Ligand conjugated tumor targeted nanoparticle drug delivery system of vincristine: 3^2 Full factorial design and *in vitro* evaluation

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ABSTRACT

An active targeting system is more preferred to enhance intracellular uptake of drug within tumor tissues, while it is highly restricted in normal tissues. Various targeting moieties or ligands against tumor cell-specific receptors have been immobilized on the surface of nano-particulate carriers to deliver them within cells via receptor-mediated endocytosis. This study investigated the preparation and characterization of a targeted system represented by folate-conjugated vincristine sulfate-loaded polymeric nanoparticles for breast cancer. Conjugation of folic acid to PLGA was achieved by coupling di-block copolymer with folic acid. The vincristine sulfate loaded nanoparticles, prepared by solvent evaporation method were characterized by particle size analysis, entrapment efficiency and in-vitro drug release. The optimization of formulation was done by three square full factorial design. Phase ratio and sonication time were the independent variables, while particle size and entrapment efficiency were responses. The optimized nanoparticles showed a particle size of 200.3 nm and an entrapment efficiency of 54.33%. The ANOVA results of particle size showed a Model F-value of 104.45, implies the model is significant. And for entrapment efficiency, the Model F-value of 59.55 implies the model is significant. By in-vitro drug release studies, the optimized formulation showed sustained release characteristics following Non-Fickian type of diffusion controlled release.

Key words: Targeted drug delivery system, Nano particles, Design of Experiments.

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INTRODUCTION

Although, chemotherapy is one of the most widely used method against cancer, its clinical application is limited by low selectivity towards the cancerous and non-cancerous cells. Targeted delivery systems are thus preferred, to improve the therapeutic indices of the anti-cancer drugs both by increasing the selectivity and decreasing toxicity. Drug-loaded nanoparticles of bio-degradable polymers can be delivered to specific sites by active targeting through conjugation of targeting molecules [1,2].

The active targeting approach is a most important strategy in chemotherapy to deliver anti-cancer drug loaded nanoparticles. In active targeting, the targeting ligand attaching onto the surface of nanoparticles, which specifically binds to receptor structure overexpressed at the target site. This targeting mechanism increases selective cellular binding and internalization through receptor-mediated endocytosis. Active targeting increases tumor internalization and target specificity, while it decreases targeting towards non-cancerous cells and it increases the therapeutic indices of anti-cancer drug by improving therapeutic efficacy [3–6].

Vincristine sulfate (VCR), is an effective chemotherapeutic agent that has been used widely for the treatment of a number of human cancers including acute leukemia, malignant lymphoma, and breast cancer. It can exert its antitumor activity by interacting with tubulin which causes disruption of microtubules of the mitotic apparatus, thereby arresting cell division in metaphase [7].

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Various targeting agents or ligands have been immobilized on the surface of nano-carriers to deliver them within cells via receptor-mediated endocytosis. Among them folic acid is an important targeting moiety and widely used one among this. The folate receptor, a tumor marker that binds to vitamin folic acid with high affinity. Folic acid is a glycosylphophatidylinositol-linked membrane glycoprotein, mostly used ligand as it is overexpressed in various tumors including ovarian, breast, colorectal, renal and neuroendocrine metastases, but is not present in normal cells [5,8]. Folic acid makes the carrier target potential, provides deeper cellular internalization and nucleus directed release due to caveolin assisted receptor mediated endocytosis [9–11]. By folate receptor mediated endocytosis, folic acid is internalized in cytoplasm and folic acid's α isoform is highly advantageous in breast cancer. Folic acid is widely used in the delivery of anti-cancer drugs due to its smaller size, low cost, non-immunogenic nature and high tumor specificity [11].

In this study, we have developed folate-decorated biodegradable poly (lactide-co-glycolide) (PLGA) nanoparticles by solvent evaporation method [12–16]. The formulation variables were optimized by three square full factorial design to obtain favourable particle size and encapsulation efficiency. The *in vitro* drug release behavior of vincristine sulfate was studied in phosphate buffer pH 7.4.

MATERIALS AND METHODS

Vincristine sulfate was a gift from Cipla pharmaceuticals, Mumbai, India. Polylactide-co-glycolide (50/50 DLG 3A, MW 33,300) was obtained from Sigma Aldrich, USA. Folic acid, N-hydroxysuccinimide and 1-ethyl-3(3-dimethyl aminopropyl) carbodiimide (EDC) were purchased from Himedia, Mumbai, India. Ethylene Diamine for synthesis, triethylamine, DMSO and Dichloromethane A.R were purchased from Merck chemicals, Mumbai, India. All other chemicals used were of reagent grade.

3.1. Synthesis PLGA–FA
A folate-conjugated diblock copolymer was synthesized by modifying the method developed be Saxena et al. [15]

3.2. Nanoparticles preparation
The folate decorated vincristine sulfate loaded polymeric nanoparticles were fabricated by a modified oil-in-water (O/W) single emulsion solvent evaporation/extraction technique [17]. Accurately weighed amounts of PLGA–EDA–FA polymer and vincristine sulfate were dissolved in dichloromethane. Drug solution was added to polymer solution with gentle stirring to dissolve the contents. The resulting organic phase was added slowly to aqueous phase containing poly vinyl alcohol (PVA) as stabilizer and sonicated using a probe sonicator at an output of 40W in an ice bath. The formed o/w emulsion was gently stirred at room temperature by a magnetic stirrer for upto 12 h for complete evaporation of organic solvent. Nanoparticles were separated by centrifuging the resulting suspension at 15,000 RPM for 20 minutes at 4^0C and washed with distilled water, thrice, to remove the emulsifier and adsorbed drugs. The washed nanoparticles were then freeze-dried [12,18–20].

3.3. Pre-optimization studies
Pre-optimization studies were carried primarily to choose the independent factors and their levels. The main objective was to obtain the nanoparticles with smaller particle size and high entrapment efficiencies. To study the factors that affect the particle size, various formulation were prepared with varying phase ratio. The prepared formulations were evaluated by measuring the particle size by dynamic light scattering (DLS) analysis and entrapment efficiency. During the pre optimization studies the organic to aqueous phase ratio and sonication time are considered as independent variables. The organic to aqueous phase ratio was varied fom 1:2, 1:3, 1:4 and 1:5 respectively. Sonication time was varied from 4, 8 and 12 min during the pre-optiization studies [21,22].

3.4. Optimization of nanoparticles by three square full factorial design
Based on the pre-optimization studies three square full factorial designs with 13 runs were used for optimization. The phase ratio, sonication time was found to have greater effect on particle size and entrapment efficiency. Based on the data from the pre-optimization, three levels were chosen and the design matrix was fixed. The particle size and percentage drug entrapment was chosen as the responses on which the success of the formulation depends. The three square design matrix and the factors with their levels are shown in Table.1 and 2 respectively.

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Table.1: Factors and Levels used in the design

Independent variables	Levels		
	-1	0	+1
Phase ratio	1:3	1:4	1:5
Sonication time(min)	4	8	12
Dependent variables	Y1 - Particle size		
	Y2 - Percentage drug entrapment		

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Table.2: Three square design matrix

Formulation code	Factor 1 Phase ratio	Factor 2 Sonication time(min)
F1	1:4	4
F2	1:4	8
F3	1:3	8
F4	1:5	8
F5	1:4	8
F6	1:3	4
F7	1:4	8
F8	1:5	4
F9	1:4	12
F10	1:3	12
F11	1:4	8
F12	1:5	12
F13	1:4	8

4. CHARACTERIZATION OF NPs
4.1. Shape and particle size distribution
Freeze-dried nanoparticles were dispersed in deionized water. Their mean particle diameter and the width of the particle distribution were determined by photon correlation spectroscopy using a Zetasizer 3000 (Malvern Instruments, Worcestershire, UK). The shape and surface morphology of NPs were determined by TEM analysis.

4.2. Encapsulation efficiency
The amount of encapsulated vincristine sulfate in the nanoparticles was evaluated by a direct method. 5mg of the freeze dried nanoparticles were vortexed with 2ml of DCM for 1hr and was filtered through 0.22μ membrane filter. Then the drug content in the filtrate was analyzed by ultraviolet (UV) spectrophotometer at 296 nm against dummy nanoparticles, which had also been prepared as reagent blanks and treated similarly to the, drug-loaded nanoparticles. The percent encapsulation, a measure of encapsulation efficiency, was calculated as the ratio of the drug content in the freeze dried powder to the initial drug amount added (11,22–24).

$$Encapsulation\ efficiency = \frac{entrapped\ drug}{total\ drug\ added} * 100$$

4.3. *In-vitro* drug release studies
Drug release from the nanoparticles was studied using a dialysis technique. 5 mg sample of nanoparticles was resuspended in 1 ml of phosphate buffer solution at pH 7.4 and placed in a dialysis bag (Spectra/Por®, molecular weight cut off 12000 Da) sealed at both ends. The dialysis bag was soaked in 100 ml of phosphate buffer solution (pH 7.4) and maintained at 37°C ± 0.5°C and 100 ± 5 rpm shaking in a shaker. At predetermined time intervals, individual samples were taken and was replaced with fresh phosphate buffer solution to maintain the sink condition (4,24–28). The data obtained from *in-vitro* release studies of folate conjugated vincristine sulfate loaded PLGA nanoparticles were fitted to various models such as zero order, first order, Higuchi and Korsmayer Peppas to ascertain the kinetic modelling of drug release (21).

RESULTS AND DISCUSSION

5.1. Preparation and optimization of nanoparticles.
The PLGA- -FA was synthesized according to the procedure. The nanoparticles were prepared by solvent evaporation method. And the pre-optimization studies, done using organic to aqueous phase ratio and sonication time as independent variables. To obtain the nanoparticles with smaller particle size and high entrapment efficiencies the phase ratio of 1:4 and sonication time of 8 min selected from pre-optimization studies. The optimization were done using three square full factorial design with 13 runs. The composition of optimized formulation was of phase ratio 1:4.6 and sonication time 12 min. And from the optimization studies, the optimized nanoparticles showed a particle size of 200 nm and an entrapment efficiency of 54.33%. The ANOVA results of particle size showed a Model F-value of 104.45, implies the model is significant. And for entrapment efficiency, the Model F-value of 59.55 implies the model is significant.
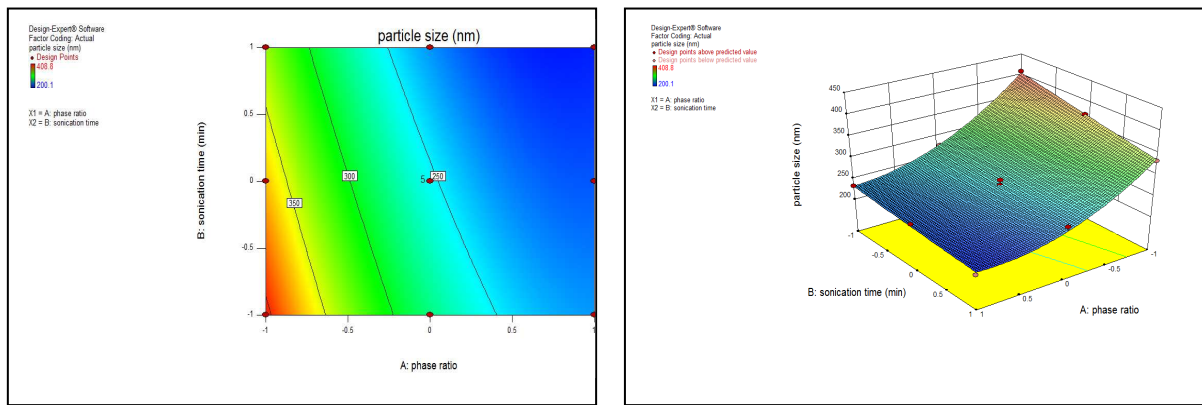
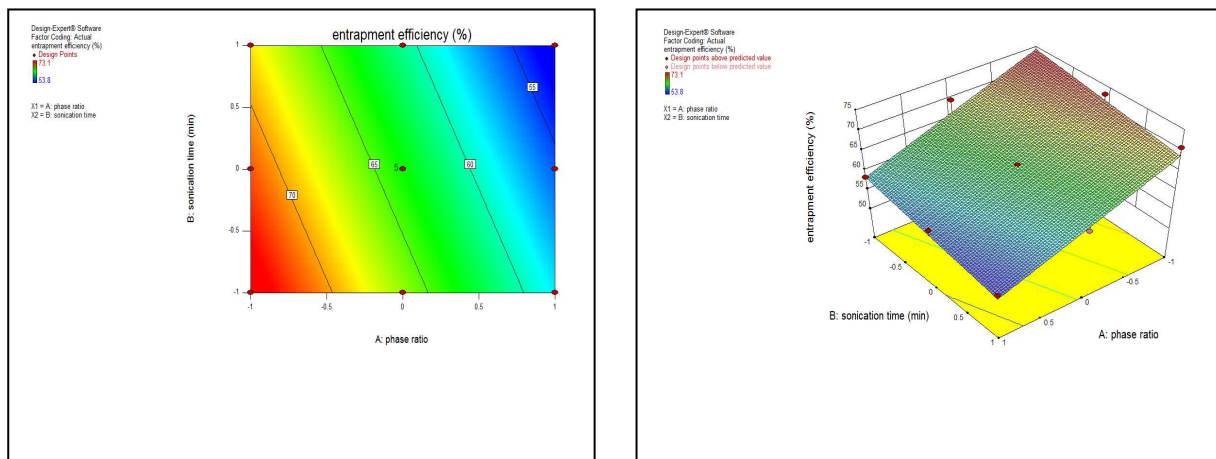**Figure.1. Contour plot and response surface plots for the response, particle size**

Figure.2. Contour plot and response surface plots for the response, entrapment effieciency

5.2.Shape and particle size distribution

The optimized nanoparticles were evaluated for particle size by dynamic light scattering measurements and showed average particle size of 200.3 nm. Shape and surface morphology of nanoparticles determined by TEM analysis and results revealed that the prepared NPs are spherical shape and it is suitable for nanoparticle drug delivery.

		Size (d.nm):	% Intensity:	St Dev (d.n...
Z-Average (d.nm): 200.3	Peak 1:	226.3	100.0	82.02
PdI: 0.097	Peak 2:	0.000	0.0	0.000
Intercept: 0.932	Peak 3:	0.000	0.0	0.000
Result quality : Good				

Size Distribution by Intensity

Record 63: SA9 2
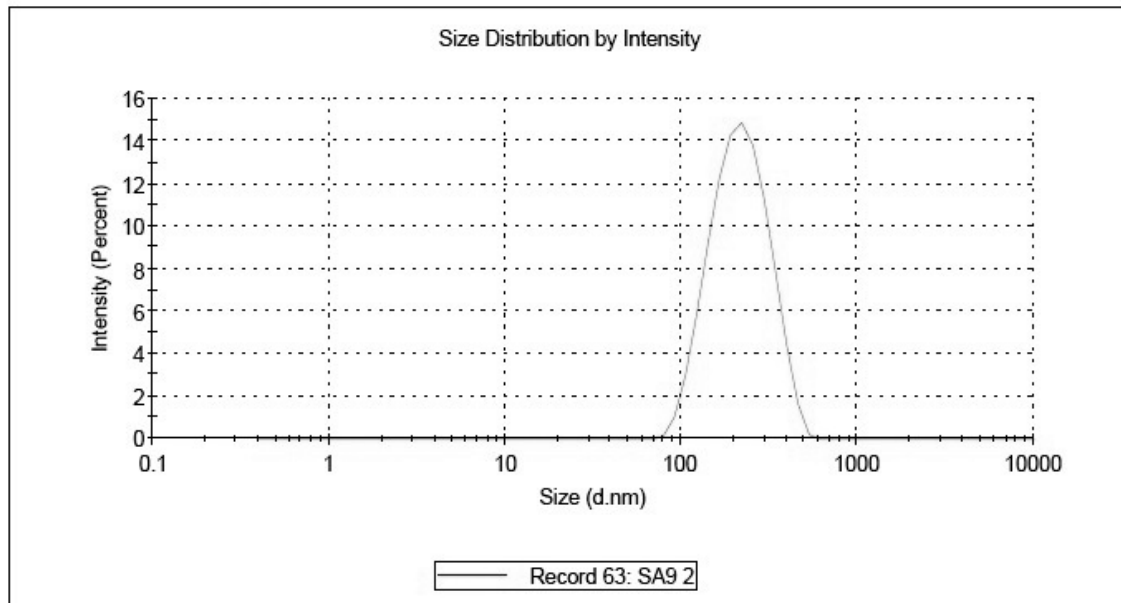
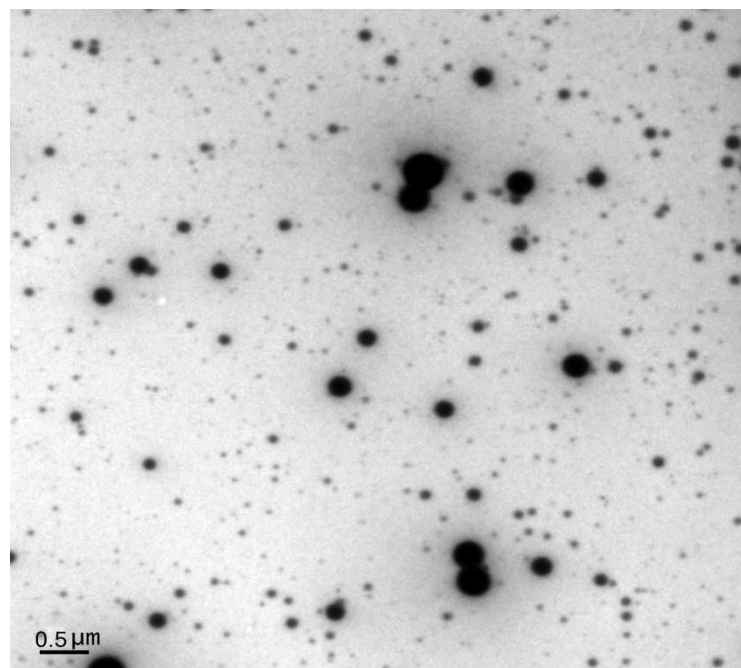**Figuer.3: Particle size distribution curve of optimized formulation**

0.5 μm

Figure 4: TEM image of NPs

5.3. *In- vitro* drug release studies
The *in vitro* drug release study was done using dialysis bag method in PBS pH 7.4. And from the evaluation and results, the drug release from the formulation was found to be sustained and 95.51 % release was obtained by 24 hours.
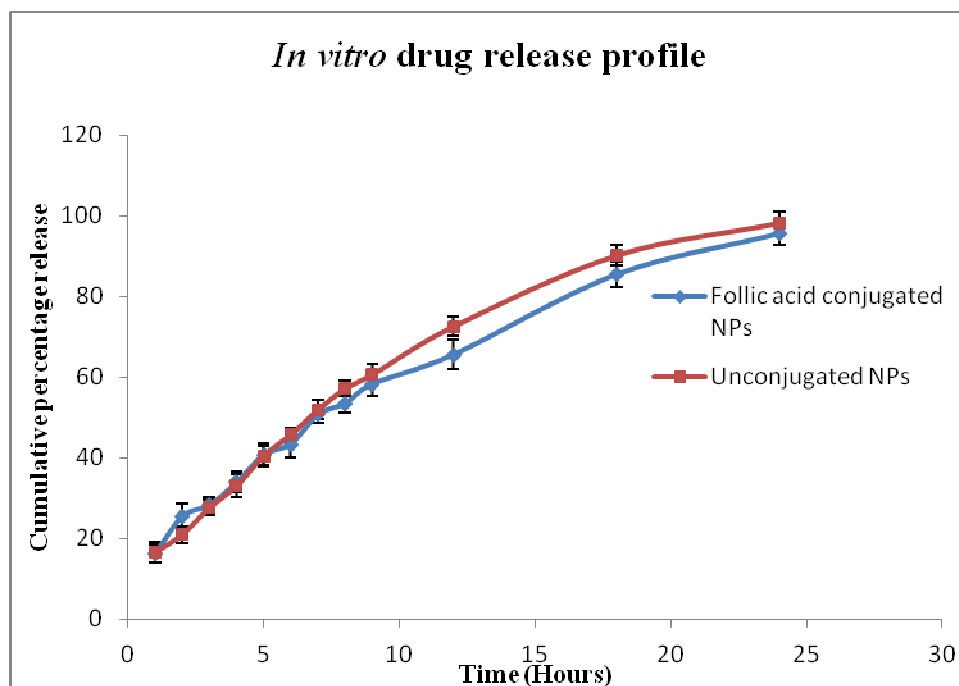
Figure 5: Correlation co-efficient value for dissolution data

Table 3: Diffusion exponent for dissolution data

Formulation	Correlation co-efficient R^2 value			
	Zero order	First order	Higuchi model	Peppas model
Folate conjugated vincristine sulphate NP"s	0.9571	0.8024	0.9954	0.9948

Table 4: Diffusion exponent for dissolution data of formulation

Formulation	Diffusion exponent (n) of Peppas model
Optimized	0.5666

The correlation co efficient values indicate that the release profile of folate decorated PLGA nanoparticles loaded with vincristine sulphate were fit into zero order kinetics than first order kinetics. The drug release was diffusion controlled as indicated by the higher R^2 values in Higuchi model. Since, the 'n' values obtained from the Korsmeyer–Peppas model were greater than 0.45, the mechanism of drug release from the PLGA NPs were non-fickian diffusion.

CONCLUSION

In this study, we have synthesized PLGA-FA conjugate as per the procdure. The vincristine sulfate loaded nanoparticles, prepared by solvent evaporation method and optimized by three square full factorial design. The composition of optimized formulation was of phase ratio 1:4.6 and sonication time 12 min and characterized for particle size, entrapment efficiency and in vitro drug release kinetics. The particle size was found to be 200.3 nm and drug release kinetics follows zero order kinetics following non-fickian type of diffusion controlled release.

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