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Systematic Development of Cyclic RGD Anchored Emulsomes for Tumor Specific Delivery of Paclitaxel

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ABSTRACT

Introduction: Paclitaxel is the most widely used taxane for treatment of tumor. It is marketed as Taxol® wherein Cremophor EL and ethanol (50:50 v/v) are used as solubilizer. Cremphor is associated with severe side effects rendering Taxol® clinically unacceptable. To overcome this limitation emulsomes are proposed to provide a biocompatible platform with the potential to encapsulate lipophilic drug in higher amount within its lipid core. Pharmaceutically stable emulsomal formulation can be prepared without the need of additional surfactant or solubilizer.

Objective: The purpose of the present study was to design systematically optimized paclitaxel (Ptx) loaded, c(RGD) anchored emulsomes for effective tumor therapy to provide non-toxic alternative to the presently cremophor based Ptx formulation (Taxol).

Method: Design expert® 11 software was used for identifying the most significant variables using Taguchi orthogonal design (L8^2 array) followed by implementation of Box-Behenken design (3-level-4-factor) for precise optimization using PL:SL(X_1), TL:SL (X_2), Aqu:org phase volume (X_3) and sonication time (X_4) as independent variables. The response variables observed included particle size (PS), percentage entrapment efficiency (EE) and cumulative percentage drug release (DR).

Results: Emulsomes has average particle size of 192.6±0.450nm with PDI 0.226±0.055, zeta potential of -33.86±0.15mV and entrapment efficiency of 75.9±3.55%. Formulation showed sustained drug release profile over 24 h at physiological pH and tumor pH. Slow release pattern could allow multi drug resistance (MDR) evasion, therefore enhancing the therapeutic efficacy of Ptx at lower dose. FACS analysis using A549 cell line showed higher uptake of RGD coated emulsome over plain emulsomes justifying the role of overexpressed integrin receptor in mediating receptor mediated endocytosis (RME).

Conclusion: RGD anchored emulsomes could serve as biocompatible tumor specific delivery nanocarrier with improved drug entrapment and controlled release characteristics.

Key Words: Emulsomes, Paclitaxel, c(RGD), QbD, Integrin, Tumor

INTRODUCTION

Paclitaxel (Ptx) is an approved first-line drug against breast and ovarian cancers. Despite of its well-evidenced therapeutic potentials, extensive clinical use is restricted owing to the poor water solubility of drug.[1] Clinically Taxol® is solubilized in 1:1 (v/v) mixture of Cremphor EL and ethanol. Cremophor- EL imposes serious side effects like hypersensitivity, nephrotoxicity and neurotoxicity and at higher dilutions it antagonizes the effect of Paclitaxel. Thus, a variety of nanocarriers have been developed in search of pharmaceutically acceptable formulation.[2] However, most of these nanoparticles, presents obstacle for pre-clinical translation because of intrinsic toxicities of the synthetic material employed in manufacturing. Thus, biomimetic drug delivery vehicles emerging from rich pre-existing bio-source, are being explored for their potential to provide safe, biocompatible and readily acceptable delivery vehicles. A core- shell based nanosized system which mimics biological composition of chylomicron will provide a bio-compatible drug delivery system for hydrophobic drugs.

Emulsomes, a new class of novel lipoidal nanocarrier bearing resemblance to chylomicron (natural lipoprotein) are capable of loading both hydrophilic and hydrophobic drug. They are comprised of two structural components, a lipidic core made from solidified fatty acid and phospholipid/s forming stabilizing envelope around the core. Our research

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team has reported the potential of emulsome based formulations for delivery of antiviral,[3] antifungal,[4,5] antipsoriatic,[6] antileshmaniasis[7, 8] and anticancer agents[9] through various routes of administration. The investigations have ascertained their higher loading capability for poorly-water soluble bioactives with subsequent protracted and sustained release of incorporated therapeutic agent from the core. Futhermore, the lipid matrix minimizes the chance of drug leakage and coalescence during systemic circulation, addressing the stability issues often observed in case of liposomes based nanocarriers.

Incorporation of active targeting functionality onto the surface of emulsomes ensures tumor specific delivery of Ptx avoiding the exposure to healthy tissue. Integrins composed of α and β subunits are highly expressed in cancer cells and present in negligible amount in normal cell. These are involved in mediating cell interaction with extracellular matrix components like fibronectin, vitronectin and collagen through their Arg-Gly-Asp (RGD) peptide motif which further promotes cell adhesion, migration, proliferation and metastasis processes vital for tumor survival.[10] Therefore, synthetic peptide comprising RGD sequence either in linear or cyclic form is easily recognized by tumor cells and is extensively utilized for integrin targeting.[11]

The present study aims at formulating sterically stabilized cRGD conjugated Ptx bearing emulsomes for effective solid tumor therapy (R-Ptx-Es). Optimization of the formulation was performed using Design expert® 11software to evaluate the impact of independent variables (formulation variables and process variables) for obtaining the best formulation in terms of entrapment efficiency, drug release and PDI. These carriers can overcome the solubility issues of Ptx as the drug gets entrapped predominantly within the lipophilic core and additionally intercalate within phospholipid bilayers thereby; improved entrapment efficiency over liposomes can be achieved. Emulsomes are expected to offer a physiologically stable system, with minimum leakage of drug and concomitant sustained release from the solidified core, thereby evading the problems of systemic toxicity and rapid drug elimination associated with parentral administration of most of the nanocarriers.

MATERIAL AND METHOD

Paclitaxel was gifted by Neon laboratories ltd. Mumbai, India. Soya Phosphatidylcholine (PC) and DSPE-PEG-COOH were offered by Lipoid Gmbh, Germany. Cyclo(-RGDfk) was provided by Peptide Specialty Laboratory, Germany. Cholesterol, Tristearin, 1-Ethyl -3-(3-dimethylamino) propyl carbodiimide (EDC) and N-hydroxysuccinimide (NHS) were purchased from Sigma Aldrich Chemie, Gmbh. Cell line A549 was purchased from NCCS Pune. All other reagents and chemicals used were of analytical grade and purchased from local chemical supplier.

Experimental design for optimization of the formulation

Plain drug loaded emulsomes P-Ptx-Es were optimized by screening out the formulation and process related variables to achieve best average particle size, PDI, entrapment efficiency and cumulative percentage drug release for site directed delivery of Ptx emulsomes (Ptx-Es).

Therefore, Taguchi orthogonal design at two levels (low and high) for selected variables i.e. Phospholipid to solid lipid ratio (PL:SL, w/w), Phospholipid to DSPE-PEG (PL:DSPE-PEG molar ratio), total lipid to solid lipid ratio (TL:SL, %), aqueous to organic phase ratio (Aqu:org phasev/v), stirring speed (r/pm), sonication time (sec) surfactant concentration (v/v), was applied to study the impact of selected variables on three response variables namely average particle size-PS (nm), entrapment efficiency-EE (%) and polydispersity index-PDI. It was implemented to rule out the insignificant factors from the experimental design in order to reduce the complexity in optimization design

The significant variables as per Taguchi's orthogonal design were investigated using Box-Behnken. The design was appropriate for investigating 3-D and 2-D contour response surfaces and for creating second order polynomial order. Independent factors PL:SL ratio (X_1), PL:DSPE-PEG ratio (X_2) and Aqu:org phase volume (X_3), TL:SL (X_4) were analyzed for their effect on dependent factors, PS, EE and cumulative percentage drug release. Each design variable represented by −1, 0 and +1, analogous to the low, middle and high values respectively.

Formulation of plain and c(RGD)fk conjugated emulsomes

Tristearin based non-liganded and liganded emulsomes designated as P-Ptx-Es and R-Ptx-Es respectively were prepared by single emulsification-solvent evaporation method with slight modifications.[12] The P-Ptx-Es were composed of tristearin:soya PC:Chol:DSPE-PEG-COOH in different ratios. Precisely, all the lipid components were dissolved in chloroform (organic phase). The organic solution was then added drop wise at the rate of 1ml/min to the aqueous phosphate buffer saline pH 7.4 (aqueous phase) with concomitant stirring. Ptx (5mg/ml), dissolved in 50:50 v/v mixture of methanol:PBS pH 7.4 was added to aqueous phase. After the addition of organic phase to aqueous phase, the mixture was subjected to overnight stirring to allow the evaporation of organic phase and self-assembling of emulsomes. The resultant emulsomes dispersion was sonicated using probe sonicator (Sonifield, Mumbai) under ice bath for attaining desirable size range, passed through the Sephadex-G 50 column

to remove the untrapped drug, lyophilized using 2% mannitol and finally stored in refrigerator. Mannitol was added as cryoprotectant to the emulsomes at sugar to lipid ratio of 5:1. The emulsome samples were frozen at -80°C for 5 h followed by lyophilization at condenser temperature of 50-55 °C for 48 h and 5 Pa.

RGD-conjugated emulsomes were prepared by post conjugation method.[13] Carboxylic group located at the distal ends of DSPE-PEG-COOH were conjugated with amine functionality of c(RGD) through amide bond formation involving carbodidimide chemistry. Firstly, DSPE-PEG was replaced by 3 mol% of DSPE-PEG-COOH and emulsomes were formulated as stated above. Then, excess of DCC and NHS (6 molar) were added to activate the –COOH group with the resultant formation of succinimidyl ester. RGD (100 mg) dissolved in PBS pH (7.4) was added to the above solution and allowed to stand overnight at room temperature to allow for conjugation through amide bond formation. To remove the unreacted components the reaction mixture was dialyzed using a dialysis membrane with molecular weight cutoff 12000 Da.

CHARACTERIZATION OF OPTIMIZED PTX-ES

Morphological Characterization of Emulsomes

The average particle size, surface zeta potential and polydispersity index values of emulsomes were determined using photon correlation spectroscopy (Malvern zetasizer Nano-ZS, UK). Measurements were conducted in triplicate at room temperature after diluting lyophilized samples with distilled water.

The shape and surface morphology of the formulation were observed by using electron microscopic techniques. Transmission Electron Microsocopy (TEM-4X, JEOL, Japan) using phosphotungastic acid (1%) based negative staining method for capturing 2-D images while the 3D view of the prepared emulsomes was photographed by Scanning Electron Microscopy, (SEM, NOVA NanoSEM 450).

Entrapment Efficiency

1 ml of the dispersion was treated with 1 ml of 0.1%v/v triton X- 100 and centrifuged to pelletize the structural components. Supernatant was used to estimate the liberated drug content by HPLC (Shimadzu) at 229 nm using following formula:

%Entrapment Efficiency (EE)=(Experimental drug loading/Theoritcal drug loading)×100

In Vitro Drug Release

In vitro drug release study was performed using dialysis tube method in buffer media with pH (7.4) and phthalate buffer pH (4.0) resembling physiological and tumor pH respectively. Briefly, 1 ml of the emulsome dispersion was taken in the dialysis bag with MWCO of 10,000 and dipped into 50 ml of PBS pH 7.4 maintained at 37 ±1 ºC. Samples were withdrawn at the predetermined intervals and replaced by the same volume of fresh release medium. The amount of Ptx released was quantified by using HPLC (229 nm).

Cell culture

A549 cell line was selected for tumor studies. The cells were cultured in RPMI supplemented with 10% Fetal Bovine Serum (FBS), 1% streptomycin/penicillin, 2mM of glutamine, maintained at 37±1°C and 5% CO_2 under humidified conditions.

FACS analysis for quantitative cellular uptake

The cellular uptake by cell lines (cancer cells) was determined quantitatively through FACS analysis of treated A549 cells as reported by Minnelli et al., 2018.[14] A549 cells at the density of $5x10^5$ cells were seeded in a 96 well plate. FITC loaded formulations namely FPtx-Es and FR-Ptx-Es were added and incubated for 2h at 37ºC. Then after, cells were washed thrice with PBS to remove unbound emulsomes, trpsinized and centrifuged at 1500 r/pm for 2min at 4 ºC. Finally, the pelletized cells so obtained were suspended in 500µl PBS and analysed using FACS (FACA, Caliber, BD Science, USA).The fluorescence intensity was quantified relative to untreated cells which served as control.

Assesment of cellular toxicity of Ptx-Es

Inhibition of cell proliferation was studied by tetrazolium salt (3-[4,5-dimethylthiazol-2-yl]-2-5-diphenyl tetrazolium bromide, MTT assay. Briefly, cells ($1x10^5$cells/well) were seeded in a 96 well flat bottom plates and allowed to grow for 48 h. Then, the cells were incubated with increasing concentration of Ptx solution and Ptx emulsome formulation for 24 h and 48 h. After 24 h, the supernatant was removed, MTT and culture medium (100 µl each) were added to each well, incubated for 4 h at 37°C and 5% CO_2 atmosphere. The unreduced MTT and medium were then discarded. Each well was washed with 200 µl of PBS, and 200 µl of DMSO was added to dissolve the MTT formazan crystals. Plates were shaken for 20 min and absorbance was read at 560 nm using a microplate reader (Molecular Devices Corporation, USA). The IC50 values (i.e., concentration resulting in 50% growth inhibition) of paclitaxel were graphically calculated from concentration-viability curves, considering the optical density of the control well as 100% viable.

Biocompatibility study

Biocompatibility of emulsomes was performed on Ptx free emulsomes. Cells were then treated with emulsomes (Es), cRGD modified emulsomes (R-Es) and Cremophor-EL:ethanol at concentrations can contain equivalent amount

of paclitaxel. Cell viability was then determined to observe the cytotoxic effect of nanocarriers.

RESULTS

As per the Pareto chart and the half-normal plots obtained from the Taguchi OA design the factors namely, PL:SL, PL:DSPE-PEG, Aqu:org phase and sonication time contributes significantly over the rest of the factors to effects the responses as displayed in (fig. 1 and table 1). Four factors were put into the BBD at low (-1) and high level (1) followed by the input of response variables, PS (Y_1), EE (Y_2) and DR (Y_3). .Table 2 summarizes the various constraints and their coded factor levels chosen for study. Table 3 records F value and P value for the ANOVA used for factorial selection. The non-significant lack of fit, model F values of 110.59, 30.67 and 36.59 for PS, EE and PDI respectively and the p values<0.05 suggested that the model and the model terms used were significant. The R^2 value closer to one is considered as good for the suggested model. The difference of less than 0.2 between the values of predicted R^2 and adjusted R^2 values as shown by the Fit statistics shows precision and aptness of opted model (Table 4). The model were significant as suggested by good F-values and P value of <0.05. Following the input of the independent variables the quadratic model resulted in 29 observations for each response with various combinations of factorial levels (table 5).

The 2D and 3D response surface depicts the effects of constraints on the response variables, EE, PS and Drug release (fig 2, 3 and 4). The response surface plots were used to study the interaction effects of two independent variables on the responses or dependent variables, when a third factor kept at constant level. The following quadratic model was generated by the design for the observed response.

$$Y=\beta_0+\beta_1X_1+\beta_2X_2+\beta_3X_3+\beta_4X_4+\beta_5X_1X_2+\beta_6X_1X_3+\beta_7X_1X_4+\beta_8X_2X_3+\beta_9X_2X_4+\beta_{10}X_3X_4+\beta_{11}X_1^2+\beta_{12}X_2^2+\beta_{13}X_3^2+\beta_{14}X_4^2$$

Where Y is the measured response of the dependent variable associated with each factor level combination; X_1, X_2,X_3 and X_4were the coded levels of independent variables, the terms X_1X_2….and X_i^2(i=1,2,3,4) represent interaction and quadratic terms respectively. Table 6 summarizes the calculated value of β coefficient (β_0 to β_{14}) for the value of Y. The equation in terms of coded factors can be used to make predictions about the response for given levels of each factor with the high levels of the factors coded as +1 and the low levels coded as -1. Positive value of coefficient before the independent variable signifies favorable effect and vice versa for the negative value of coefficients.

Effect of independent variable on responses

The size of emulsomes ranged from 145±2.5 to 240±3.6 nm over 29 runs suggesting that the size was affected by the selected independent variables. The model applied proved important as proposed by the higher F value of 54.49, there is only 0.01% chance that large F-value due to noise (Table 2). p values of <0.05 for PL:SL, PL:DSPE-PEG, Aqu:org phase volume and sonication time implies their significance on size.

The model was significant for optimizing the percentage entrapment efficiency (EE) of emulsomes as suggested by F value of 24.07 indicating that there is only a 0.01% chance that a high F-value could occur due to noise. Lack of fit was non-significant with R^2, predicted and adjusted R^2 values observed in range (Table 2).

Optimized Formulation

Optimized formulation was obtained by evaluating the design factors through numerical and graphical optimization. The Box-Behnken design generated 100 solutions with desirability 1 for various compositions of factors in the design space. Desirability 1 is an indicator of best fit. Amongst these suggested values, the factors combination meeting the criteria for best response values in terms of required size range with maximum entrapment efficiency and slower sustained release were separated. The size of nanocarrier plays a vital role in overcoming macrophagic uptake, maintaining longevity in systemic circulation as well as retention in the tumor tissue. Reports have suggested the size range of 90-200 nm serves as optimal range for elevating tumor accumulation and prolonging circulation time.[15] Hence, formulations with size range below 200 nm showinmaximum entrapment efficiency with desirability 1 was selected as final optimized emulsome formulation for paclitaxel. 32 solutions with entrapment efficiency above 70% for particles in preferred size range were obtained (table 7). Hence, the final optimized tristearin based emulsome possessed the size of 192.6±0.450nm, entrapment efficiency of 75.9±3.55% and cumulative percentage drug release of 42.4±5.56 % physiological pH from P-Ptx-Es after 24 h. Fig. 5 shows the plots of design space for all the responses of optimized formulation.

Morphological Studies of Optimized Formulation

Optimized formulation had average hydrodynamic diameter of 192.6±0.450 nm, PDI 0.226±0.055 with the zeta potential value of -33.86±0.15 mV. Fig. 6 comprises of morphological features P-Ptx-Es.

In Vitro Drug Release Profile at Different pH

The drug release profile was obtained by estimating the cumulative percentage drug released over seven days at different pH conditions. Fig. 7 shows the drug release profile of Ptx from emulsomes with cumulative percentage drug release of 42.4±5.56 %, 31.8±4.2% at pH 7.4 from P-Ptx-Es and R-Ptx-Es respectively and 67.5±2.4, 55.3±3.2 from P-Ptx-Es

and R-Ptx-Es respectively at pH 4.0. On 7th day the percentage drug release at pH 7.4 was found to be 75.24±1.2% and 66.5±2.5% from P-Ptx-Es and R-Ptx-Es respectively and 80.0±2.6 and 76.5±4.2 5 from P-Ptx-Es and R-Ptx-Es at pH 4.0.

In Vitro Assesment of Cellular Toxicity of Ptx Emulsomes

The antiproliferative activity of different Ptx-Es formulation on A549 cell line was observed at 24 and 48 h in concentration range of 0.1µg/ml to 10 µg/ml. To further validate the advantage of Ptx loaded emulsomes the concentration required to induce death of 50% (IC_{50}) of the incubated cells over a designated period of time was evaluated (fig. 8A, 8B and 8C. The percentage cell viabilities and IC_{50}values of A549 cells treated with free Ptx, P-Ptx-Es and R-Ptx-Es are shown in respectively. IC_{50} value of the Ptx, P-Ptx-Es and R-Ptx-Es were found to be 17.4±0.02 µg/ml, 13.3±0.05µg/ml and 5.4±0.021µg/ml after 24 h of treatment. After 48 h incubation, inhibitory IC_{50} values of Ptx encapsulated in cRGD modified emulsomes decreased from 5.2±0.24 to 1.4±0.20 µg/ml. and were found to be 8.9 folds and 5.8 fold lower than plain Ptx and Ptx emulsomes respectively.

Biocompatibility study revealed none of the formulations showed anti-proliferative effect except cremophor EL/ethanol (Fig. 8D) owing to non-selective cellular toxicity of the organic solvent.

FACS Analysis for Quantitative Cellular Uptake

Flow cytometric analysis was performed to estimate the cellular uptake quantitatively. Cellular uptake of the targeted emulsomes was compared with the plain emulsomes as shown in fig. 9. Maximum shifting in intensity was recorded in the cells treated with R-Ptx-Es. The cellular uptake of 15.5±1.5%, 54.0±2.2% and 97.2±1.8% was observed for plain FPtx, FP-Ptx-Es and FR-Ptx-Es respectively after 2h.

DISCUSSION

The present study was aimed at preparing the optimized tristearin based emulsomes for tumor specific delivery of Paclitaxel. The modified single emulsification-solvent evaporation method was employed for the preparation of plain Ptx loaded (P-Ptx-Es) and RGD coated emulsomes (R-Ptx-Es). The optimization was carried out using Quality by design approach, Design-expert® ver. 11 software. Box-Behnken design facilitates comprehensive interpretation of the data in a relatively complicated design but it requires greater number of experiments to be conducted for each variable. Hence, the design matrix was simplified by conducting preliminary screening of the variables to remove the insignificant variables, by applying Taguchi design which considers all the variables at their extremities, minimizing the number of experiments and displays the most significant factors for analysis by Box-Behnken design.

The method adopted for preparation of emulsomes was found to be satisfactory resulting in formation of spherical and uniform nanocarriers. The SEM image of optimized emulsomes formulation revealed the spherical geometry of Ptx-Es while the TEM photomicrographs revealed smooth surface with dark solid core surrounded by light phospholipid boundary.

On increasing the PL content the size was found to increase. This could be attributed to the multilayered phospholipid envelope formation around the tristearin core. While on increasing the DSPE-PEG molar ratio in the phospholipid envelope, a decreasing trend in size was noted. Negative surface produced on PEGylation promotes the repulsive interaction, preventing particle from being aggregated resulting in smaller size and better PDI as well.[16] On increasing the sonication time from 10 sec to 50 sec considerable reduction in size was observed as expected.

As per the Pareto chart entrapment efficiency was reliant on the PL:SL content and Aqu:org phase volume. The entrapment efficiency increased with the increasing proportion of PL:SL ratio as well as TL:SL owing to the extra space for drug accumulation, created by multilayers of PL.[17] In contrast, increasing the aqueous phase volume had a negative effect on the entrapment efficiency of emulsomes, increased partitioning of drug in the volume of aqueous medium.[18] The overall range for %EE varied from 54.0±4.5 to 79±2.8%.

The emulsomes showed slower drug release profile than the plain paclitaxel solution, attributed to the slow diffusion of the drug from the solid lipid core and the surrounding phospholipid layers.[19] Decreasing trend in drug release was observed with consecutive increase in the ratio of the three factors. This could be due to increased path length for the diffusion of drug vis a vis the opposition for entry of water molecules offered by multiple phospholipid layers. Lower release value at physiological pH justifies the minimum drug leakage from emulsomes, thereby rendering the Ptx emulsomes safe for parentral administration. The graph presents initial burst release followed by the sustained release of paclitaxel from emulsomes in all the formulations at both physiological and tumor pH. This could be ascribed to the entrapment of drug in the PL bilayers resulting in the former phase with drug release at high concentration succeeded by the slow and sustained release from the fatty core of emulsomes. The drug release was observed to be follow pH dependent character with faster release at pH 4 than at pH 7.4 invariably from all the formulations. Such a drug release behavior predicts the capability of emulsomes to offer tumor pH specific drug release while remaining stable in systemic pH resulting in minimized toxic effects as reported by Paliwal et al for a tumor specific pH sensitive liposomes.[20]

Drug release from R-Ptx-Es was observed to be slower than Ptx-Es, probably due to longer diffusion path from the fatty core. In vitro drug release study showed that the intracellular therapeutic concentration of drug could be maintained for prolonged period of time which in turn could help in overcoming MDR in chemotherapy. Thus, emulsomes can be further investigated for the cytosolic delivery of anticancer agents within therapeutic range.

The results revealed concentration as well as time dependent growth suppression on treatment with Ptx loaded emulsomes. R-Ptx-Es showed higher cytotoxicity than Ptx and P-Ptx-Es which could be attributed to their higher accumulation through αvβ3-mediated endocytosis facilitated by the cRGD-integrin receptor interaction, confirming the role of c(RGDf)K in targeting effects. Inefficiency of efflux transport system (e.g. P-gp efflux transporter) for the expelling c(RGDf)K modified drug carrier may attribute to the comparatively higher cytotoxic effects of R-Ptx-Es than P-Ptx-Es. Moreover, the higher cytotoxicity of R-Ptx-Es also indicates no loss of anti-cancer activity of Ptx after modification of emulsomes with the peptide. The uptake of RGD coated emulsomes was 3.2 times and 1.5 times than the plain Ptx and plain emulsomes respectively. The results were in accordance to the previously reported study performed by other authors which indicates the role of c(RGD)fk in integrin mediated endocytosis by cancer cell[21] and suggests acceptability of emulsomes as biocompatible alternative to the present cremophor EL/ethanol based Taxol.

CONCLUSION

The primary aim of the present study was to investigate the feasibility of emulsomes to be formulated with such dimensions and functionalities so as to meet the requirements for tumor targeting. Adopted method for formulation favored the production of uniform emulsomes with nanometric size range. Pegylated lipid gets easily accommodated along with the PL envelops providing sterically stabilized nanosystem along with the free ends for anchoring c(RGD) functionality for targeting the integrin receptors overexpressed on tumor cell. Quality by Design (QbD) approach which is usually applied for getting best formulation compositional and process parameters can be applied for emulsome optimization as well. Systematic optimization was carried out for the chosen factors based on the desired response variables and the results so obtained proved the applied models to be significant as suggested by p value (<0.05), F value and regression values near to one. In vitro drug release studies revealed that emulsomes were capable of offering sustained and prolonged drug delivery for longer duration of action. The c(RGD) proved to be efficient in delivering the carrier specifically to the tumor cell as suggested by higher uptake values from quantitative cellular uptake study by FACS. Hence, emulsomes have emerged to be a biocompatible better choice over other vesicular and colloidal carriers for lipophilic drug delivery. Overall, the composition and manufacturing technique of the emulsomes make practicable the production of a stable final product that could be an economically interesting alternative to the current paclitaxel formulations. However, current study provides the basis overview of the Ptx emulsome formulation, the formulation is still needed to be probed by undertaking *in vivo* studies in order to validate the ex vivo results as well as its clinical acceptance.

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Author contribution: Dubey S: Performed all the experiments, collected and analyzed the data. Wrote the paper.

Sharma R: Assisted implementation of Design of Experiment (DoE) based optimization and its analysis.

Vyas SP: Designed and supervised all the experiments and the collected data.

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Table 1: Taguchi Orthogonal Array (Toa) Design

Factor	Name	Units	Type	Minimum	Maximum
A	PL:DSPE	molar ratio	Categoric	1	5
B	PL:SL	w/w	Categoric	0.6:1	1.4:1
C	TL:SL	%	Categoric	1:1	1:5
D	Aqu:org phase	v/v	Categoric	1:1	5:1
E	Sonication time	Sec	Categoric	10	30
F	Stirring speed	Rpm	Categoric	1000	5000
G	Surfactant	%	Categoric	0.1	0.5
Response	Name	Units	Observations	Analysis	
R1	PS	Nm	8	Factorial	
R2	EE	%	8	Factorial	
R3	PDI		8	Factorial	

Abbreviations: PL- Phospholipid; DSPE-1,2-Distearoyl-sn-glycerol-phosphoethanolamine; SL-solid lipid; TL-Total lipid; Aqu-aqueous; org-organic.

PS-Particle size; EE-Entrapment efficiency; PDI- Polydispersity index

Table 2: Box-Behnken Design

Name	Goal	Lower Limit	Upper Limit	Lower Weight	Upper Weight	Importance
A:PL:SL (X_1)	is in range	-1	1	1	1	3
B:PL:DSPE-PEG (X_2)	is in range	-1	1	1	1	3
C:Aqu:org phase volume (X_3)	is in range	-1	1	1	1	3
D:TL:SL (X_4)	is in range	-1	1	1	1	3
Response						
PS (Y_1)	is in range	145	240	1	1	3
EE (Y_2)	maximum	54	79	1	1	3
DR (Y_3)	minimum	37	62	1	1	3

Abbreviations: DR-Drug release

Table 3: Anova Table

Source	Sum of Squares	F-value	p-value	
Response 1: PS				
Model	5068.50	110.59	*0.0014	Significant
A-PL:DSPE	1128.12	98.45	*0.0022	
B-PL:SL	3570.12	311.57	*0.0004	
D- Aqu:org phase	325.12	28.37	*0.0129	
F-Stirring speed	45.12	3.94	0.1414	
Residual	34.37			
Cor Total	5102.88			
Response 2: EE				
Model	80.50	30.67	*0.0032	Significant
B-PL:SL	18.00	20.57	*0.0105	
C-TL:SL	50.00	57.14	*0.0016	
D- Aqu:org phase	12.50	14.29	*0.0194	
Residual	3.50			
Cor Total	84.00			
Response 3: PDI				
Model	0.0155	36.59	*0.0023	significant
A-PL:DSPE	0.0090	63.45	*0.0013	
D- Aqu:org phase	0.0058	41.22	*0.0030	
E-Sonication time	0.0007	5.10	0.0868	
Residual	0.0006			

The Model *F-value 110.59, 30.67 and 36.59 implies the model is significant and there is only a 0.14%, 0.32% and0.23% chance respectively that an F-value this large could occur due to noise.
P-values less than 0.0500 indicate model terms are significant
* Indicates significant factors for each response
Abbreviations: PL- Phospholipid; DSPE-1,2-Distearoyl-sn-glycerol-phosphoethanolamine; SL-solid lipid; TL-Total lipid; Aqu-aqueous; org-organic.

Table 4: Observed Parameters For Response Analysis

Source	Sequential p-value	Lack of Fit p-value	R^2	Adjusted R^2	Predicted R^2	F-value	
	Response 1: PS						
Quadratic	< 0.0001	0.2848	0.9320	0.9476	0.9320	54.49	Suggested
	Response 2: EE						
Quadratic	< 0.0001	0.5056	0.9601	0.9202	0.8149	24.07	Suggested
	Response 2: DR						
Quadratic	< 0.0001	0.9168	0.9818	0.8818	0.7912	15.92	Suggested

Table 5: Response Factors For Box-Behnken Design

Response	Name	Units	Observations	Analysis	Min.	Max.	Mean	Std. Dev.	Model
Y1	Particle size (PS)	nm	29	Polynomial	145	240	190.97	25.63	Quadratic
Y2	Entrapment efficiency (EE)	%	29	Polynomial	54	79	69.47	8.03	Quadratic
Y3	Drug Release (DR)	%	29	Polynomial	37	62	48.55	8.07	Quadratic

Table 6: Quadratic Model Summary

β coefficient	Response variables		
	Particle size (nm)	Entrapment Efficiency (%)	Drug Release (%)
β_0	+192.60	+75.90	+42.50
β_1	+38.08	+5.58	+1.17
β_2	+1.92	+1.42	-1.67
β_3	-2.42	-0.5000	+0.9167
β_4	-1.92	+1.17	-0.7500
β_5	+8.75	-0.7500	-0.2500
β_6	+0.5000	+0.0000	+5.75
β_7	-2.50	-0.5000	+1.50
β_8	+1.75	+2.00	-0.7500
β_9	-1.25	-0.5000	-0.5000
β_{10}	-1.0000	-1.00	+0.2500
β_{11}	-1.55	-13.70	+14.47
β_{12}	-3.55	-1.20	-0.0333
β_{13}	+1.95	+0.4250	-1.41
β_{14}	-0.8000	-1.08	+1.84

Table 7: Desirable Solutions

S. No	PL:SL	PL:DSPE-PEG	Aqs:org phase	TL:SL	PS	EE	DR	Desirability	
	1.000	3.000	30.000	30.000	192.600	75.900	42.400	1.000	Selected
	1.000	3.000	50.000	10.000	194.250	74.583	44.250	1.000	
	1.000	3.000	50.000	50.000	188.417	74.917	43.250	1.000	
	1.000	3.000	10.000	10.000	197.083	73.583	42.917	1.000	
	1.000	1.000	30.000	10.000	187.000	70.542	46.125	1.000	
	1.000	5.000	10.000	30.000	193.583	75.042	39.125	1.000	
	1.000	5.000	30.000	10.000	193.333	74.375	43.792	1.000	
	1.000	1.000	30.000	50.000	185.667	73.875	45.625	1.000	
	1.000	5.000	30.000	50.000	187.000	75.708	41.292	1.000	
	1.000	1.000	50.000	30.000	184.917	71.208	44.292	1.000	
	1.340	3.300	47.000	28.000	225.613	70.982	57.334	1.000	
	0.894	4.864	19.404	29.939	179.959	73.309	41.893	1.000	
	1.186	2.687	21.457	37.418	209.348	76.145	44.737	1.000	
	1.122	2.724	26.916	11.856	205.256	74.266	45.629	1.000	
	0.991	1.839	39.723	39.450	187.392	74.048	43.832	1.000	
	1.171	2.175	43.028	41.509	202.517	74.381	48.827	1.000	
	0.977	1.531	20.672	43.112	289.036	75.698	43.146	1.000	
	1.283	1.613	26.395	33.698	211.564	72.371	50.860	1.000	
	1.072	1.582	14.366	14.074	199.177	74.265	42.897	1.000	
	1.072	1.582	14.366	14.074	199.177	74.265	42.897	1.000	
	1.000	5.000	10.000	30.000	193.583	70.042	39.125	1.000	
	1.000	5.000	30.000	10.000	193.333	74.375	43.792	1.000	
	1.000	1.000	30.000	50.000	185.667	73.875	45.625	1.000	
	1.000	5.000	30.000	50.000	187.000	70.708	41.292	1.000	

Table 7: ***(Continued)***

S. No	PL:SL	PL:DSPE-PEG	Aqs:org phase	TL:SL	PS	EE	DR	Desirability
	1.000	1.000	50.000	30.000	184.917	71.208	44.292	1.000
	1.340	3.300	47.000	28.000	225.613	75.982	57.334	1.000
	1.186	2.687	21.457	37.418	209.348	76.145	44.737	1.000
	1.122	2.724	26.916	11.856	205.256	74.266	45.629	1.000
	1.171	2.175	43.028	41.509	202.517	74.381	48.827	1.000
	1.283	1.613	26.395	33.698	211.564	72.371	50.860	1.000
	1.000	5.000	30.000	50.000	287.000	70.708	41.292	1.000
	1.000	1.000	50.000	30.000	284.917	78.208	44.292	1.000

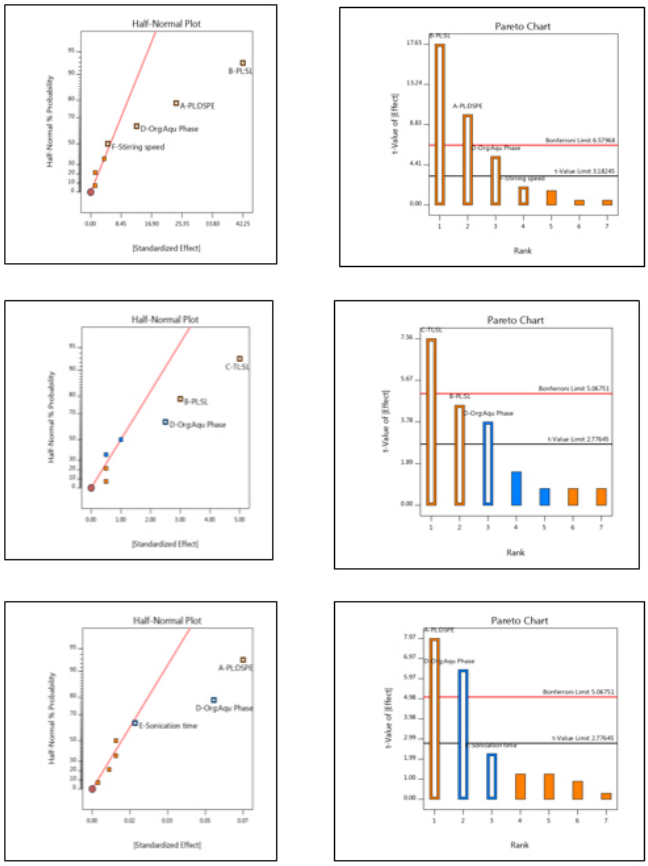

Figure 1: Half normal plots and Pareto charts depicting the significant factors affecting (a) Particle size (b) Entrapment Efficiency (c) PDI.

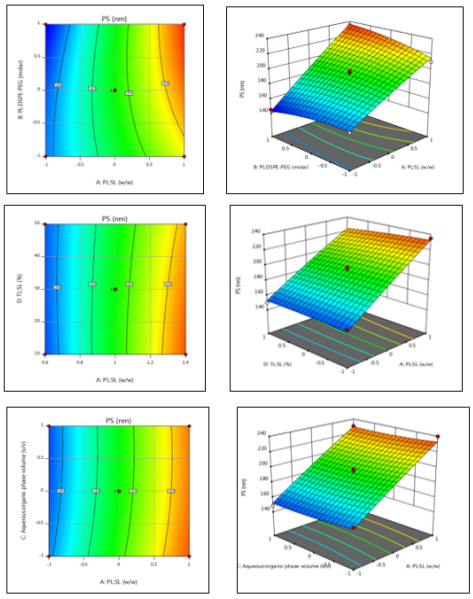

Figure 2: 2D and 3D contour plots showing effect of PL:DSPE-PEG, TL:SL and Aqu:org phase volume on particle size (PS).

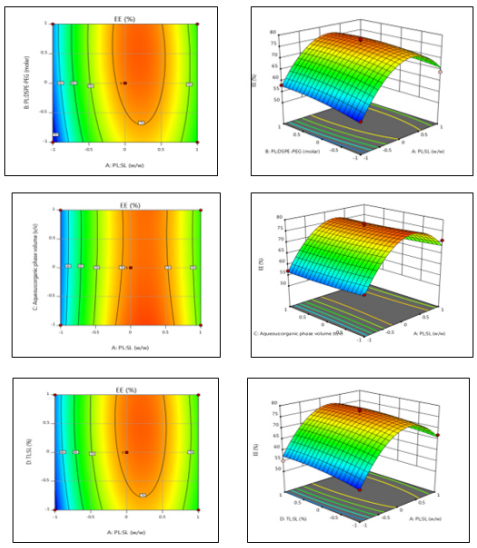

Figure 3: 2D and 3D contour plots showing effect of PL:DSPE-PEG, Aqu:org and TL:SL on entrapment efficiency.

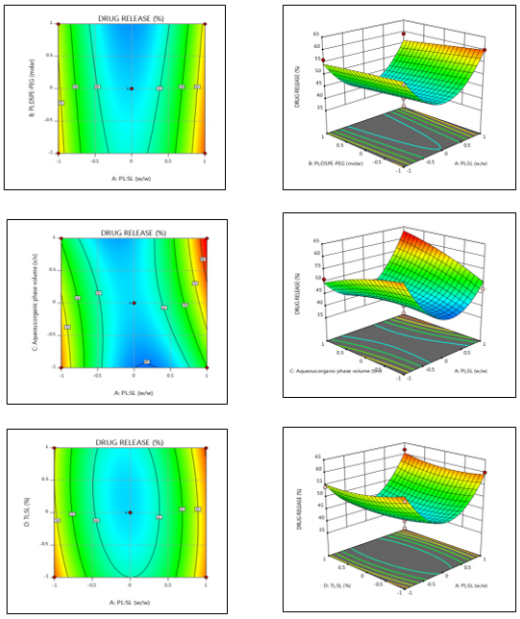

Figure 4: 2D and 3D contour plots showing effect of PL:DSPE-PEG, Aqu:org and TL:SL on drug release.

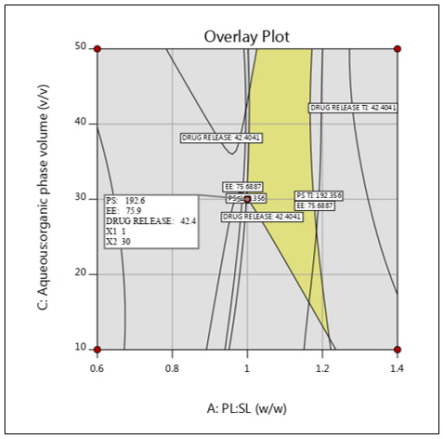

Figure 5: Overlay plot of the optimized R-Ptx-Es formulation showing optimized variables in flag.

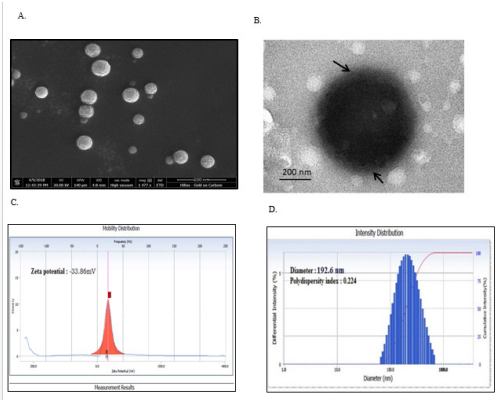

Figure 6: A. SEM photomicrograph of emulsomes; B. TEM photomicrograph of emulsomes. Arrow indicates phospholipid envelop; C. Size distribution; D. Zeta potential.

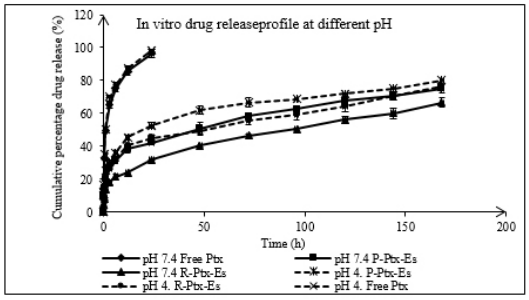

Figure 7: In vitro drug release profile of various Ptx loaded emulsomes at different pH. Data represents mean±S.D. (n=3).

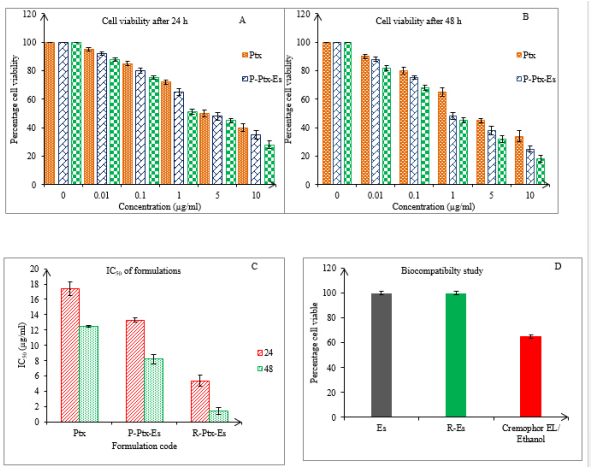

Figure 8: A. Percentage cell viability at 24 h after treatment with various drug concentrations. B. Percentage cell viability at 44 h after treatment with various drug concentration. C. IC_{50} values of formulations at 24 h and 48 h after treatment. D. Biocompatibility study of formulated emulsomes an cremphore. Data represents mean±S.D. (n=3).

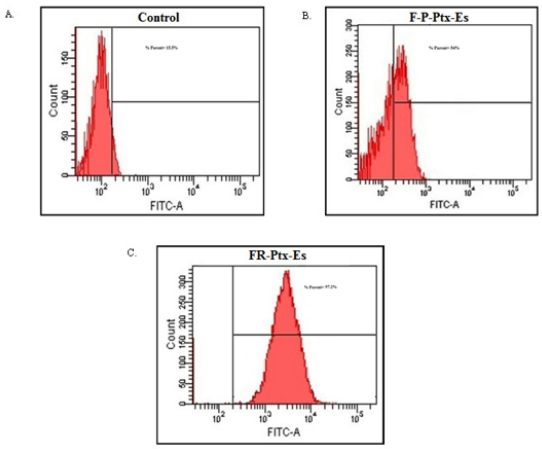

Figure 9: Cellular uptake by FACS after 2 h. A. Ptx; B. FITC loaded plain FPtx-Es; C. FITC loaded FR-Ptx-Es; D. Biocompatibility study of emulsomes.