ORIGINAL ARTICLE

Design of Three-factor Response Surface Optimization of Camouflaged Capecitabine Nanoerythrovesicles

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Abstract

Aim: Circulation half-life has become one of the major design considerations in nanoparticulate drug delivery systems. By taking cues for designing long circulating carriers from natural entities such as red blood cells (RBCs) have been explored for many years. Among all the cellular carriers including leukocytes, fibroblast, islets, and hepatocytes, RBCs offer several distinctive features. Nanovesicles (NVs) represent a novel transporter for cell signals to modify functions of target cell. Therefore, NVs play many roles in both physiological and pathological process. **Materials and Methods:** This report highlights biogenesis, composition, and biological rules of erythrocytes-derived NVs (EDNVs). Furthermore, we address utilization of EDNVs as novel drug delivery cargo as well as therapeutic target. EDNVs are biocompatible, biodegradable, efficient drug loading target specificity, and prolonged biological half-life. It is also rich in phospholipids, proteins, lipid raft, and hemoglobin. **Results and Discussion:** In this study, nanosize lipoprotein membrane vesicles (EDNVs) bearing capecitabine were prepared by sonication method. An optimized EDNV was obtained through implementation of Box-Behnken experimental design. Developed capecitabine nanoerythrosomes conjugate formulation were preliminary optimized on the basis of vesicle morphology, size and size distribution, loaded drug concentration, and *in vitro* release studies. **Conclusion:** Targeting efficiency of drug-loaded erythrocytes over free drug is higher, which may provide increased therapeutic index and drug targeting to various organs. It may help in the reduction of dose required for the therapy and there by dose-related systemic side effects could also be minimized.

Key words: Box-Behnken design, capecitabine, erythrocytes-derived nanovesicles, red blood cells

INTRODUCTION

Capecitabine is a prodrug that is converted to fluorouracil in the body tissue following the oral as well as parental administration. It is widely used in the treatment of metastatic colorectal cancer and breast cancer. The adverse effects associated with capecitabine including bone marrow depression, cardiotoxicity, diarrhea, nausea and vomiting, and dermatitis. Hence, formulating capecitabine as a controlled release dosage form would provide greater or longer *in vitro* and *in vivo* antitumor activity, thereby reducing its toxic side effects. Moreover, its adverse effects are enhanced because the drug must be frequently administered at high doses, due to its limited oral bioavailability. To reduce capecitabine side effects and increase its therapeutic efficacy, several studies have suggested different formulations for capecitabine administration such as transdermal systems, nanoparticles microparticles, and capecitabine-loaded erythrocytes.[1] Pharmacokinetic studies revealed that these formulations achieved sustained release, dose reduction, improved stability erythrosomes are preferred as a drug delivery system because they are biocompatible and biodegradable, have a long half-life, and can be loaded with a variety of drugs. Anticancer, antiviral, and antiparasitic drugs are examples of therapeutic agents that have been loaded into nanoerythrocytes. Nanoerythrosomes were used either as a

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carrier for sustained release of the drugs or to accomplish targeted delivery of the drugs to infected organs.

The safety and utilization of nanoerythrocytes as carrier systems have been potentially explored.[2-4] The major problem encountered in the use of biodegradable natural cells as drug carriers is that they are removed *in vivo* by the reticuloendothelial system (RES) as a result of modifications that occur during the loading procedure in cells. Although this expands the capability of erythrocytes to target the RES, it seriously limits their life-span as long-circulating drug carriers in circulation and, in some cases, may pose toxicological.[5]

Utilization of erythrosomes as a drug carrier in humans also has the inherited problems of transfusion of blood from one to another, possible contamination due to the origin of the blood, the equipment used, and the loading environment.[5] Therefore, screenings of these carriers for the absence of diseases as well as rigorous controls are required for the collection and handling of nanoerythrosomes to eliminate any risk of contamination.[6] Furthermore, erythrosomes as a drug carrier raise other potential concerns due to the changes in their biochemical nature. Drugs or other bioactive agents can be loaded in nanoerythrocytes either by physical methods or by chemical methods.[7] For successful entrapment into the erythrocytes, the drug should have a degree of water solubility and resistance to degradation within erythrocytes. Certain drugs have been entrapped in erythrocytes by endocytosis, including vinblastine, chlorpromazine, hydrocortisone, propranolol, tetracaine, retinol, and capecitabine.[8] Anchoring the drugs to red blood cell (RBC) membranes with the help of nanotechnology is a powerful method to load the drug that shows binding affinity toward the membrane protein and remains effective during the long circulation lifetime of the carrier. RBCs can be engineered to load specific drugs to facilitate the localized delivery of chemotherapeutic drugs in target-specific organs, which can prolong period of time in circulation and to overcome systemic toxicity. The conjugation of the drug on cell membrane surface enhances their survival and avoids drug leaching during the transit time. Biodegradable lipoproteosomes are small vesicles that are produced from RBCs by hypotonic lysis method to produce erythrocyte ghosts by removing their hemoglobin content.[9,10] Subsequently, these erythrocyte ghosts were extruded to form small vesicles having a mean diameter of about 100 nm.[10]

The use of Box-Behnken experimental design is required to map the optimal composition range for process parameters; this technique is mainly used to map the optimum nanoemulsion, tablets, suspensions, etc.[11-15]

In the present work, the preparation of capecitabine-loaded nucleotide excision repairs (NERs) is discussed and use of BBD explored to map the optimal composition range and can be used to show the influence of changes in the volume fractions of the different tonic phases on the drug content and entrapment efficiency of the NERs. Therefore, the present study aims to improve physicochemical properties and anticancer activity of capecitabine. The capecitabine was conjugated on the carrier membrane for sustained and targeted drug delivery, to avoid drug leakage, to improve the circulatory time of carrier, to increase the stability, and to reduce cost and toxicities of capecitabine therapy which would present a significant advantage over many conventional systemically administered formulations. The study focuses on development and optimization of NERs-based formulation of capecitabine by probe sonication method and subsequently drug loading with the help of hypotonic preswelling method.[16]

MATERIALS AND METHODS

Materials

Analytical grade materials were used for study. Capecitabine (Reliance Life Science, India) were received as gift sample. Acetonitrile, chloroform, glutaraldehyde, distilled water, and phosphate buffer pH (7.4) were also used throughout the study. All other chemicals and reagent were of analytical grade and were used without further purification.

Isolation of ghost

The whole blood obtained from registered blood bank (Galaxy Hospital, Maharashtra, India) was centrifuged at 3000 rpm for 5 min at 4 ± 1°C in cooling centrifuge (Remi Corp., Mumbai). The serum and buffy coats were removed by washing three times with normal saline. The washed erythrocytes were hemolyzed with distilled water and centrifuged at 2000 rpm for 15 min and stored at 4°C until further use.[17] Erythrocytes were hemolyzed by incubating them sequentially in 50 and 30 mOsm hypotonic solutions, prepared from isotonic normal saline solution (~300 mOsm). The hemoglobin in the supernatant was removed after centrifugation and cream-colored pellet was resuspended in hypotonic solutions and subjected to centrifugation again. The colorless ghosts thus obtained were incubated in hypertonic solution for 60 min at 37°C for resealing.[18] The resulting sealed ghosts were washed 3 times with isotonic normal saline and stored at 4°C until further use.

Preparation of nanoerythrosomes

Nanoerythrosomes were prepared by reducing the size of erythrocyte ghosts containing capecitabine. Two size reduction methods were used to prepare nanosized erythrosomes: Bath sonication (Sonic, Unique Biologicals, Kolhapur, India) and probe sonication (Remi 210, Remi Corp., Mumbai). Sonication was performed for 15 min at 25°C, and the drug was analyzed as indicated above. The

drug loading in membrane ghost was done using hypotonic Preswell dilutional method [Figure 1]. Glutaraldehyde solution was used as cross-linking agent, resealed using hypertonic solution, and incubated for 15 min, under refrigerated condition at 4 ± 1°C. Sonication process was done for 25 min.[19,20]

Box-Behnken experimental design

The objective of the present study selected as maximizing the drug content while entrapment efficiency. Hence, Box-Behnken statistically designed with 3 factors, 3 levels, and 15 run was selected to statistically optimized the formulation parameters and evaluate the main, quadratic, and interaction effects of the preparations on the drug content and entrapment efficiency of NERs. 3 factors, 3 level designs were used to explore the quadratic response surface and for constructing the polynomial models thus helping in optimizing a process using a small number of experimental runs.[21,22] The experimental design consists of a set of points lying at the midpoint of each edge and the replicated center point of the multidimensional cube.

The independent and dependent variables are listed in Table 1. The polynomial equation generated by this experimental design is as follows:

$$Y_i = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2$$

Where, Y_i is the dependent variables, b_0 is the intercept, b_1 to b_{33} are the regression coefficients computed from the observed experimental values of Y from experimental runs; X_1, X_2, and X_3 are the independent variables that were selected from the preliminary experiments.

$X_1 = (A - X_0)/\Delta X$; X_1 = Coded value of the variables A; X_0 = Value of A at the center point; ΔX = Step change and so on where A, B, etc., are the input variables.

Table 1: Box Behnken experimental design for preparation of capecitabine loaded nanoerythrosomes

Run order	Independent variables			Dependent variables	
	(X_1)	(X_2)	(X_3)	(Y_1)	(Y_2)
1	1	0	1	52.80	47.2
2	−1	0	−1	86.2	13.8
3	0	0	0	77.6	22.4
4	−1	0	1	77.20	22.80
5	−1	−1	0	91.50	8.50
6	0	−1	−1	72.5	27.5
7	0	0	0	77.6	22.4
8	1	0	−1	85.20	12.80
9	0	1	1	85.8	11.5
10	1	1	0	91.95	8.1
11	0	1	−1	51.74	48.3
12	0	0	0	77.6	22.4
13	−1	1	0	85.2	14.8
14	0	−1	1	78.5	21.50
15	1	−1	0	87.84	12.2

Independent variables	Levels			Dependent variables
X_1=Sonication time (min)	5	10	15	Y_1=Drug content (%)
X_2=Hypotonic solution %	0.6	0.7	0.8	Y_2=Entrapment efficiency (%)
X_3=Hypertonic solution %	1	1.1	1.2	

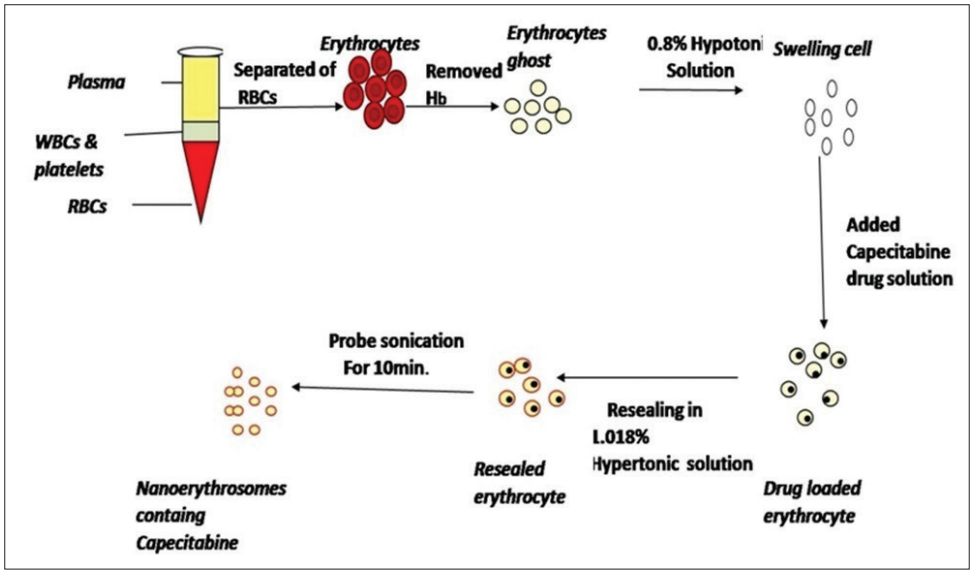

Figure 1: Preparation of capecitabine-loaded nanoerythrosomes

Independent variables were hypotonic solution (X_1), hypertonic solution (X_2), and sonication time (X_3). The dependent variables were drug content (Y_1) and entrapment efficiency (Y_2). The range of independent variables in study [Table 1] along with their low, medium, and high levels, which were selected based on the result from preliminary experiments.

Statistical analysis

Statistical analysis of the Box-Behnken design batches was performed by multiple regression analysis using Relia soft DOE++ software. The models were evaluated in terms of R^2 values, and statistically significant coefficient and various feasibility and grid searches were conducted to find the optimum parameters. To graphically demonstrate the influence of each factor on the response, the response surface plot was generated using the Relia soft DOE++ software.[23]

Optimization data analysis

The computation for optimized formulation was carried using Relia soft DOE++ software. The optimized formulation was obtained by applying constraints (goals) on dependent and independent variables. After developing the polynomial equation for the response drug content and entrapment efficiency with the independent variables, the formulation was optimized for the both responses. Optimization was performed to find out the level of independent variables (X_1, X_2, and X_3) that would yield a maximum value of drug content constraints on entrapment efficiency.[24]

Characterization of NERs

Drug content and entrapment efficiency

Drug content was determined by deproteinized loaded cells done using acetonitrile followed by centrifugation, nanoerythrosomes was added in acetonitrile (10 mL) and centrifuged.[25] The supernatant was collected and the drug content was estimated using ultraviolet (UV)-visible (Cary 60, 2100, Agilent Technology, Germany) spectrophotometric method at 245 nm.

$$\% \text{ Drug content} = \frac{\text{Amount of drug loaded}}{\text{Amount of drug added}} \times 100$$

$$\% \text{ Entrapment efficiency} = \frac{\text{Amount of drug added} - \text{amount of drug loaded}}{\text{Amount of drug added}} \times 100$$

Hemolysis

The tendency of NERs to hemolyze blood was studied by incubating NERs with the whole blood and subsequently measuring the degree of hemolysis. NERs containing capecitabine were added to whole blood at either 1:1 or 2:1 ratio and incubated at 37°C for 30 min in the dark followed by centrifugation for 10 min at 5000 rpm. The amount of hemoglobin released was determined in the supernatant by measuring the absorbance at 245 nm using a UV-visible spectrophotometer.[26] A completely lysed blood sample (prepared by adding distilled water to whole blood) was used as a control. The percent hemoglobin release was calculated using the following equation:

$$\text{Percent hemolysed} = \frac{(\text{A540 of sample} - \text{A540 of background})}{(\text{A540 of100\% Hemoglobin})} \times 100$$

A540 is the absorbance at 540 nm.

Hematological indices

Control erythrocytes, blank erythrocytes, and capecitabine-loaded NERs were hematologically characterized. The mean corpuscular volume (MCV), the mean corpuscular hemoglobin (MCH), the MCH concentration (MCHC), and the hematocrit were measured using a hematology analyzer.[27] To estimate the morphological variation between normal and capecitabine-loaded NERs, both normal and capecitabine-loaded erythrocyte samples were examined using a scanning electron microscope (SEM). NERs were diluted 1:10 ml using autologous plasma and then mixed by several gentle inversions. The mixture was aliquoted into Eppendorf tubes. The samples were incubated at 37°C and rotated vertically. Samples were removed at 0.5, 1, 2, 4, 8, 12, 24, and 48 h and then centrifuged at 3000 rpm for 5 min. 100 µl of the supernatant was separated for capecitabine assays, and the remaining portion was centrifuged for 5 min. The supernatant was used for hemoglobin analysis using measuring the absorbance at 540 nm. The results are expressed as percentages of the absorbance of a completely hemolyzed sample.[28]

Osmotic shock

Capecitabine contained NERs were incubated with distilled water for 15 min followed by centrifugation at 3000 rpm for 10 min and may cause the release of drug, and it was estimated using the UV-Visible spectrophotometer at 245 nm.[29]

Osmotic fragility

The osmotic fragility test was used to assess the ability of erythrocyte membranes to resist lysis caused by exposure to solutions of NaCl ranging from 0.0 to 0.9 g%. A 25 µL NERs sample was added to each of a series of 2.5 mL saline solutions containing 0.1-0.9 g% NaCl. After gentle mixing and standing for 15 min at room temperature, the NERs suspensions were centrifuged at 5000 rpm for 5 min. The absorbance of the supernatant was measured at 540 nm. The

released hemoglobin was expressed as percentage absorbance of each sample in reference to a completely lysed sample prepared by diluting packed cells of each type with 1.5 ml of distilled water.[30]

Turbulence fragility

Aliquots of 0.5 mL of packed erythrocytes of each of the three types were suspended in 10 ml of phosphate-buffered saline (PBS) in polypropylene test tubes and shaken vigorously using a multiple test tube orbital shaker at 2000 rpm for 4 h. To evaluate the time course of hemoglobin release, 0.5 ml portions of each suspension were withdrawn at 0, 1, 2, 3, 4, and 5 h. The samples were centrifuged at 1000 g for 10 min, and the absorbance of each supernatant was determined spectrophotometrically at 245 nm. The percent hemoglobin release was determined relative to that of a completely lysed suspension with the same cell fraction (i.e., 0.5 ml packed cells added to 10 ml of distilled water). To compare the turbulence fragilities of the different types of erythrocytes, the turbulence fragility index was used.[31,32] This value is calculated as the shaking time required to produce 20% hemoglobin release from erythrocytes.

In vitro drug release

Release of capecitabine from NERs was studied using the dialysis (Slide-A-Lyzer, 3500 MWCO, Thermo-Scientific) method at 37 ± 2°C and was compared with the pure drug solution. Briefly, the dialysis bags were first hydrated for 30-60 min with PBS (pH 7.4) and NERs (500 µL) were loaded carefully using a syringe without puncturing the dialysis membrane. Then, the tubes were immersed in 100 ml of release medium PBS (pH 7.4). While stirred the release medium using the magnetic stirrer at 150 rpm/min, 1 ml samples were withdrawn at predetermined time intervals. From the release medium and the same volume was replaced with fresh medium.[15,33-35] The sample was analyzed at 245 nm using the UV-visible spectrophotometer (Shimadzu Corporation, Japan).

Vesicle size

Vesicle size and polydispersity index of drug-loaded nanoerythrosomes were measured using dynamic light scattering method using Zetasizer, ZEM5002 (Malvern Instruments Ltd., Worcestershire, UK) at a fixed angle of 90°.[36]

RESULTS AND DISCUSSION

In the present investigation, we sought to develop and explore the potential of NERs for delivery of an anticancer drug, capecitabine. Thus, we have performed a series of *in vitro* studies to optimize drug loading of capecitabine encapsulated NERs.

Isolation of ghost

To prepare cells with surface pores, we incubated erythrocytes in hypotonic solutions of varying strengths. By controlling the osmolarity, cells with surface pores between 10 and 500 nm can be prepared. When cells were incubated in 30 mOsm solution, we obtained cell ghosts completely devoid of hemoglobin and intracellular organelles; hemoglobin depleted cells had numerous pores on the surface. The pores on ghost cell membranes were then closed by incubating in hypertonic solution (PBS ×10) at 37°C that resulted in spherical ghost cells. After optimization of preparation parameters, we examined the morphological features of resealed ghosts under a microscope. Hypertonic solution mediated resealing was efficient in retaining spherical morphology of the NERs.

Drug loading

The loaded amount, the entrapment efficiency, and the percent cell recovery were determined.[37] The UV method was used to estimate the capecitabine content of the supernatants after the incubation of erythrocytes with capecitabine. The obtained data indicate that 70 µg of capecitabine was loaded, with an entrapment efficiency of 29.3%. This amount is notable in comparison to those values reported in the literature for a variety of drugs. The observed cell recovery of approximately 85.94% is comparable to the recovery results for various drugs reported in other studies.[32-34]

Design of experiment

The traditional approaches to developing a formulation are to change one variable at a time. By this method, it is difficult to develop an optimized formulation, as the method reveals nothing about the interaction among the variables. The use of experimental design allows for testing a large number of factors simultaneously and precludes the use of a huge number of independent runs when the traditional step-by-step approaches are used.[11] Systematic optimization procedures are carried out by selecting an objective function, finding the most important or contributing factor, and investigating the relationship between response and factors by the so-called surface response methodology.

Nanoerythrosomes were prepared by Preswell dilution techniques and optimized the process using Box-Behnken experimental design. The objective functions for the present study were selected as maximizing the drug content and entrapment efficiency as responses depending on three independent variables sonication time, hypotonic solution, and hypertonic solution at three different levels. Hence, Box-Behnken statistical design with 3 factors, 3 levels, and 15 runs was selected to statistically optimize the formulation parameters and evaluate the main, interaction, and quadratic

effects of the formulation ingredients on the drug content and entrapment efficiency of nanoerythrosomes.[12] 3-factor, 3-level design was used to explore the quadratic response surfaces and for constructing polynomial models thus helping in optimizing a process using a small number of experimental runs.

Statistical analysis of the Box-Behnken design batches was performed by multiple regression analysis using Relia soft DOE. The contribution of each factor with different levels to the response was evaluated with two-way analysis of variance (ANOVA). The models were evaluated in terms of statistically significant coefficients and R^2 values.[13,14] The experimental design consists of a set of points lying at the midpoint of each edge and the replicated center point of the multidimensional cube [Table 1].

Data analysis

All the batches of prepared within the experimental design yielded NERs, and these were evaluated for drug content and entrapment efficiency. The Box-Behnken experimental design has the advantages of requiring fewer experiments (15 batches) than would a 3^2 full factorial design. Transformed values of all the batches were shown in Table 1. The all selected dependent variables obtained at various levels of the three independent variables (X_1, X_2, and X_3) were subjected to multiple regression to yield a second polynomial equation.[15]

Probability plots

Normal probability graph explains the whether the residuals follow a normal distribution, in which case the points will follow a straight line. Expect some scatter even with normal data. Look only for definite patterns like an "S-shaped" curve, which indicates that a transformation of the response may provide a better analysis, the plot shown by drug content and entrapment efficiency [Figures 2 and 3]. From this concluded that the normal probability distribution the blue spot indicates the nonsignificant effect on variable distributed around the straight line.[14]

Pareto chart

The ANOVA Pareto chart was used to investigate the standardized effect of the independent variables and their interaction on the dependent variables as drug content (Y_1) and entrapment efficiency (Y_2), which depicts the main effect of the independent variables and interactions with their relative nonsignificance on the Y_1 and Y_2. The length of each bar below significance or critical line detonated by blue in the chart indicates the standardized effect of that factor in the responses.[14] Factor remains inside the reference line indicate that these terms contribute the least in prediction of responses so form the Pareto chart concluded that for linear, interaction, and quadratic effect showed nonsignificance effect on drug content (Y_1) and entrapment efficiency (Y_2) response [Figures 4 and 5].

Contour plot

Two-dimensional contour plots [Figures 6 and 7] are useful to study the single and interaction effect of the factor on the responses at one time and the third factor was kept at a constant level.[23] All the relationships among the three variables are linear up to certain range the effect of X_1 and X_2 with their not interaction on drug content at a fixed or the level X_3. The plots were found to be linear up to 95.96%

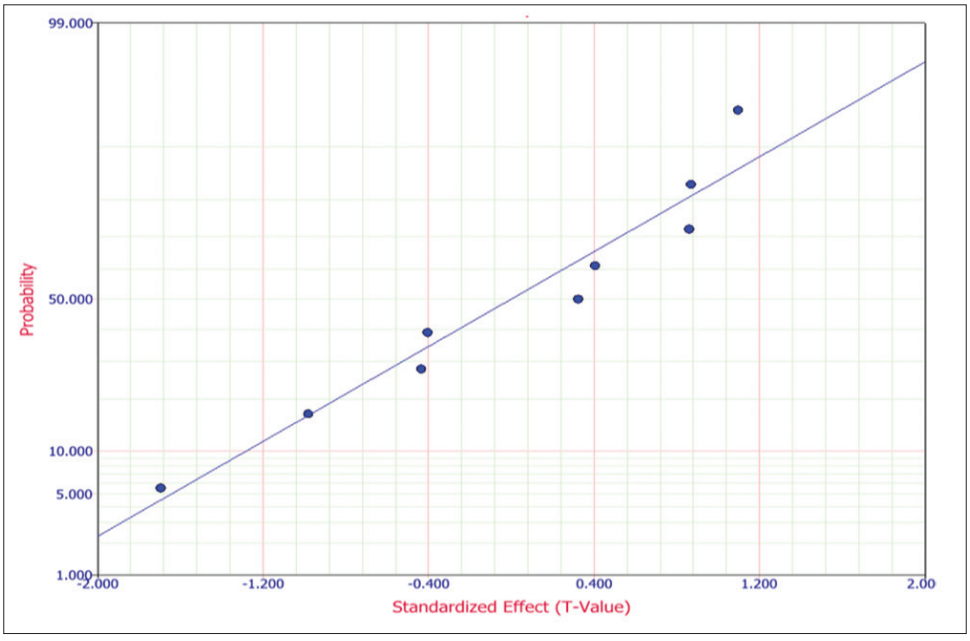

Figure 2: Normal probability plot of drug content of capecitabine-loaded nucleotide excision repairs

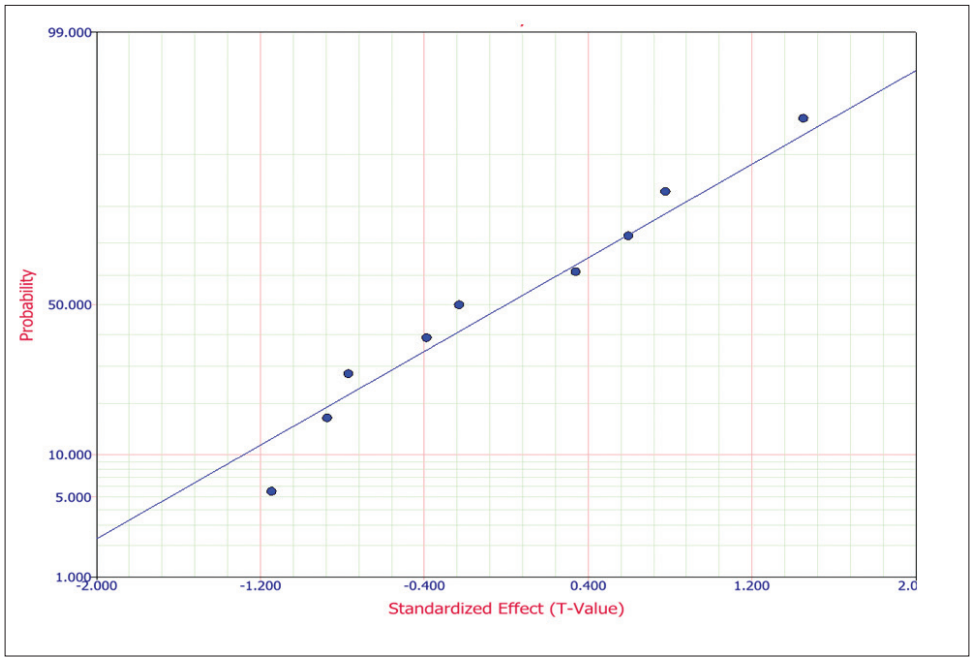

Figure 3: Normal probability plot of entrapment efficiency of capecitabine-loaded nucleotide excision repairs

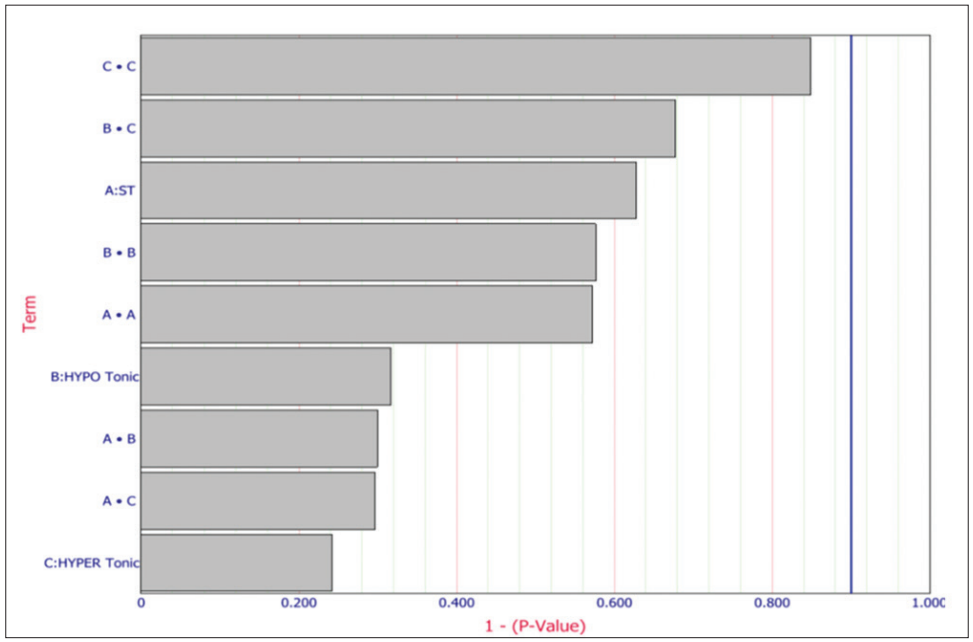

Figure 4: Analysis of variance Pareto chart showing the standardized effect of independent variables and their interaction on drug content

indicating a linear relationship between X_1 and X_2. Similarly, all values for reminded dependent variables. An optimum value of drug content could be obtained with and X_1 level range from 93.82% to 95.96% and X_2 at 18.98% to 20.99%.

Interaction plot

It was showed that change in sonication time of independent variables on the response is easy to interpret one-factor interaction from this plot.[24] It will be appeared with two nonparallel lines, hence indicating that the effect of one-factor independent on the response [Figures 8 and 9]. From this concluded that drug content and entrapment efficiency indicating nonsignificance effect on the response.

ANOVA, pure error, and lack of fit

The results of ANOVA demonstrate that the model was nonsignificant for all dependent variables [Tables 2 and 3]. Regression analysis was carried out to determine the

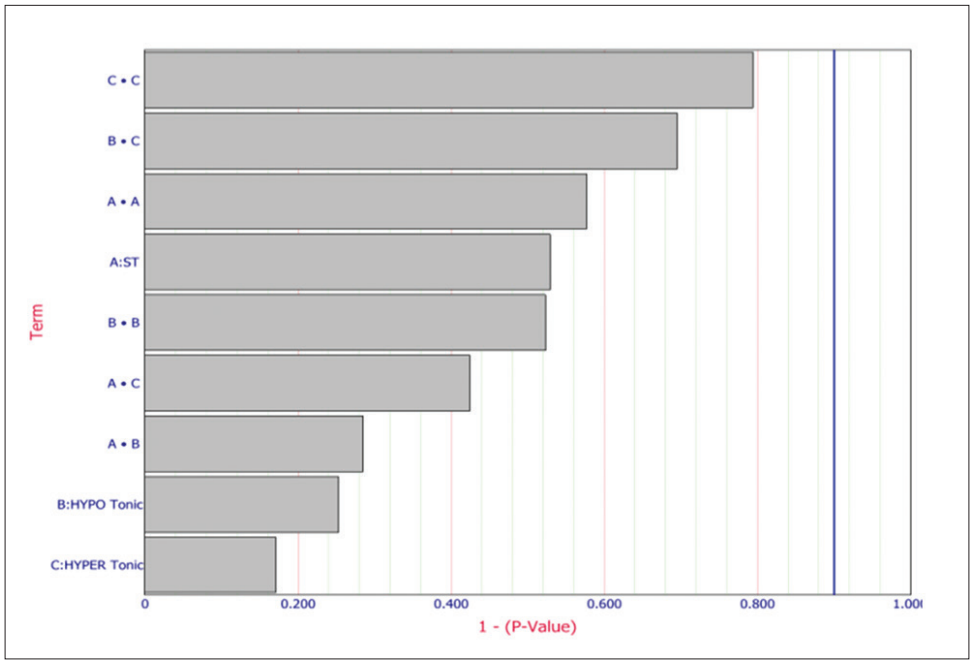

Figure 5: Analysis of variance Pareto chart showing the standardized effect of independent variables and their interaction on entrapment efficiency

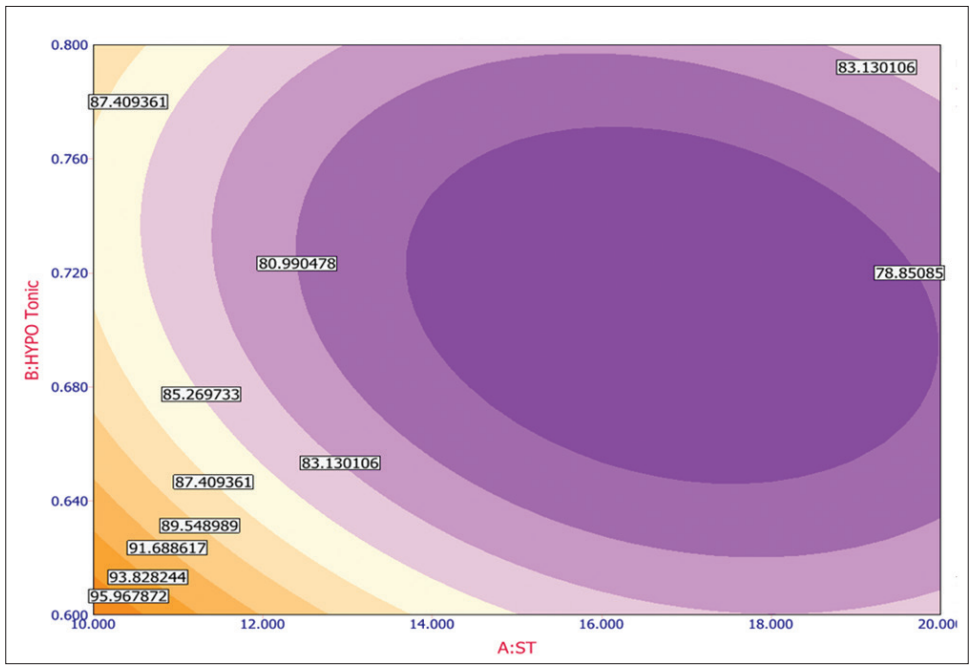

Figure 6: Contour plot showing effect of sonication time (X_1), concentration of hypotonic solution (X_2), and concentration of hypertonic solution (X_3) on drug content

regression coefficient, and all the independent variables were found to be nonsignificant for all response variables. The linear as well as quadratic model was found to be not significant for Y_1 and linear model for Y_2. So, above result indicates that both the factors not play an important role in the formulation of NERs containing capecitabine. The pure error and lack of fit [Tables 2 and 3] can provide a mean response and an estimate of pure experimental uncertainty. The residual is the difference between observed and predicted values.[23,24]

The ANOVA for the dependent variables demonstrates that the model was not significant for all response variables. The effects are like the concentrations of hypotonic solution and sonication time were found to be not significant along with its quadratic and interaction terms for all the dependent variables. Hence, the above results lead us to believe that the all independent variables are not play important role and optimal concentration in NERs gives rise to optimum entrapment efficiency and drug content.

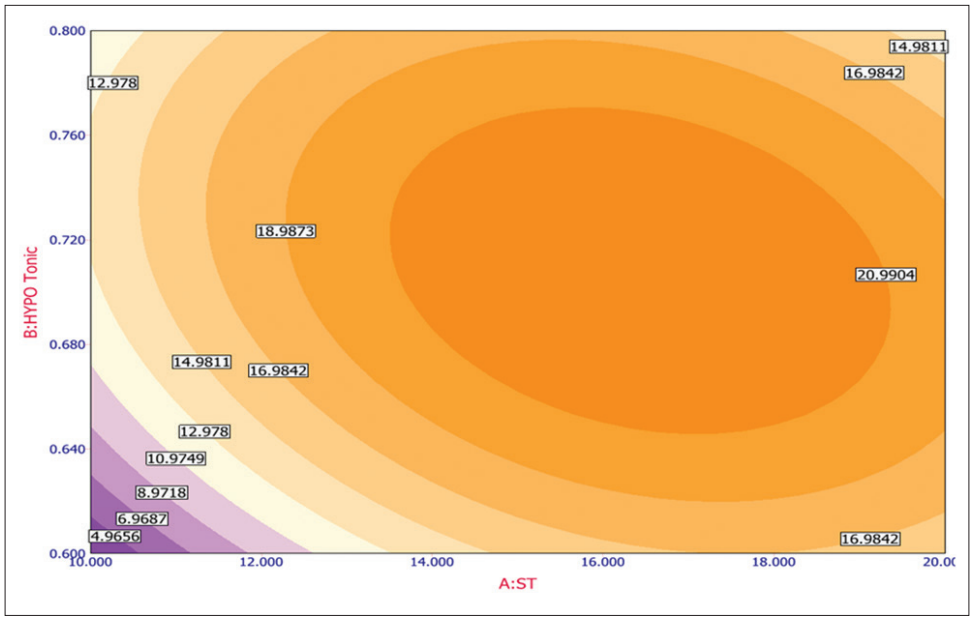

Figure 7: Contour plot showing effect of sonication time (X_1), concentration of hypotonic solution (X_2), and concentration of hypertonic solution (X_3) on entrapment efficiency

The data for pure error and lack of fit provide a mean response and estimate of pure experimental uncertainty. The residual value represents the difference between observed and predicted value respectively. The computed F-value was lower than critical F-values, which denotes non significance with regard to lack of fit [Tables 2 and 3].

The three replicated center point in Box-Behnken experimental design made it possible to assess the pure error of the experiments and enabled the models lack of fit to be checked. In this study, the model was checked for lack of fit for the all the responses. For lack of fit *P* values, we obtained are not showed for response Y_1 and Y_2, and hence, the current model provided a satisfactory fit to the data and had no lack of fit.[11-15] The statistical nonsignificance of each effect was tested by comparing the mean square against and estimates of the experimental error. It was noted X_1, X_2, and X_3 with their interaction effect other than X_1X_2 and quadratic effect had *P* value more than 0.05, indicating nonsignificance effect of this variables in prediction of *X*, whereas linear effect X_1 interaction effect X_1 X_3 and quadratic effect of X_{22}, *X*33 indicating nonsignificance effect of this variables in prediction of response Y_2 [Tables 2 and 3] because of having *P* value is also more than 0.05. Standard error indicates the standard deviation of the coefficient.

Table 2: ANOVA of drug content of capecitabine loaded NER's

Source of variation	*F* value	*P* value
Model	0.835433	0.617109
A: Sonication time	0.960108	0.37216
B: Hypotonic concentration	0.187541	0.683016
C: Hypertonic concentration	0.105897	0.758041
AB	0.165959	0.700584
AC	0.16247	0.70355
BC	1.205795	0.32221
A2	0.742942	0.428114
B2	0.758579	0.423611
C2	2.865784	0.151216
Residual		
Lack of fit	-	-
Pure error	-	-
Corre total		
S=12.77	PRESS=13059	
R^2=60.06%	R^2(adjusted)=0%	
R^2(pred)=0%		

NER: Nucleotide excision repairs, ANOVA: Analysis of variance

Polynomial equation in terms of coded values

The negative coefficient of X_1 suggests decrease in sonication time and has inversely proportional relationship with drug content, and same coefficient is observed with X_2, clearly indicates that individual effect of X_1 and X_2 have negative coefficients. However, in case of X_3, it has positive effect. Likewise interaction effect and quadratic effect of X_1, X_2, and X_3 on drug content showed the positive coefficient except the interaction effect of X_1X_3 and quadratic effect of X_3.

$$\text{Drug content } (Y_1) = 77.6 - 4.42X_1 - 1.95X_2 + 1.47X_3 + 2.60X_1X_2 - 2.57X_1X_3 + 7.01X_2X_3 + 5.73X_{11} + 5.79X_{22} - 11.25X_{33}$$

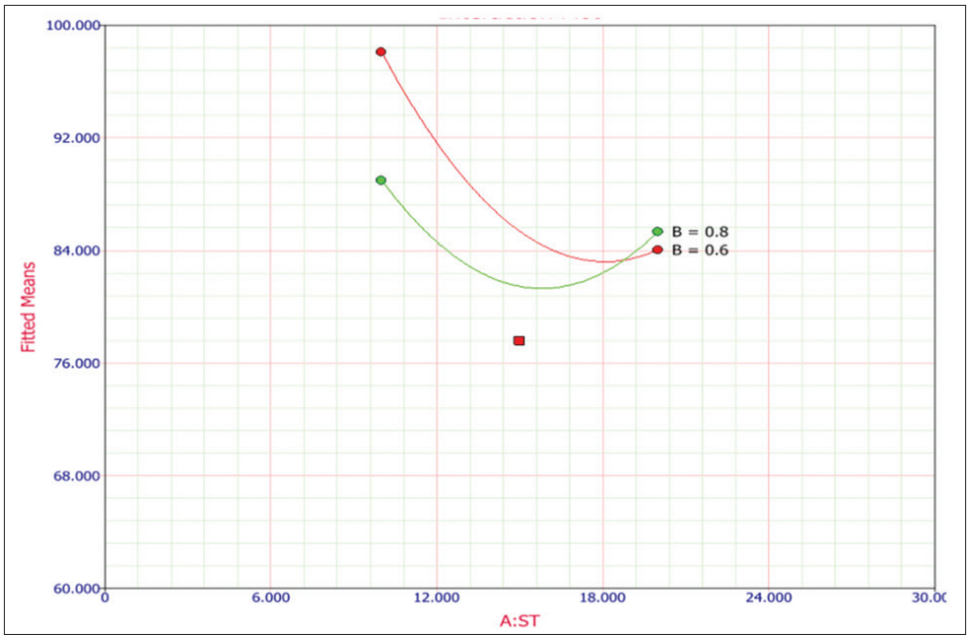

Figure 8: Interaction plot showing effect of sonication time on drug content (Y_1)

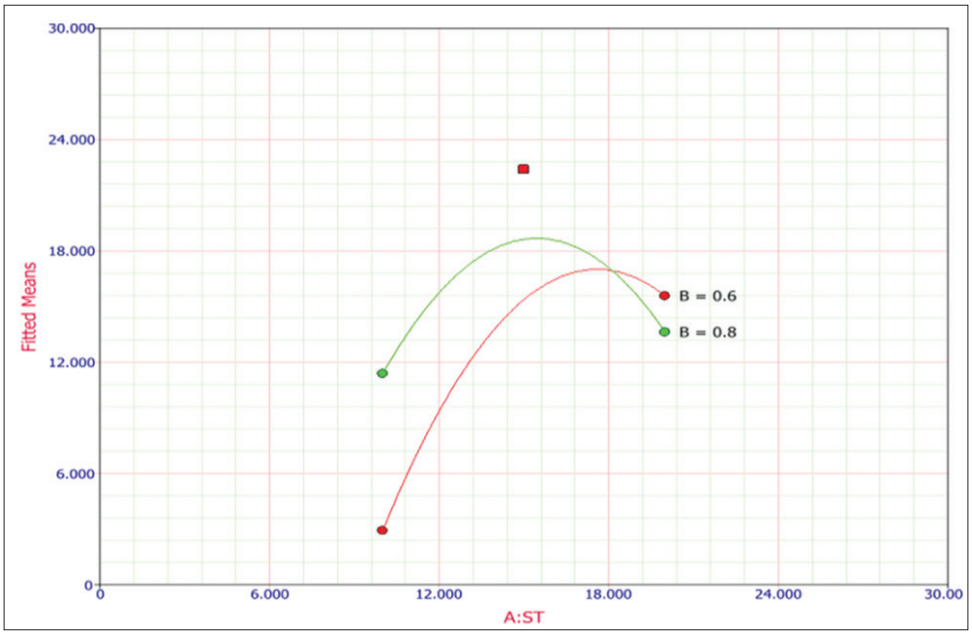

Figure 9: Interaction plot showing effect of sonication time on entrapment efficiency (Y_2)

The positive coefficient of X_1 suggests increase in sonication time and has directly proportional relationship with entrapment efficiency, and the same coefficient is observed with X_2, clearly indicates that individual effect of X_1 and X_2 have positive coefficients. However, in case of X_3, it has a negative effect. Likewise interaction effect and quadratic effect of X_1, X_2, and X_3 on entrapment efficiency showed the negative coefficient except the interaction effect of X_1X_3 and quadratic effect of X_3.

Entrapment efficiency $(Y_2) = 22.4 + 3.71X_1 + 1.62X_2 - 1.08X_3 - 2.6X_1X_2 + 4.02X_1X_3 - 7.7X_2X_3 - 6.11X_{11} - 5.38X_{22} + 10.18X_{33}$

The three replicated center point in Box-Behnken experimental design made it possible to assess the pure error of the experiments and enabled the models lack of fit to be checked. In this study, the model was checked for lack of fit for the all the responses.[14] For lack of fit *P* values, we obtained are not showed for response Y_1 and Y_2, and hence, the current model provided a satisfactory fit to the data and had no lack of fit.

Optimal solution

After using the desirability approach, optimal solution suggested by Relia soft DOE ++ was used for further study

[Figure 10]. Coded and actual values of independent variable clearly state that when hypotonic solution concentration increases drug content of system increases, and then later, it get decreased up to 10 min of sonication time.[24] In the preparation, the hypotonic solution from 0.6% to 0.8% showed better drug content, for 1.018 concentrations and 0.8% hypotonic solution showed excellent entrapment efficiency. The optimized solution [Table 4] predicts that 10 min sonication time, 0.8% hypotonic solution, and 0.8% hypertonic solution concentration as independent variables and 72.38% drug content and 28.74% entrapment efficiency for preparation of nanoerythrosomes.

Characterization of NERs

Hemolysis

An increase in erythrocyte hemolysis destabilizes the hemestructure in hemoglobin molecules, leading to a release of free iron ions that generate more free radicals. Moreover, the presence of capecitabine in the media surrounding the erythrocytes promotes the production of reactive oxygen species (ROS). Furthermore, several studies have reported that capecitabine stimulates ROS production. The *in vitro* hemolysis study was performed at different time intervals of standard ghost sample and optimized capecitabine-loaded nanoerythrosomes.[26] The % HR observed [Figure 11] maximum hemolysis was observed at 4 h. The results may conclude that, nanoerythrocytes does not loss the cell integrity after increase in their time of shaking.[26] This study carried out at 4 h and amount of percent hemolysis was found to be 33% at 4 h.

Hematological indices

The major hematological indices of the control, ghost cell, and optimized capecitabine-loaded NERs are shown in Table 5. These parameters, which are measured as part of routine clinical hematology tests, may provide some useful estimates of the biological state of the erythrocytes.[27,28] The results of the present study showed that significant changes in erythrocyte volume were caused by the entrapment process in Ghost and optimized capecitabine loading, as indicated by the MCV values. However, both the MCH and the MCHC decreased following the exposure of the erythrocytes to the loading procedure, in ghost and capecitabine-loaded erythrocytes. The overall loss of hemoglobin from the erythrocytes upon loading procedure was expected because the procedure is destructive in nature. In similar studies, all of these parameters were found to be lower in carrier erythrocytes than in normal unloaded cells.

The cholesterol and protein level in nanoerythrocytes was estimated using the UV-visible spectrophotometer. The cholesterol levels of nanoerythrocytes membrane were found to be 102.53 mg/dl, and protein level was found to be 14.80 g/dl. Protein value which was found to be a normal range (not more than 13.24 ± 1.82 mg/dL)[27,28] and it may conclude that protein was present in the NERs. the cholesterol level was significantly less than the normal value (not more than 128.17 ± 14.2 mg/dL) from this concluded that the

Table 3: ANOVA of entrapment efficiency of capecitabine loaded NER's

Source of variation	*F* ratio	*P* value
Model	0.703861	0.695568
A: Sonication time	0.607328	0.471054
B: Hypotonic concentration	0.116358	0.746885
C: Hypertonic concentration	0.052113	0.828468
AB	0.148938	0.715427
AC	0.356937	0.576238
BC	1.306296	0.304823
A2	0.759864	0.423245
B2	0.5903	0.477003
C2	2.110733	0.205998
Residual		
Lack of fit	1.598221E+31	0
Pure error	-	
Core total		
S=13.474105	PRESS=14524	
R^2=55.89	R^2(adjusted)=0%	
R^2(pred)=0%		

NER: Nucleotide excision repairs, ANOVA: Analysis of variance

Table 4: Optimal solution showing values of independent and dependent variables suggested by desirability approach of software

Factor	Value	Response	Value
A: Sonication time	10	Drug content	72.38
B: Hypotonic solution concentration	0.8	Entrapment efficiency	28.74
C: Hypertonic solution concentration	1.018		

Table 5: Hematological parameters of control erythrocytes, ghost erythrocytes and capecitabine loaded NER's

Test	Ghost	Capecitabine loaded NER's	Control
MCV (fL)	67.0±3	69.1±9.9	90.9±1.2
MCH (pg)	25.3±0.5	29±1.1	31.6±2.5
MCHC (g/dL)	33.4±1.3	31.1±4.6	33.2±1.1

MCV: Mean corpuscular volume, MCH: Mean corpuscular hemoglobin, MCHC: Mean corpuscular hemoglobin concentration

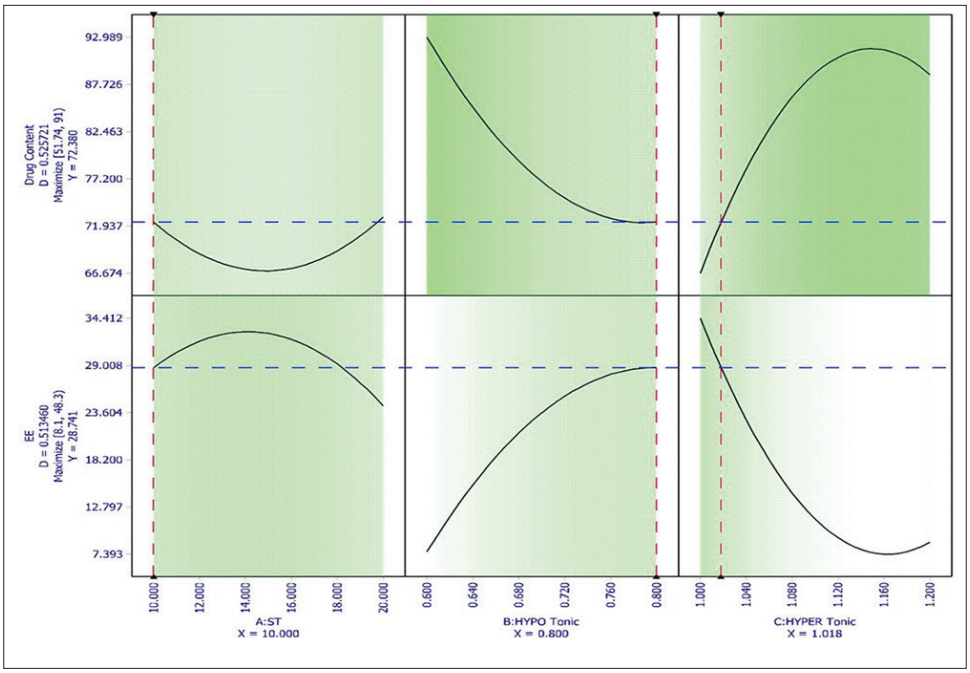

Figure 10: Desired optimal response showing the effect of change in concentration of sonication time (X_1), concentration of hypotonic solution (X_2), and concentration of hypertonic solution (X_3) on independent variables

formation of erythrocyte membrane during this process also removed the fatty substance from the cell.

Osmotic shock

The capecitabine content of NEs was determined using UV-Visible spectroscopy method at 245 nm. The changes in osmotic condition of cell were resembles to change their integrity. When drug-loaded nanoerythrosomes were incubated with distilled water, the cell was completely ruptured and there was complete release of drug from the cell.[29,38] This indicates that there was complete lysis of the cell when formulation was incubated with water for osmotic shock study. Hence, change their osmotic condition then drug released was increased at 90% in distilled water as compared to isotonic condition [Figure 12]. Therefore, the storage of carrier erythrocytes in isotonic suspensions may be more suitable in lower temperatures.

Osmotic fragility

In the developed osmotic lysis method for encapsulation of capecitabine, osmolality of the buffer used is crucial. Developed formulation of nanoerythrosomes in that drug was encapsulated using preswelling method. From this concluded that drug-loaded nanoerythrosomes are swelled at higher concentration of hypotonic solution; hence, it was minimum drug entrapped in the cell and hence maximum drug retained in the solution [Figure 13], however, it clear that low concentration of sodium chloride solution indicated grater drug release in the cell to through formulation, 0.2% sodium chloride have entrapment efficiency decreased

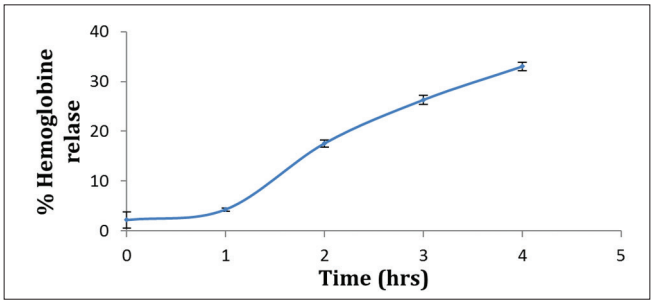

Figure 11: Hemolysis profile of nanoerythrosomes loaded with capecitabine. Data represent the mean ± standard deviation (n = 3)

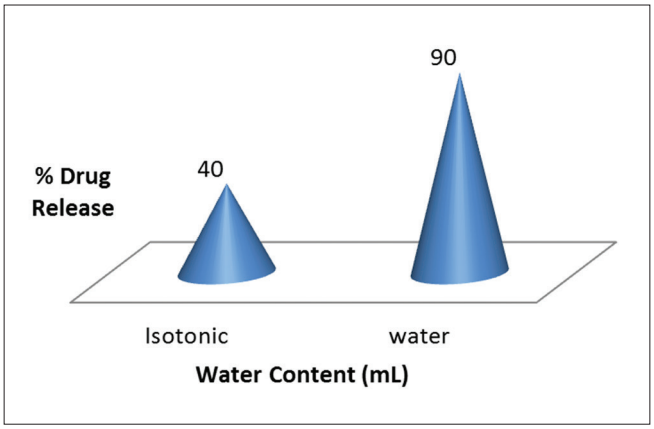

Figure 12: Osmotic shock studies of capecitabine-loaded nucleotide excision repairs

in the resealed erythrocyte.[30,38,39] A hypotonic solution of concentration 0.3% w/v induced cell swelling and the formation of pores that allowed the drug to penetrate the

erythrocyte [Figure 14]. However, hemolysis of erythrocytes was greater with a low concentration of sodium chloride. It indicates that at 0.2% NaCl, there was less resistance of cells to hemolysis as compared to other concentrations of sodium chloride used for the study.

Turbulence fragility

The turbulence fragility test is used to exploit the mechanical strength of the erythrocyte membranes. In the present study, this test was mainly carried out by shaking the cell suspensions vigorously. The hemoglobin released was measured at different times. The results indicated that the turbulence fragility of the capecitabine-loaded NERs was greater than that of the control erythrocytes. The turbulence fragility index values for unloaded, control, and capecitabine-loaded erythrocytes were 3, 2, and 1 h, respectively. Similarly, other studies have shown that the turbulence fragility of the erythrocytes as drug vehicles increases significantly relative to that of normal control cells.[31,32] These results indicate that the resistance of the erythrocytes to vigorous turbulent flow shows a decreasing trend from control cells to capecitabine-loaded erythrocytes. These results indicate that erythrocytes become more fragile during the loading process and that this fragility is enhanced by capecitabine encapsulation.

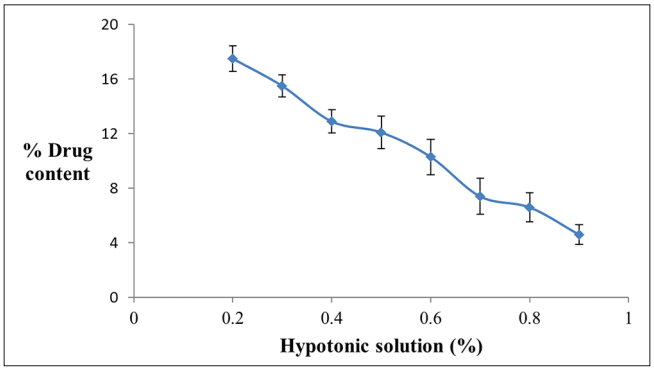

Figure 13: Effect of osmotic fragility on drug content of capecitabine-loaded nucleotide excision repairs

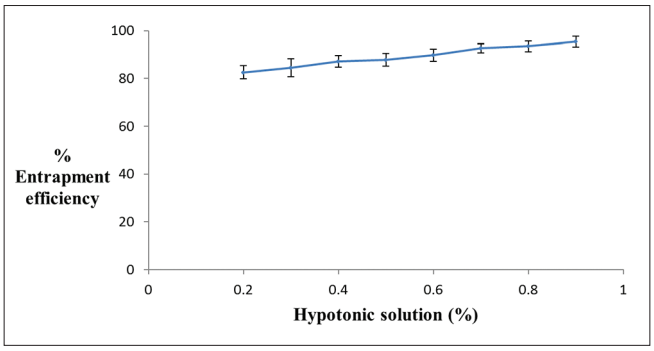

Figure 14: Effect of osmotic fragility on entrapment efficiency of capecitabine-loaded nucleotide excision repairs

In vitro drug release

The *in vitro* drug release from capecitabine solution, optimized capecitabine-loaded NERs were studied at 37°C ± 2 in PBS buffer [Figure 15]. When the release of plain capecitabine was evaluated using dialysis bag as barriers, ~100% drug was available in the receiver chamber only after 1 h, suggesting that bags were not controlling the passage of drug molecules from donor to receiver chambers.[21] No degradation in the release media is expected since capecitabine remained stable over an extended period (~24 weeks) at various storage conditions. However, a slower release may stem from the compact structure of nanoerythrosomal membrane that is composed of natural lipids, cholesterol, and surface proteins.[15,33,38] The percent cumulative drug release from the capecitabine solution was found 71.19 % to be after 8 h, capecitabine-loaded nanoerythrosomes to be 48.49% after 8 h. The release was better controlled from capecitabine-loaded NERs compared to standard drug solution. From the above results, it is very clear that the drug-loaded NERs would show very slow release of capecitabine in the blood circulation, targets more to tumor tissues, and therefore, meets the requirements for an effective drug delivery system.

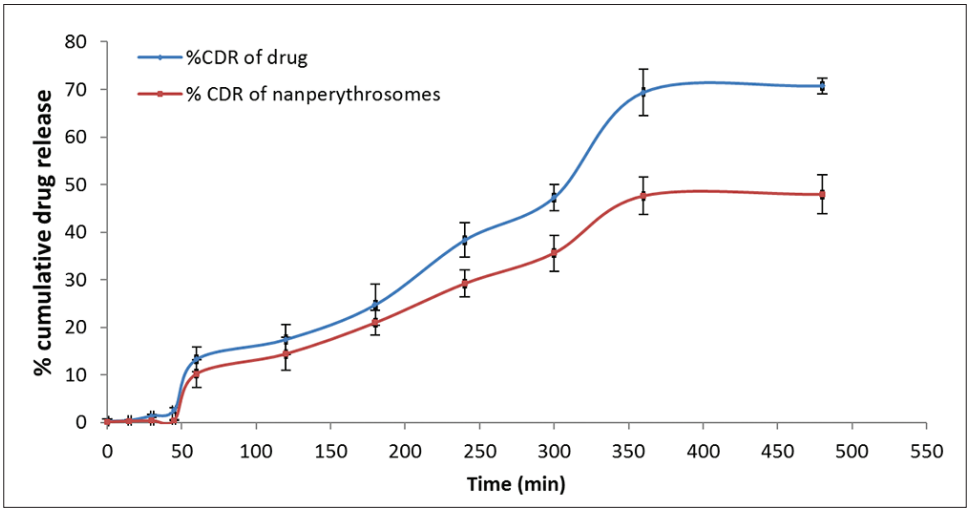

Figure 15: Percent cumulative drug release of capecitabine and optimized capecitabine-loaded nucleotide excision repairs

Shape and surface morphology

To investigate the possible morphology changes of nanoerythrocytes on loading process, samples of resealed nanoerythrocytes were observed under the SEM [Figures 16 and 17]. As from the concluded that, the loading process with drug and different cross-linking agent resulted in the formation of cup-form nanoerythrocytes very disperse sizes. Native human nanoerythrocytes show the expected biconcave morphology under SEM. After drug loading and treatment with glutaraldehyde, a slight change in their shape was observed with the same magnification ×1500. This shows that nanoerythrocytes undergo considerable morphology change during the loading process, which is confirmed by particle size.[37] From this find out the no observed effect on the morphology of the carrier cells and the changes of the drug loading encapsulation cell. The main morphological change in capecitabine-loaded NERs, as revealed by scanning electron microscopy [Figure 17], was the transformation of loaded cells from biconcave (normal) to near spherocytes. Attainment of a spherical shape due to drug loading makes the erythrocytes more fragile.[37] The fragile cells may be destroyed and rapidly cleared from the circulation by macrophages. Further studies are required to elucidate and demonstrate erythrophagocytosis of capecitabine-loaded NERs.

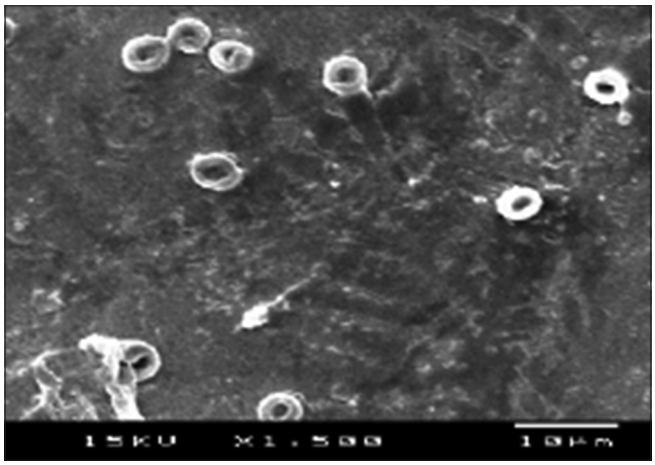

Figure 16: Photomicrograph image (scanning electron microscope) of unloaded/ghost nanoerythrosomes

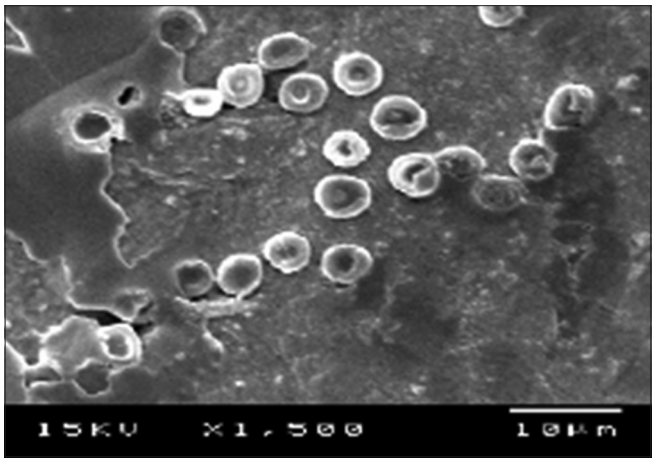

Figure 17: Photomicrograph image (scanning electron microscope) of capecitabine-loaded nanoerythrosomes

Vesicle size

We used different sizing methods to assess their influence on the homogeneity and entrapment efficiency of the formulations. Sizing with sonication produced polydispersed particles (polydispersity index >0.5) and drastically reduced the entrapment efficiency. The particle size of capecitabine-loaded NERs was found to be 2.96 nm, and protein disulfide isomerase was found to be 0.097 [Figure 18]. The polydispersity index of capecitabine-loaded nanoerythrosomes was less than one and concludes that NERs formed are mono dispersed or of uniform size. The sizes of NERs were found in nanometer; therefore, we could expect better accumulation at tumor by EPR effect. Further, sonication had minimal effect on entrapment efficiency, suggesting little or no disruption of cells. This is consistent with the flexible structure of erythrocytes that continually travel through narrow capillaries and slits of sinusoids in the physiological system. In fact, structural flexibility is a very important feature of erythrocytes that determines the fate of cells and a slight deviation in terms of shape or rigidity can lead to clearance of cells by macrophages.[40,41] Thus, nanoerythrosomes are expected to be optimal for *in-vivo* efficacy and are likely to avoid clearance by alveolar macrophages.

CONCLUSION

Nanoerythrosomes is a suitable carrier for the preparation of capecitabine-loaded nanoerythrosomes prepared by sonication methods. Prepared carriers were optimized for many formulation variables such as morphology of vesicles, size and size distribution, polydispersity, integrity of membrane, loaded drug concentration, release rate, and *in vivo* bioavailability. The developed capecitabine-NERs carrier revealed nonaggregated, polydispersed vesicles with smooth surfaces. Preparations containing glutaraldehyde as cross-linking agent showed maximum drug entrapment efficiency. Capecitabine-NERs formulation was best fit for zero-order kinetics and capable of controlled release of drug for 8 h. Capecitabine-NERs formulation showed good redispersibility with normal saline which is desirable for parenteral administration. Targeting efficiency of drug-loaded erythrocytes over free drug is higher, which may provide increased therapeutic index and drug targeting to various organs. It may help in the reduction of dose required for the therapy and thereby dose-related systemic side effects could also be minimized.

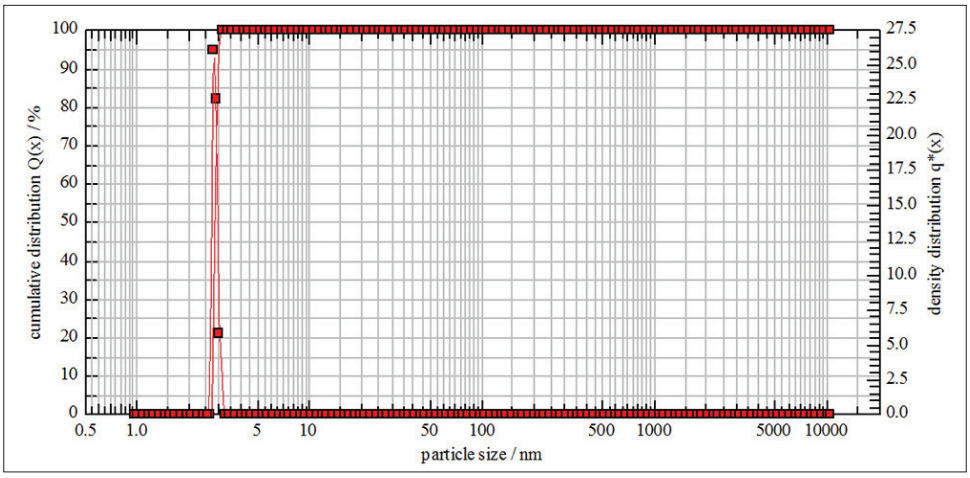

Figure 18: Vesicle size distribution of capecitabine-loaded nanoerythrosomes

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