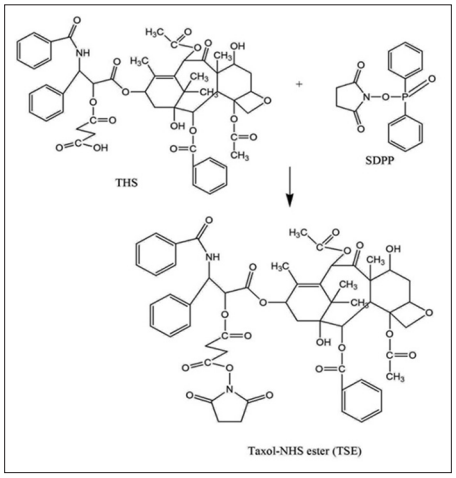

Figure 3: Preparation of Taxol–NHS ester

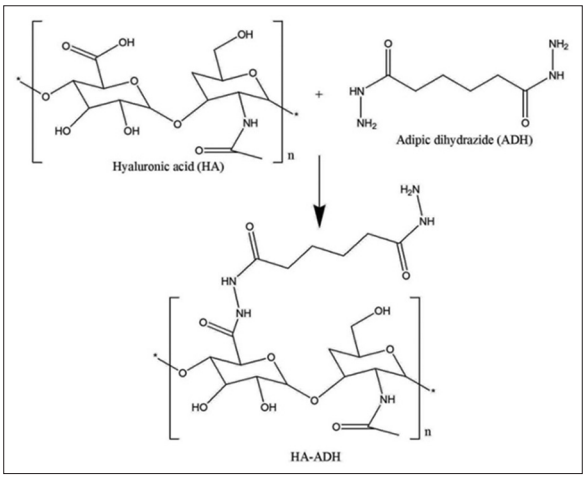

Figure 4: Synthesis of adipic dihydrazide functionalized hyaluronic acid

and water. The TSE solution was added to the HA-ADH solution. The reaction was performed at RT for 24 h under continuous stirring as shown in Figure 5. After the completion of the reaction, the residue was collected under vacuum in the dried form. The product was purified using column chromatography, and the confirmation of the conjugate structure was performed using IR spectroscopy.

Preparation of NPs

The HA-Ptx-loaded NPs were prepared by the modified nanoprecipitation method.[16,17] In this method, three process variables were selected as the CPPs for NPs formulation, namely polymer-to-conjugate (w/w) ratio (5/1, 10/1, and 15/1), surfactant concentration (0.5%, 1.0%, and 1.5%), and stirring speed (500, 1000, and 1500 rpm), and their responses particle size and EE are recorded in Table 1. Briefly, the polymer and conjugate were dissolved in an organic phase, while surfactant, in different concentrations, was dissolved in an aqueous phase consisting of pH 7.4 phosphate buffer saline (PBS). Then, the organic phase was added dropwise to aqueous phase with a syringe under constant stirring (REMI, Mumbai, India) at room temperature. Organic solvent was removed by continuous stirring overnight on a magnetic stirrer at room temperature. The formulations were sonicated under probe sonicator (Soniweld, Imeco Ultrasonics, Mumbai, India) for 5–10 min at the cold temperature. NPs were then recovered from the nanodispersion by centrifugation (Spinwin, India) at 10,000 rpm for 30 min and washed 2–3 times with distilled water. Finally, dispersion was lyophilized (Heto Dry Winner, Denmark) for 24 h to yield freeze-dried NPs. The particles were characterized for the surface morphology, size, and EE.

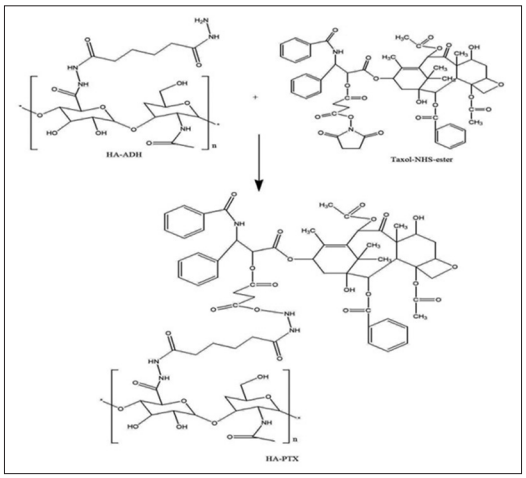

Figure 5: Synthesis of hyaluronic acid -paclitaxel

The QTPP for the optimized formulation was selected as the lowest particle size and highest EE because low particles offer the advantage of large surface area which helps in better penetration to the site of action, i.e., tumor cells. Higher EE shows the higher drug loading and reduces the loss of the drug during the formulation process.

Optimization of NPs using Box–Behnken experimental design

DoE method was used for the optimization of NPs preparation, and the BBD design is a suitable approach for determining the quadratic response surfaces with fewer runs under DoE method. The independent variables and levels were selected by preliminary studies. The polymer/conjugate ratio (A), surfactant concentration (%) (B), and stirring speed (rpm) (C) were selected as the independent variables, while particle size (nm) (Y1) and EE (%) (Y2) were selected as the dependent variables. All other parameters (solvent addition rate, stirring time, sonication amplitude, temperature, etc.) were kept constant to minimize fluctuations. The design consisted of total 17 runs in which five replicated center points were chosen to provide better results by estimating the experimental errors. The factors were varied on three levels according to the experiments as represented in Table 1. The interactive terms and polynomial equation produced by the experimental design are as follows:

$$Y = b_0 + b_1A + b_2B + b_3C + b_{12}AB + b_{13}AC + b_{23}BC + b_{11}A^2 + b_{22}B^2 + b_{33}C^2$$

Where Y is the dependent variable (response), b_0 is the intercept, and b_i (b_1, b_2, b_3, b_{12}, b_{13}, b_{23}, b_{11}, b_{22}, and b_{33}) are the estimated coefficients for the independent factors and interactions thereof. These interactive terms are used for determining the response value while simultaneous changes in the independent variables. Statistical analysis and response surface plot were performed using Design Expert© 10 software (Trial version; Stat-Ease, Inc., USA).

Table 1: Independent and dependent variables with their levels

Independent variables	Low (–1)	Medium (0)	High (+1)
Polymer/conjugate (ratio)	5	10	15
Surfactant concentration (%)	0.5	1.0	1.5
Stirring speed (rpm)	500	1000	1500
Dependent variables	Constraint		
Particle size (nm)	Minimum		
EE (%)	Maximum		

EE: Entrapment efficiency

Statistical calculation and data interpretation

The experiments were performed in triplicate (n= 3) so as to present the results as the mean value ± standard deviation (SD). Quadratic model was selected on the basis of statistical data, such as r-square, p-value, and lack of fit. Analysis of variance (ANOVA) analysis was used for evaluating the significant effect of independent variables on the responses.

Characterization of NPs

Determination of particle size of NPs

Particle size of HA-Ptx-loaded NPs was determined using Malvern Zetasizer (Malvern Instruments, UK) at room temperature in triplicate. The values of particle size were measured as mean diameter (nm) ± standard deviation.

Determination of percentage EE

The % EE is determined by centrifugation method[18,19] in which the supernatant was collected for the estimation of conjugate in the NPs using the following equation:

$$\text{\% Entrapment Efficiency (\%EE)} = \frac{\text{Total conjugate added- Conjugate in supernatant}}{\text{Total conjugate added}} \times 100$$

The NPs dispersion was centrifuged for 30 min at 10,000 rpm using centrifuge (Spinwin, India). Supernatant was collected and diluted with PBS pH 7.4. The conjugate in the supernatant was determined using UV-spectroscopy at 227 nm, and % EE was determined by the given equation.

Surface morphology of NPs

The shape and surface morphology of NPs were determined using TEM (Technai, FEI, USA) and SEM (Nova NanoSEM 450, FEI, USA).

The sample for TEM study was prepared by taking a drop of NPs dispersion onto a carbon- coated copper grid and remove out the excess of sample by soaking it with filter paper. Then, the grid was allowed to air dry thoroughly, and 1% phosphotungstic acid was added on the grid as the negative staining of the sample. Finally, the samples were evaluated using TEM.

Sample for the SEM was prepared using the sample holder with proper cleaning before preparation of the sample. Subsequently, a drop of the dispersed formulation was placed onto the sample holder. It fixes the sample on the holder by making it completely dry. The sample holder should be completely dry because sample chamber of the microscope operates under vacuum and is very sensitive to moisture present in the sample, which may cause the destruction of the sample. Then, the sample was placed in the microscope chamber for the microscopic evaluation of the NPs.

In vitro release studies

In vitro release study was performed for CNPs using the dialysis bag method with dialysis membrane (HiMedia, Mumbai) of molecular weight cutoff between 12,000 and 14,000 Da. The release rate from NPs depends on several factors, namely polymer degradation or erosion, molecule diffusion from the matrix, desorption of adsorbed conjugate, and solubility of the conjugate. In the method, the NP dispersion was filled in the dialysis tube and immersed in PBS (pH 7.4) under continuous magnetic stirring. The sampling was taken at the preset internal of time, i.e., 1, 2, 4, 6…. and up to 24 h.[18] The samples were filtered and diluted to determine the conjugate concentration using UV spectrometer. The kinetic studies were used to estimate the release characteristics of the formulation. The study included five models, namley zero order, first order, Higuchi's equation, Korsmeyer–Peppas model, and Hixson–Crowell cube root law. Among all the models, the best model was selected on the basis of regression coefficient (R^2) value, i.e., near to 1. The model describes the release mechanism of the conjugate from the formulation.

In vitro cytotoxicity study

The optimized formulations were subjected to cytotoxicity study using sulforhodamine blue (SRB) assay on the MCF-7 cell line at the ACTREC, Mumbai. The cell lines were cultured in RPMI-1640 medium containing 10% fetal bovine serum and 2 mM L-glutamine. For the present screening experiment, cells were inoculated into 96-well microtiter plates in 100 µL at plating densities. After cell inoculation, the microtiter plates were incubated at 37°C, 5% CO2, 95% air, and 100% relative humidity for 24 h before addition of experimental samples.

Samples were dissolved in DMSO to make 100 mg/ml and diluted to 1 mg/ml using water and finally added into the microtiter wells within the four concentration ranges, i.e., 10, 20, 40, and 80 µg/ml. After 48 h of the experiment, the assay was completed with the addition of 30% cold trichloroacetic acid and incubated at 4°C for 1 h. The supernatant was removed, and plates were washed with water 5 times and air dried. 0.4% (w/v) SRB solution was prepared with 1% acetic acid, and the solution was added into each well plate and incubated at room temperature for about 20 min. The plates were washed 5 times with 1% acetic acid, to recover unbound dye, and air dried. 10 mM Trizma base was added to elute out subsequent bound dye, and the absorbance was determined at 540 nm with 690 nm standard wavelength. Cytotoxicity was estimated with percentage (%) growth of cells in each well plate. The % growth was determined using the formula given below:

$$\% \text{ Growth} = \frac{\text{Average absorbance of test well plate}}{\text{Average absorbance of control well plate}} \text{ X } 100$$

RESULTS AND DISCUSSION

Characterization of HA-Ptx conjugate

The conjugate was characterized using FTIR spectroscopy. Figure 6a-c shows the IR spectra of Ptx, HA, and HA-Ptx conjugate, respectively. The characteristic peaks of the conjugate were present in the spectra [Figure 6c] which confirms the amide bond formation between the HA and Ptx. These peaks are present at 3509, 3445, 1730, and 1648 cm^{-1} which indicate that the N-H, C=O and ester bond stretching, respectively. The other peaks in the spectra represent the structure of HA and Ptx.

Optimization of NPs using BBD method

Modified nanoprecipitation method was used to prepare CNPs, but various factors affect the formulation characteristics and properties of the NPs. Among the various factors, three main parameters, namely polymer/conjugate ratio, surfactant concentration, and stirring speed, were selected as the independent variables for the optimization process using BBD method using Design-Expert 10 software. Dependent variables depend on the independent variables and determine the characteristics of the formulation. Two dependent variables, namely particle size and % EE, were used for the characterization and optimization of the formulation. In the study, a set of 17 experiments with five center points were developed for CNP formulations. All the formulations were characterized, and data are shown in Table 2. An ANOVA was used to eliminate less significant factors and evaluate the effect of the most significant factors at 95% confidence interval. The ANOVA analysis also produces the quadratic polynomial equations for each factors, with the plus and minus signs which indicate the direct and inverse proportional effect of the parameters on the formulation properties, respectively. The lack of fit F-value was also determined for the each response and found to be not significant which means the BBD model was well-fitted model for the optimization. Contour and response surface plot was plotted which shows the relationship between the parameters and the responses as shown in Figure 7.[20,21]

Determination of particle size

Variation in the formulation and process variables leads to change in the particle size of formulation and was found to be in the range of 120.2–195.5 nm. F-value and *P*-value found to be as 103.08 and <0.0001, respectively, through ANOVA analysis. The results of F-value and *P*-value suggest that the quadratic model is best fitted and significant model for particle size as given in Table 3. Furthermore, the large lack of fit value (i.e., >0.05) shows that the model is best for the optimization of formulations when the particle size is selected as the response. The regression coefficient value (R^2) was found to be >0.99 which indicates the good relationship between the predicted value and the experimental values. The "predicted R^2" of 0.9041 was in reasonable difference with the "adjusted R^2" of 0.9829, i.e., the difference is <0.2. "Adequate precision" value measures the signal-to-noise ratio. The ratio was obtained as 22.878 which indicates an adequate signal. A ratio >4 is desirable. Therefore, the proposed model will be used to navigate the design space. The polynomial equation was produced with the help of factor coefficient estimate value. The values in the equation suggest that the polymer/conjugate ratio have a direct impact on the particle size due to its positive value and other two factors, namely surfactant

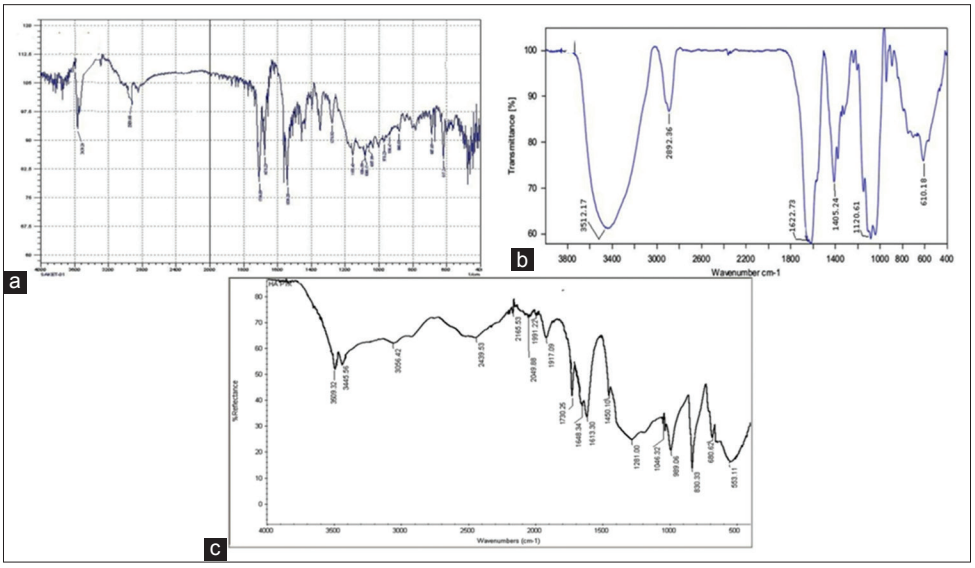

Figure 6: Infrared spectra of (a) paclitaxel (Ptx), (b) Hyaluronic acid (HA) (c), HA-Ptx

Table 2: Design matrix evaluation for response surface quadratic model

Run	Independent factors			Responses	
	A: Polymer/ conjugate (ratio)	B: surfactant (%)	C: Stirring speed (rpm)	Particle size (nm)	Entrapment EE (%)
1	15	1	500	190.3	60.2
2	10	1	1000	120.2	71.3
3	5	0.5	1000	187.4	55.2
4	15	1	1500	185.4	59.5
5	15	0.5	1000	195.5	61.2
6	10	1.5	1500	182.3	66.3
7	5	1	1500	193.2	57.8
8	10	1	1000	123.7	70.3
9	10	1	1000	124.1	71.2
10	5	1.5	1000	185.6	56.6
11	10	1.5	500	193.7	66.8
12	10	1	1000	127.3	70.7
13	10	1	1000	123.2	69.9
14	10	0.5	500	193.4	64.3
15	10	0.5	1500	187.8	65.2
16	5	1	500	185.3	55.5
17	15	1.5	1000	192.6	62.3

EE: Entrapment efficiency

Table 3: ANOVA analysis results of quadratic model

Response	F-value	*P*-value	% CV	Press	R-Squared	Adj R-Squared	Pred R-Squared	Lack of fit	Adeq Precision
Particle size	103.08	<0.0001	2.39	1481.35	0.9925	0.9829	0.9041	0.0848	22.878
Entrapment EE	77.24	<0.0001	1.36	63.68	0.9900	0.9772	0.8791	0.1221	23.625

EE: Entrapment efficiency

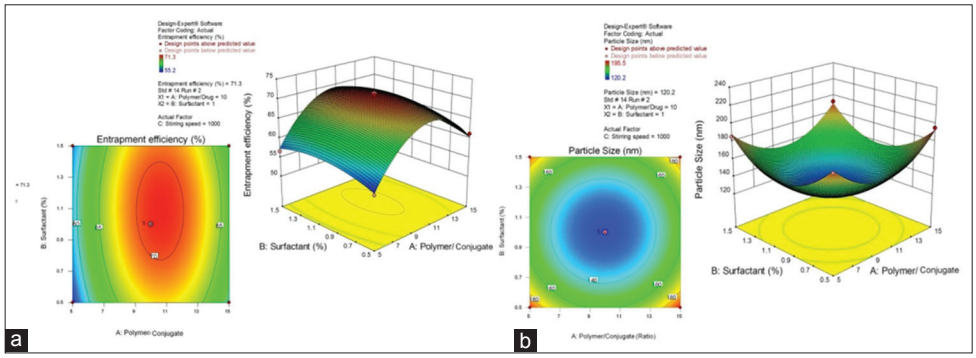

Figure 7: Contour and surface response plot of (a) percentage entrapment efficiency and (b) particle size

concentration and stirring speed, implying the negative impact on the particle size. Therefore, when the polymer/conjugate ratio was increased, their particle size was also increased which may be due to larger polymer concentration in the system. The higher concentration of the polymer leads to increase in the viscosity of the organic phase, which may be responsible for the formation of larger size droplets. Hence, the size of NPs is directly proportional to the concentration of polymer. The increase in the stirring speed leads to enhance the attrition force between the particles which may be due to the splitting up of the NPs into the smaller size. The surfactant concentration has a negative impact on the particle size which may be due to the coating with a higher concentration of surfactant, can prohibit the interaction between the particles, and thus decrease the particle size. The quadratic polynomial equation obtained from the above parameters is given as follows:

Particle size= 123.70 + 1.54 * A – 1.24 * B – 1.75 * C – 0.28 * AB – 3.20 * AC - 1.45 * BC + 32.91 * A^2 + 33.66 * B^2 + 31.94 * C^2

Determination of percentage EE

The % EE of the formulations was found to be in the range of 55.2 %–71.3 %. F-value and p-value were found to be 77.24 and <0.0001, respectively. The ANOVA analysis was used for the optimization of %EE and obtained the best fit quadratic model as shown in Table 3. The lack of fit value was found to be > 0.05, and the value was non-significant. Therefore, the quadratic model was selected as the best model for the optimization of formulation for the % EE. The regression coefficient and "predicted R^2" value were found to be 0.9900 and 0.8791, respectively, which shows a good adequacy and better fit to the model. The quadratic polynomial equation was produced using factor coefficient values and the obtained equation was used to determine the positive and negative effects of the parameters on the %EE of formulations. From the equation, it is concluded that all the three variables show the significant effect on the EE. Hence, the increase in the polymer/conjugate ratio up to optimum level leads to an increase in the %EE, which may be due to the more polymer available for the entrapment of free drug. After a definite level, an increase in the polymer concentration does not affect the conjugate entrapment in the NPs due to the unavailability of free drug conjugate for entrapment. The surfactant may also assist in the loading of the conjugate in the NPs and thus increase in the surfactant amount is responsible for the enhancement in the EE. The increase in the stirring speed results in the formation of monodisperse smaller size NPs, which have more capacity to entrap higher amount of drug when compared with the polydisperse larger particles. The derived quadratic polynomial equation for %EE is obtained as follows:

% Entrapment efficiency = 70.68+ 2.26 * A + 0.76 * B + 0.25 * C - 0.075 * AB – 0.75 * AC – 0.35 * BC – 9.63 * A^2 – 2.23 * B^2 – 2.80 * C^2

Surface morphology of NPs

The shape and surface morphology of NPs were determined using TEM and SEM. TEM studies of the Ptx-loaded optimized NPs formulation confirmed the particle spherical shape and their discrete and homogeneous dispersion within the size range of 120–230 nm [Figure 8a]. The surface morphology of the optimized NPs was also confirmed by SEM, and surface of the prepared formulation was found to be the smooth and spherical in shape [Figure 8b].

In vitro release studies

In vitro release study was performed using the dialysis bag method with the help of dialysis membrane

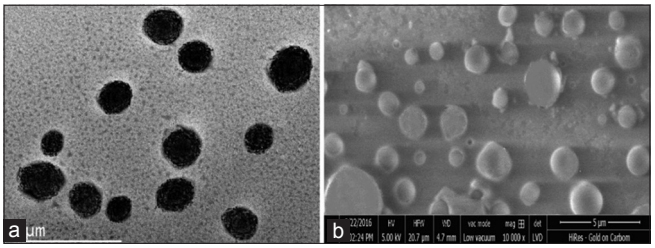

Figure 8: Photomicrograph of optimized nanoparticles (a) transmission electron microscopy and (b) scanning electron microscopy

(Mw – 12,000–14,000 Da). The release studies were carried out by plotting the graphs with five different kinetic models. These kinetic model graphs were plotted with different release data from the optimized formulation.

In zero-order model, conjugate release graph was plotted between cumulative % conjugate release and time, in first-order model, graph wasplotted between log cumulative % conjugate remaining and time, and in Higuchi's model, graph was obtained by plotting the cumulative % conjugate release and square root of time, whereas in Korsmeyer–Peppas model and Hixson–Crowell model, graph was obtained by plotting the log cumulative % conjugate release v/s log time and difference in cube root of % conjugate remaining and initial amount v/s time, respectively. All the resultant graphs are shown in Figure 9 with their respective regression coefficient (R^2) value for each model. The model showing highest regression coefficient value was selected as the best fit model for the release profile of the formulation. The regression coefficient value (R^2) and release rate constant (k) value are given in Table 4, and the highest value of the regression coefficient (i.e., 0.9965) was found with the Higuchi model. Therefore, Higuchi model was selected as the best, fittest model for release study indicates that the conjugate release from the polymeric matrix is dependent on the square root of time but not dependent on the concentration which confirmed the release from the formulation through the diffusion process.

In vitro cytotoxicity study

The cytotoxicity study shows that the CNPs have a better effect on the MCF-7 cell line when compared with the PNPs and Ptx while adriamycin (Adr) used as the standard cytotoxic agent. CNPs have the lowest IC_{50} value, i.e., <10 µg/ml concentration, because they show >50% cell growth inhibition after 48 h of the experiment at this concentration. Although PNPs and Ptx solution also inhibit cell growth, at lesser extent, they have larger IC_{50} value as shown in Figure 10. The difference in the value may be due to the HA-Ptx conjugate targets the overexpressed CD44 receptors on the tumor cell line and NPs system provide the sustained action at the site. From the cytotoxicity study, we can conclude that the CNPs are more effective for the treatment of tumor cells.

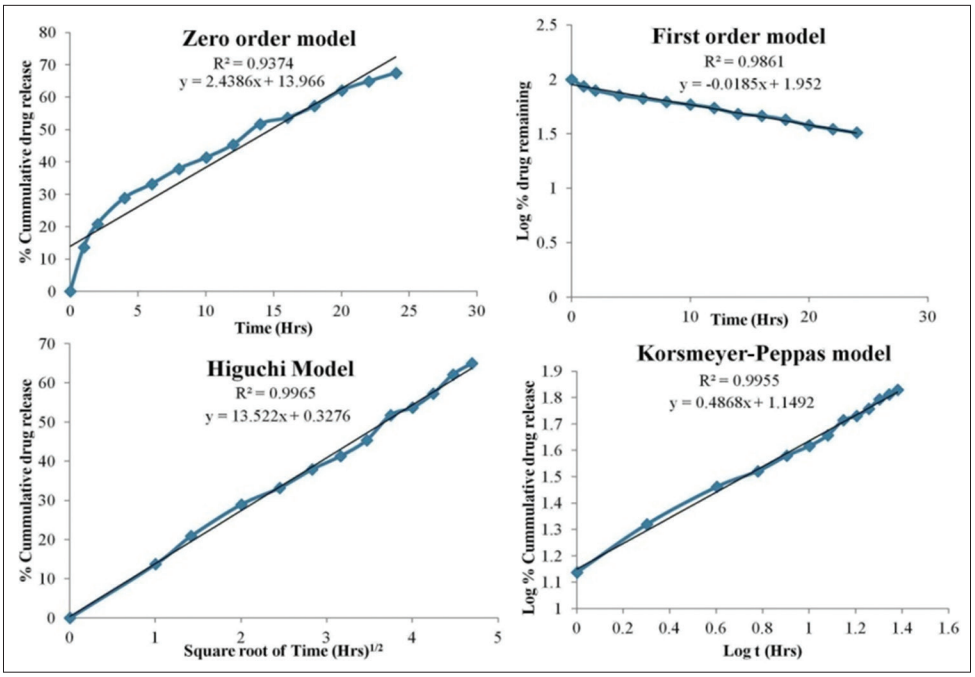

Figure 9: Different models showing *in vitro* conjugate release

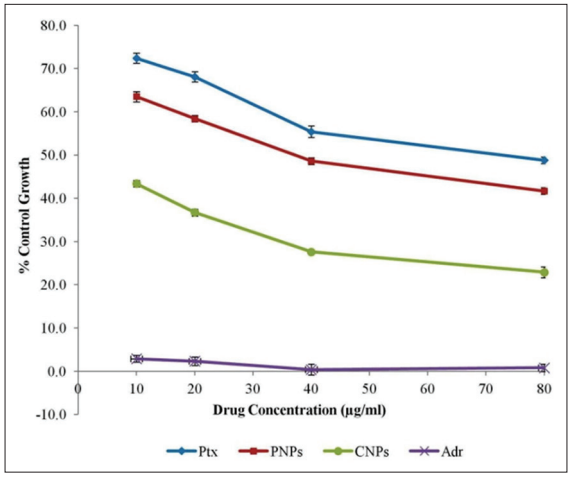

Figure 10: Cytotoxicity study graph

Table 4: Regression coefficient value (R^2) and release rate constant (k) of different models

Pharmacokinetic model	Regression coefficient value (R^2)	Release rate constant (k)
Zero order	0.9355	2.4343
First order	0.9861	-0.0185
Higuchi model	0.9965	13.561
Korsmeyer–Peppas model	0.9955	1.149
Hixson–Crowell model	0.5639	0.1021

CONCLUSION

The HA-Ptx was successfully synthesized using carbodiimide chemistry and the conjugation was confirmed by FTIR study. The CNPs were prepared using the modified nanoprecipitation method. The method provides the best result on the basis of particle size and % EE of NPs. In the experiment, 3-level BBD was used with three independent factors and two responses. The independent factors, which affect majorly on the dependent variables, were selected on the basis of the results of previous optimization studies. On the basis of the quadratic polynomial equation, optimized formulation was selected having optimized parameters such as 10:1 (polymer conjugate ratio), 1% (surfactant concentration), and 1000 rpm (stirring speed). The optimized formulation shows a minimum particle size of 120.2 nm and maximum EE of 71.3% which were selected using the BBD model. TEM and SEM showed that the NPs in the formulation were in well-defined smooth and spherical in shape. The *in vitro* kinetic study of the formulation was performed using different kinetic models. The result of the kinetic studies confirmed the drug release followed the Higuchi model, which indicates the drug release from the polymeric matrix dependent on the square root of time, depends on the diffusion process. The cytotoxicity result showed the higher interaction between HA-Ptx and the overexpressed CD44 receptors on the tumor cells, and hence, CNP formulation exhibited the lowest IC_{50} value than the other formulations. In the near future, the *in vivo* study will be performed to evaluate the therapeutical and pharmacological activity of the optimized formulation. Therefore, CNPs could be considered as a hopeful carrier system for targeted drug delivery to solid tumors.

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