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Development and optimization of Etoposide loaded nanoparticles by using DoE response surface central composite design

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Abstract: Cancer is a leading cause of death globally. The World Health Organizationestimates that 9.6 million people died of cancer in 2018. More than 70% of all cancer deaths occur in low- and middle-income countries, where resources available for prevention, diagnosis and treatment of cancer are limited or nonexistent. Present investigation aimed at formulation and optimization of nanoparticles with Etoposide, an anticancer drug. Design Expert software is used for optimization. A randomised response surface design was used for optimization procedure. Stirring speed and crosslinking agent concentration are the independent variables for the dependant variables of particle size and drug loading. Statistical data of the dependent variables obtained were subjected to the analysis of variance and were found significant at $p<0.001$ for particle size and at $p<0.0001$ for drug loading. Encapsulation efficiency of the NPs was evaluated and they were found to be between 65.87±2.78 - 88.95±1.09. The prepared NPs are with the minimum size of 98±8.76nm and a maximum size of 786.24±6.89nm. Through factorial design, it was optimised that 500rpm (at a level of 1) and the maximum concentration of glutaraldehyde at 25% (at a level of 1) will result a least mean particle size of 150.13nm and maximum drug loading of 86.24%.

Key words: Bovine serum albumin, Design of Experiment, Etoposide, Nanoparticles and Optimization

Introduction

Polymeric nanoparticles (NPs) are the biodegradable colloidal particles which can carry drug by adsorption or chemical bonding. NPs are advantageous in improving the bioavailability ofpoorly absorbed drugs, to provide a controlled release, and also for assured cell targeting (1). Due to nanosize, the targeting into various cancers has been shown to be promising with NPs[2] (2). Therapeutic effect of NPs depends on several process and formulation factors. In evaluating the multiple effects, traditional experiments have the disadvantage of being timeconsuming and requiring more effort and materials. The design of experiments (DoE) is an approach for effectively and efficiently exploring the cause and effect of relationship between numerous process variables and the output. The goal of the experimental design is to find out, with the minimum number of experimental runs, the effect of various independent variables on the final product. Etoposide (ETP) is derived from podophyllotoxin and has anticancer activity by the induction of DNA damage caused by topoisomerase II (3). Bovine serum albumin (BSA) is one of the biodegradable polymer used for the preparation of NPs. The factorial design is an important tool in optimizing polymeric NPs in order to modulate their physicochemical characteristics and ultimately their therapeutic response. The objective of the present work isto design and optimize NPs for ETPby using DoE study and to find out the best possible formulation (4).

Materials and Methods

Materials: ETP is a kind gift sample from MSN laboratories, Hyderabad. Bovine serum albumin is from Sigma Aldrich, Mumbai. All the other chemicals and reagents used are of analytical grade.

Methods

Experimental design: A randomised response surface design was used for the optimization procedure. Mathematical

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modelling and evaluation of the ability to fit to the selected model was performed with statistical analysis through Design-Expert® Software (Stat-Ease, Inc). A central composite design was used in this 2^3 factorial study and 2 factors were evaluated at randomised 3 levels (5 centre points and 8 non centre points). The selected variables and their levels were given in Table 1.

Table 1: Randomised Response Surface design parameters indicating the levels of variables

Variable	Levels		
	-1	0	+1
Stirring speed in rpm	100	250	500
Glutaraldehyde%	5	15	25

The percentage of glutaraldehyde and stirring speed were selected as independent variables. Particle size and drug loading efficiency were as dependent variables. The batches thus prepared by factorial design are evaluated and the effect of individual variable was studied according to the central composite randomized design (5).

The equation for the design of present investigation can be written as,

$$Y= C+ \beta_1A+ \beta_2B+ \beta_3AB+ \beta_4A^2+ \beta_5B^2$$

In this equation, *Y* is the dependent variable, C is the intercept and it is the arithmetic mean response of the 13 runs. $\beta_1$to β_5 are the estimated coefficients for the corresponding factors A and B. Analysis of Variance (ANOVA) was performed, and P-value with 95% confidence interval was evaluated to determine the significance of each coefficient term. To determine the fitting extent of experimental data, regression coefficient R^2 along with predicted and adjusted R^2 were determined (6).

Preparation of NPs

ETP loaded BSA-NPs were prepared by desolvation method with the addition of acetone as the desolvating agent. 1% BSA solution was prepared in double distilled water. ETP dissolved in acetone was added drop wise at a rate of 1ml/min in to BSA solution under a constant stirring rate until the solution becomes turbid. Finally, 0.01ml of glutaraldehyde was added and stirred for 3H to harden the NPs by intra particle crosslinking. The BSA NPs formed were purified by two cycles of centrifugation and re-dispersion to remove unreacted chemicals and free BSA molecules. For each centrifugation step, the BSA NP solutions were centrifuged at 20,000g (Remi RM-12C, Remi Electrotechnik ltd, India) for 30 min. The supernatant was subjected for encapsulation efficacy and the pellet was collected for further characterization (7).

FTIR studies

The physicochemical compatibilities of the drug and the used excipients were tested by FTIR. Potassium bromide, an infrared transparent matrix, at 1:10 (Sample: KBr) ratio was used for the study. The KBr discs were prepared by compressing the powders at a pressure of 5 tons for 5min in a hydraulic press. Scans were obtained at a resolution of 4 cm−1, from 4,000 to 400 cm−1

DSC analysis

The thermal behaviour of drug individually and as NPs was studied using DSC (Mettle Toledo DSC823, Switzerland). The heating rate and nitrogen purges were 10 K/min and 20 ml/ min, respectively.

Drug loading efficiency

To check the drug loading efficiency, the free drug present in the supernatant after centrifugation was measured in UV Spectrophotometer (Elico SL 159) at 283nm. The loading efficiency was calculated using the formula.

Drug loading efficiency (%) =

(Total Drug-Drug in supernatant)/ (Total drug) × 100

Zeta Size

Mean particle size of the NPs was determined by Photon Correlation Spectroscopy (PCS) attached with a Malvern Zetasizer Nano ZS (Malvern Instruments, Malvern, UK). The prepared NPs were dispersed in deionized water and sonicated for 30 min. The resultant dispersions were analyzed for mean particle size and particle size distribution.

Scanning Electron Microscopy

The size-controlled BSA NPs were examined in a low vacuum scanning electron microscope (S-3700N, Hitachi Science Systems, Ltd., Japan). Shade dried particles were deposited on aluminum stubs using double-faced adhesive and coated with gold-

palladium under an argon atmosphere using a gold sputter module in a high-vacuum evaporator before observation.

Results

Experimental design

In this work, a randomised response surface design was conducted to assess theinfluence of two different parameters on the properties of BSA NPs containing ETP. The individual effectsof each parameter and whether there was interaction betweenparameters was verified. The response surface quadratic models were generated using Expert Design Software. These were subjected to multiple regressions by quadratic order to yield polynomial equations. Experimental trials were performed at all 13 possible combinations prepared according to the model and the results were given in Table 2.

Table 2: Randomised Response Surface design parameters indicating the levels of variables

Run	Stirring speed (rpm)	% Glutaraldehyde	Particle size (nm)	Drug loading efficiency (%)
1	0	0	686±12.78	65.87±2.78
2	1.41	0	105±8.76	85.95±1.09
3	1	-1	445±10.73	77.45±3.45
4	0	1.41	568±3.53	75.87±5.23
5	0	0	786±6.98	65.87±2.98
6	0	-1.41	495±8.45	69.23±4.32
7	0	0	786±6.98	65.87±7.43
8	1	1	98±8.76	88.95±3.76
9	-1	1	448±5.73	75.45±2.34
10	0	0	686±12.78	68.95±5.23
11	-1	-1	332±3.98	77.45±3.24
12	-1.41	0	323±7.53	76.23±6.27
13	0	0	686±12.78	65.87±5.98

ANOVA results for the optimization of particle size were summarized in Table 3. Contour plot and the 3D surface response graph for particle size were given in Figure 1 and 2. Statistical data of the dependent variables obtained were subjected to ANOVA and were found to be significant at $p<0.001$. Overall interaction was found to be significant at $p<0.05$ indicating, effect of both the variables in combination also significant on the mean size of particles. Lack of fit value $p>0.05$ indicates, the noise of the experiments is nonsignificant.

Table 3: ANOVA responses for the factorial design for particle size of NPs

Source	Sum of squares	DF	Mean of square	F value	P value	
Model	6.246E+05	5	1.249E+05	29.03	0.0002	Significant
A-Stirring speed	37168.81	1	37168.81	8.64	0.0218	
B-Glutaraldehyde	2040.40	1	2040.40	0.4741	0.5133	
AB	53592.25	1	53592.25	12.45	0.0096	
A^2	4.941E+05	1	4.941E+05	114.79	< 0.0001	
B^2	80765.65	1	80765.65	18.77	0.0034	
Residual	30127.78	7	4303.97			
Lack of Fit	18127.78	3	6042.59	2.01	0.2493	Not significant
Pure Error	12000.00	4	3000.00			
Cor Total	6.547E+05	12				

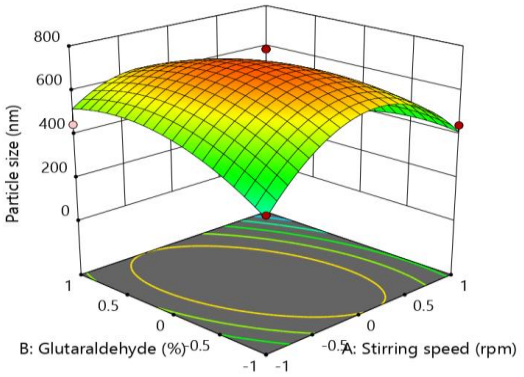

Figure 1: Response 3D plot for the effect of Stirring speed and Glutaraldehyde % on particle size of the NPs

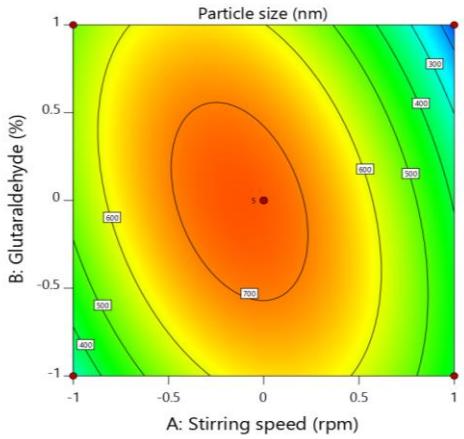

Figure 2: Contour plot for the effect of Stirring speed and Glutaraldehyde % on particle size of the NPs

Table 4: Coefficient of estimate values for the Particle size of NPs

Factor	Coefficient of Estimate	DF	SE	95%CI low	95% CI High	VIF
Intercept	726.00	1	29.34	656.62	795.38	
A-Stirring speed	-68.16	1	23.19	-123.01	-13.32	1.0000
B-Glutaraldehyde	-15.97	1	23.19	-70.82	38.88	1.0000
AB	-115.75	1	32.80	-193.32	-38.18	1.0000
A^2	-266.50	1	24.87	-325.32	-207.68	1.02
B^2	-107.75	1	24.87	-166.57	-48.93	1.02

The main effects A and B represent the average result of changing variable at a time from its low level to high level. The interaction terms (AB, $A^2$and B^2) show how the responses change when 2 variables are simultaneously changed. All the coefficients of estimates (Table 4) are negative both for main effects and for interactive effects. This indicates all the independent variables have favourable effect for the decrease in particle size.

ANOVA results for the optimization of drug loading were summarized in Table 5. Contour plot and the 3D surface response graph for particle size were given in Figure 3 and 4.

Table 5: ANOVA responses of the design for drug loading on NPs.

Source	Sum of squares	DF	Mean of square	F value	P value	
Model	687.21	5	137.44	37.43	< 0.0001	Significant
A- Stirring speed	92.77	1	92.77	25.26	0.0015	
B-Glutaraldehyde	44.57	1	44.57	12.14	0.0102	
AB	45.55	1	45.55	12.40	0.0097	
A^2	451.43	1	451.43	122.93	< 0.0001	
B^2	99.63	1	99.63	27.13	0.0012	
Residual	25.71	7	3.67			
Lack of Fit	18.10	3	6.03	3.17	0.1470	Not significant
Pure Error	7.61	4	1.90			
Cor Total	712.91	12				

Statistical data of the dependent variables obtained were subjected to ANOVA and were found to be significant at $p<0.0001$. Overall interaction was found to be significant at $p>0.05$ indicating, the effect of both the variables when combined together on drug loading is nonsignificant. Lack of fit value $p>0.05$ indicates, the noise of the experiments is nonsignificant.

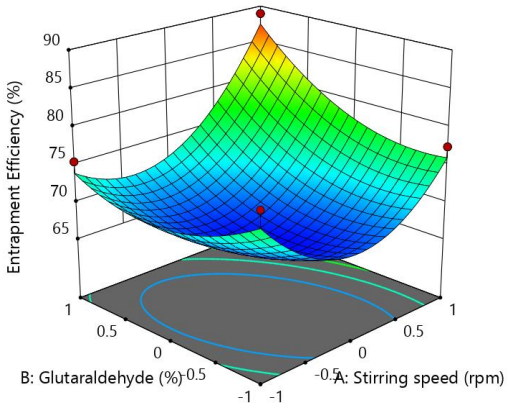

Figure 3: Response 3D plot for the effect of Stirring speed and Glutaraldehyde % on drug loading of the NPs

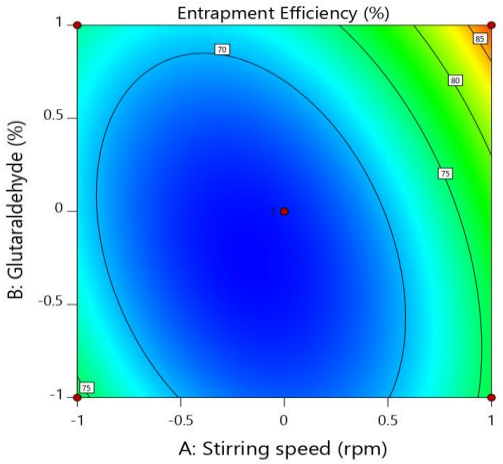

Figure 4: Contour plot for the effect of Stirring speed and Glutaraldehyde % on drug loading of the NPs

Table 5: Coefficient of estimate values for the drug loading on NPs.

Factor	Coefficient of Estimate	DF	SE	95% CI low	95% CI High	VIF
Intercept	66.49	1	0.8570	64.46	68.51	
A- Stirring speed	3.41	1	0.6775	1.80	5.01	1.0000
B-Glutaraldehyde	2.36	1	0.6775	0.7583	3.96	1.0000
AB	3.37	1	0.9581	1.11	5.64	1.0000
A^2	8.06	1	0.7265	6.34	9.77	1.02
B^2	3.78	1	0.7265	2.07	5.50	1.02

All the coefficients of estimates (Table 5) are positive both for main effects and for interactive effects. This indicates all the independent variables have favourable effect for theincrease in drug loading.

Regression analysis of variance for both particle size and drug loading was given in Table 6. The correlation coefficient for the models were also calculated which are found to be > 0.9 indicating good fit. Predicted R^2 was calculated as a measure of how good the model predicts a response value. The adjusted R^2 and predicted R^2 should be within approximately 0.20 of each other to be in reasonable agreement (8). If they are not, there might be a problem with either the data or model. Present model fit values indicate a difference of less than 0.2, indicating an agreement with the adjusted R^2 value in all responses.

Table 6: Regression analysis of the full factorial design of the NPs

Response	Particle Size	Drug loading efficiency
R^2	0.9540	0.9639
Adjusted R^2	0.9211	0.9382
Predicted R^2	0.7745	0.8028
Adequate precision	14.121	16.115

Optimised NPs

The variables used in obtaining NPs are very important in deciding the final characteristic of NPs and are responsible for the execution of therapeutic effect. NPs with small size combined with high-drug loading are the required characteristics to achieve the desired therapeutic effect. In order to continue with the choice of the optimalformulation, it was necessary to establish the stirring speed combined with amount of cross-linking agent that produced greaterinteraction and influenced the desired size and loading.

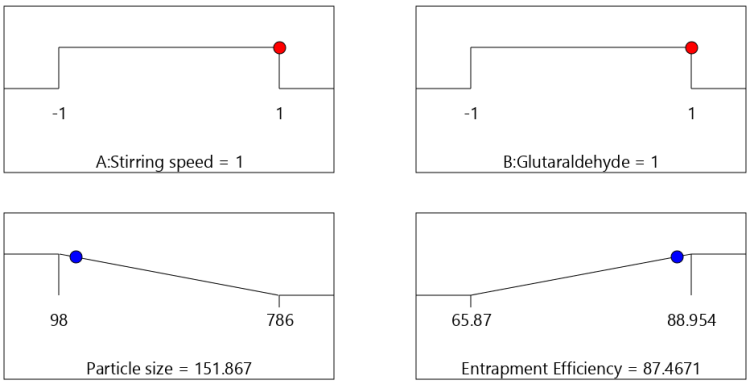

Figure 5: Optimized levels of independent variables for the minimum particle size and maximum drug loading shown in ramp plot.

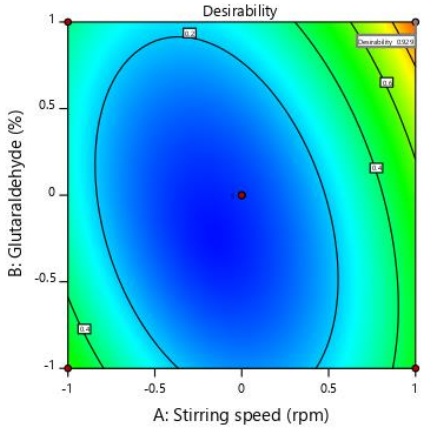

Figure 6: Optimized levels of independent variables for the minimum particle size and maximum drug loading shown in contour plot.

FTIR studies

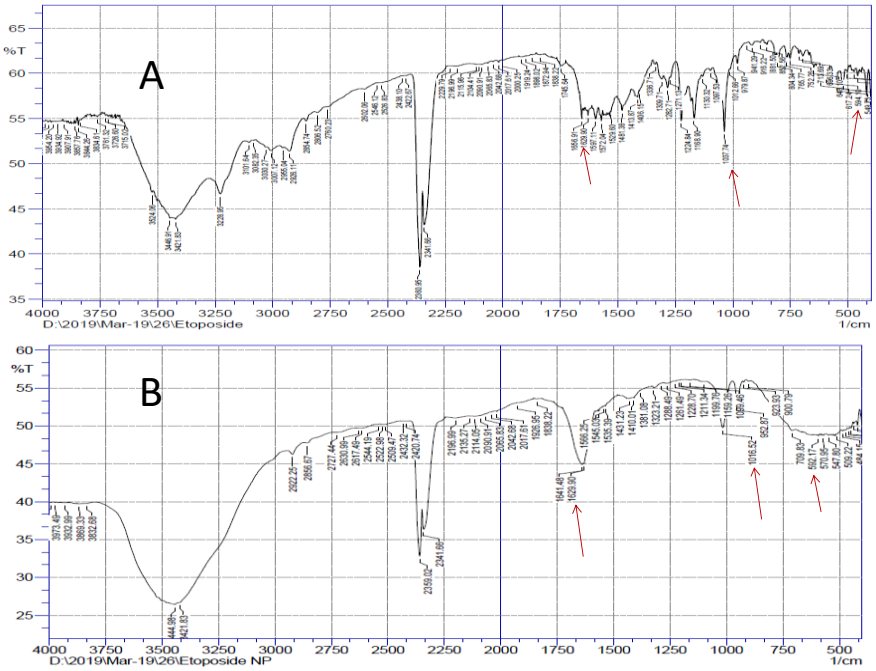

Figure 7: FTIR showing the peaks corresponding to ETP in A and ETP NPs in B

Through factorial design (Figure 5 and 6), it was found that 500rpm (at level 1) and the maximum concentration of glutaraldehyde at 25% (at level 1) with a mean particle size of 151.867nm and an drug loading efficiency of 87.467% with a desirability of 0.929.

Figure 7 shows the FTIR graphs of drug along with the polymer. The peaks corresponding to ETP at 1629cm^{-1}, 1032cm^{-1} and 592cm^{-1} are superimposable with the FTIR of ETP NPs. This is in accordance with the earlier reports (9) and confirms the absence of interactions.

Differential scanning Calorimetry

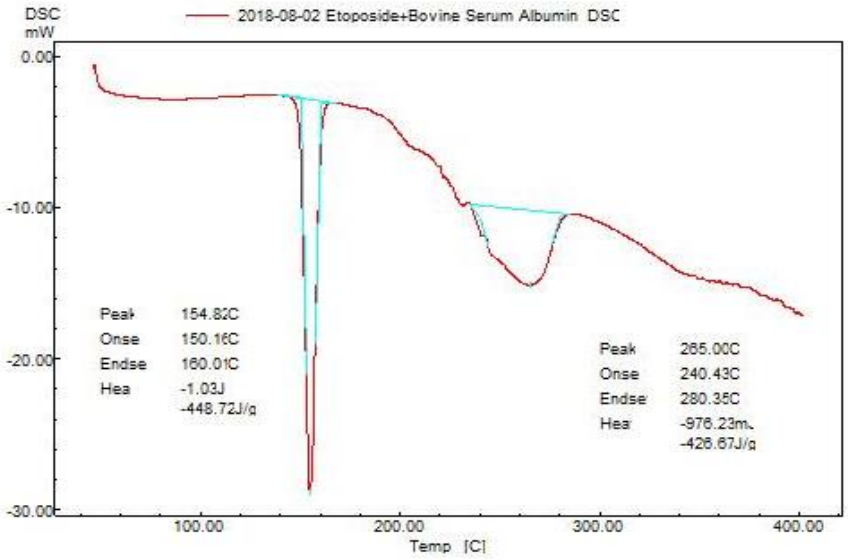

Figure 8: DSC graph showing the endothermic peaks corresponding to ETP and BSA

Thermographs obtained by DSC studies (Figure 8), revealed that the melting point of pure drug and polymer are 265˚C and 154˚C. There is no drastic difference from the literature (10).

Drug loading efficiency

Encapsulation efficiency of the NPs was evaluated and they were found to be between 65.87±2.78 - 88.95±1.09. Among the prepared NPs, 7 formulations were found to have drug loading more than 75%. Both stirring speed and concentration of glutaraldehyde were found to influence the drug loading. The combined effect was found to be nonsignificant than the individual effects.

Zeta Size of NPs

The prepared NPs are with the minimum size of 98±8.76nm and a maximum size of 786.24±6.89nm. We can see the individual and combined effects of glutaraldehyde concentration and stirring speed together significantly influencing the particle size. Particle size distribution curve of NPs is given in Figure 9.

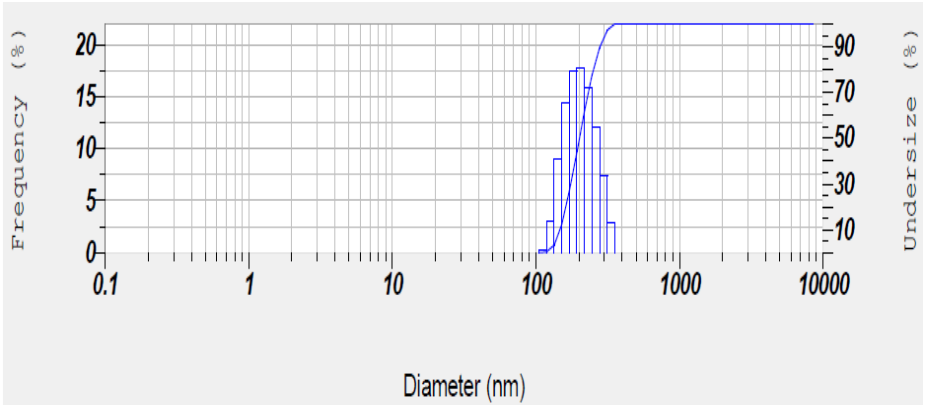

Figure 9: Mean particle size and particle size distribution of the prepared NPs

Scanning Electron Microscopy

From the SEM pictures (Figure 10) of NPs it was observed that, the NPs are almost spherical in shape and are loosely attached with each other. No aggregates were observed indicating the effect of selected stirring speed. Higher stirring speed might be the reason for creating shear enough to cleave the aggregates resulting in loosely attached NPs.

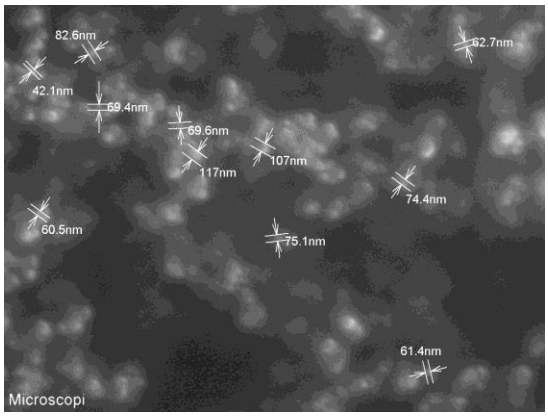

Figure 10: Scanning electron microscopic picture of prepared NPs

Discussion

Factorial analysis allowed the verification of the effect of different components of the formulation simultaneously on NPs characteristics, based on a limited number of 13 experiments. Our results indicated that the proper choice of the stirring speed and concentration of the crosslinking agent are the key factors in determining the NPs mean size and drug loading. Optimal parameters were obtained for NPs composed by keeping 500rpm as stirring speed and 25% Glutaraldehyde as crosslinking agent.

Particle size is a critical feature for NPs as it influences the circulating half-life, cellular uptake and bio-distribution especially in drug targeting. Cellular uptake is also size dependent and smaller particles could be taken up to a greater extent[1]. Present investigation aimed at the study of stirring speed as one of the independent variables affecting the particle size. In the absence of shear stress, low porosity will result. However, in the presence of shear forces, the primary particles form a network that will produce particles with high porosity (11). It also influences the viscosity of the dispersion, the greater the stirring speed, the lower the generated net shear stress (12). At high rpm, the high energy dissipation would lead to increased nucleation rates, which would lead to smaller particles (13). High stirring rate with the energy transferred to the dispersion medium also increases and the reaction solution can be dispersed in to smaller particles and thus the size is reduced. Another reason for this reduction was the anomalous diffusion of particles at higher degree of agitation reduced the growth kinetics of the particles, and resulted in the smaller sized particles (14). Results showed that smaller particles (98nm) were formed when increasing the stirring speed (500rpm).

Glutaraldehyde is one of the cross-linked agents, that is widely used for crosslinking of NPs. It's less cost and more efficiency as crosslinking agent made it popular. It can be used even for control the release rate of drugs effectively (15). It results in smooth surfaced particles (16). This is evident with the smooth surfaced particles observed in SEM picture. Interaction term (AB) for % of crosslinking agent and stirring speed was found to be significant on the size of NPs. This may be due to the reason that, stirring reduces and concentration of crosslinking agent builds up the size of aggregation and both are inter related with each other.

BSA is a protein rich in charged amino acids (lysine) and thus it allows the positive and negative charged molecules to adsorb electrostatically on to its surface. Upon the addition of the drug, it results in spontaneous formation of the NPs due to the electrostatic attraction between the charged drug particles and the BSA particles. Well-defined structure of BSA with charged amino acids; it could favor the electrostatic adsorption of negatively or positively charged molecules, inside or on the surface. Its hydrophobic cavities may facilitate the incorporation of water-insoluble drugs (17). This might be the reason for the loading of ETP on to BSA NPs. Even though the encapsulation efficiency of BSA NPs was due to the electrostatic and hydrophobic interaction between the drug and NPs, particle size will have significant influence on drug loading. Decreased particle size increases the surface area of NPs in turn increases the drug loading due to increased surface area for drug loading. This might be

the reason for the drug loading of 88.95% in NPs with a particle size of 98nm.

Mechanism of protein cross-linking with glutaraldehyde might be nucleophilic attack by the o-amino groups of the lysine residues and the N-terminal amino groups of the proteins. Amino groups about 10 percent of bovine serum albumin protein might be solidified by a condensation reaction with a reagent containing an aldehyde group (18). Glutaraldehyde leads the crosslinking of protein and also results in bridge formation between drug and protein. It can react with several functional groups of proteins, amines, thiol, phenol and imidazole because of more reactive nucleophiles (19). ETP contains phenolic group which may be prone for crosslinking by glutaraldehyde. We observed more % of drug loading in presence of the maximum concentration of the crosslinking agent may be due to decreased leakage of the drug.

Conclusion

For the diseases like cancer, time lapse in designing the formulation may increase the mortality rate. Optimization of formulation through DoE will eliminate the time lapse. Formulation of ETP NPs was optimized by using factorial design and the process parameters that can result the best responses were identified. Through factorial design, it was optimized that 500rpm (at a level of 1) and the maximum concentration of glutaraldehyde at 25% (at a level of 1) will result a least mean particle size of 150.13nm and maximum drug loading of 86.24%.

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