Original Article

Tenofovir Loaded Poly (Lactide-Co-Glycolide) Nanocapsules: Formulation Optimization by Desirability Functions Approach

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ABSTRACT

Background: Nanoparticles made of biodegradable polymers are the most effective colloidal drug delivery systems. The *in vitro* characteristics as well as *in vivo* presentation of these nanoparticles are majorly influenced by their formulation parameters. **Objectives:** The principle objective of the work was to optimize various critical formulation parameters by using statistical tools in order to develop poly (lactide-co-glycolide) (PLGA) nanocapsules loaded with tenofovir disoproxil fumarate (TDF) with effective characteristic properties. **Methodology:** In this work, biodegradable nanocapsules with PLGA for TDF were prepared by modified double emulsification technique. Concentration of PLGA, concentration of Pluronic F-68 in secondary emulsion and concentration of glycerol in external phase were selected as the formulation factors hence, a 3^3 full factorial design in response surface methodology was chosen as an experimental design. The prepared nanocapsules were evaluated for various characteristic properties like particle size, surface morphology, entrapment efficiency, drug release studies and DSC studies. The selected formulation parameters were optimized by a mathematical model called desirability functions approach with the set desirability of high entrapment efficiency, low drug release constant and less particle size. **Results and Conclusion:** Particle size, entrapment efficiency and drug release constant were found in the ranges of 249.3-376.3nm, 20.95-71.35% and 0.039-0.184 hr^{-1}. These selected formulation parameters were found to have significant (at $p < 0.05$ by ANOVA) influence on various characteristics of prepared nanocapsules. The optimized formulation found to have 63.08% of entrapment efficiency, 284.53 nm of particle size with zeta potential of –26.1 mV and 0.054 h^{-1} of release rate constant.

Key words: Tenofovir, Nanocapsules, Optimization, Response surface methodology, Desirability functions approach.

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INTRODUCTION

Polymeric nanoparticles are most effective colloidal drug delivery systems than others like liposomes, niosomes, solid lipid nanoparticles and resealed erythrocytes due to their high physical stability and amendable surface properties.[1] These can easily diffuse deeper into tissues due to their nano-size and suitable surface properties hence these can be best employed for targeted delivery of drugs directly at the site of action,[2] thus reducing side effects and also dose of the drugs. Polymeric nanoparticles prepared with biodegradable polymers like PLGA,[3] poly lactic acid (PLA),[4] poly (ε-caprolactone),[5] poly (methyl methacrylate).[6] are having additional advantages like biocompatibility, biodegradability and low/no toxicity.[7] The major physicochemical properties viz. entrapment efficiency, particle size, drug release constant and surface charge significantly influence the *in vitro* as well as *in vivo* performance of nanoparticles.[8] These physicochemical properties largely depend on the formulation as well as process parameters of nanoparticles. Hence, optimization of these formulation parameters such as nature and concentration of polymers, surfactants, solvents, stabiliz-

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ers used and process parameters such as temperature, stirring speed, in order to obtain nanoparticles with effective properties for their improved performance is of most important area of research.

Various research reports have been published in this area but still there is a large scope for exploration of new methods and optimization of related formulation and process parameters. Some of the works reported earlier were effect of stirring speed, organic phase injection rate, polymer concentration and stabilizer concentration on the polycaprolactone nanoparticles prepared by solvent displacement method reported by Waisudin Badri *et al.*[9] effect of organic to aqueous phase volume ratio, surfactant concentration, polymer concentration and type of polymer on biodegradable nanoparticles of paclitaxel prepared by emulsion solvent evaporation method reported by Navneet Sharma *et al.*[10] optimization of polymer content, stabilizer concentration, surfactant in primary emulsion and stirring speed on PLGA nanoparticles prepared by w/o/w emulsification using statistical methods reported by Pradipta Sarkar *et al.*[11] optimization of surfactant concentration, amount of PLGA and sonication cycles on nanoparticles prepared by emulsion solvent evaporation method reported by Nazimuddin Chishti *et al.*[12] But, no literature was reported yet on the influence of external phase properties of secondary emulsion on polymeric nanoparticles prepared by multiple emulsion method.

Response Surface Methodology (RSM), a statistical tool, was adopted in this work in order to obtain meaningful and significant conclusions regarding the influence of various formulation parameters at different levels on the response variables. RSM is a compilation of statistical and mathematical techniques that are useful in optimizing a process/product. RSM is known for its robustness in various applications including designing, developing, optimizing processes and new products as well.[13] Desirability function approach is the most successful mathematical/statistical approach to optimize the selected formulation and/or process parameters with target of desired values of critical quality characteristics.

In this work, we aimed to develop and optimize formulation of PLGA nanoparticles of tenofovir disoproxil fumarate (TDF) prepared by modified multiple emulsification method. Three formulation factors were selected viz. polymer concentration, concentration of stabilizer in secondary emulsion and composition of outer external phase at three levels each were taken as independent variables; three critical quality characteristics of nanoparticles viz. size, entrapment efficiency and drug release constant were selected as responses/dependent variables. The experiment was designed as a 3^3 full factorial design under RSM. The prepared nanoparticles at various combinations of factors according to the design were characterized for the response variables. Later, optimization by desirability function approach was done with the target of less particle size, more entrapment of drug and less drug release constant.

MATERIALS AND METHODS

Materials

TDF was procured as gift sample from Hetero Drugs Ltd. Poly (D,L lactide-co-glycolide) (RESOMER RG503H/ PLGA RG503H) was bought from Sigma-Aldrich, Mumbai. DMEM (Dulbecco's modified Eagles medium), [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (MTT), trypsin, EDTA Phosphate Buffered Saline (PBS) were acquired from Sigma Chemicals Co. and Fetal Bovine Serum (FBS) was bought from Gibco. 25 cm^2, 75 cm^2 flask and 96 well plated were bought from eppendorf India. Pluronic F-68, SLS, Span 80 and Chloroform were acquired from S.D Fine Chemicals, Mumbai.

Preparation of PLGA Nanocapsules of TDF

Experimental design

In this work, PLGA Nanocapsules of TDF (PLGA-TDF-NCs) were aimed to develop by multiple emulsification technique. Three formulation parameters viz. concentration of polymer (A: PLGA RG503H – 50%, 62.5% and 75%), amount of surfactant (B: Pluronic F-68, 0%, 0.25% and 0.5% w/v) in the secondary emulsion and, the external phase in the final emulsion (C: Aqueous glycerol solution, 0%, 25% and 50% v/v of glycerol in water) were selected as the factors and all were taken 3 levels each. So, the experiment was developed as a 3^3 factorial design by using Design Expert software v8.0. All the possible combinations of the factors were taken as a single block with 1 center point. The major characteristics of nanoparticles viz. particle size, entrapment efficiency and drug release constant were taken as dependent variables. Description of the factors and their levels taken was given in Table 1. The various combinations of the selected factors at taken levels were shown in Table 2.

Preparation of PLGA-TDF-NCs

PLGA-TDF-NCs were prepared by double emulsification (w/o/w) method reported by Khushwant S. Yadav *et al.*[14] after making necessary modifications. The primary internal water phase was prepared by dissolving TDF in a mixture of distilled water and methanol taken

in the ratio of 4:1. PLGA RG503H was dissolved in chloroform. 2 mL of aqueous phase was then added drop wise to 10 mL of chloroform as organic phase under stirring at 12000 rpm for 30min to obtain w/o emulsion. Then this w/o emulsion was slowly added to 20 ml of external phase (distilled water / 25 %v/v aqueous glycerol / 50 %v/v aqueous glycerol) comprising 0.0% or 0.25 % or 0.5%w/v of Pluronic F-68 while the mixture was in continuous stirring. The stirring was continued until the chloroform was evaporated. The resulted aqueous nano-dispersion was centrifuged (Sorvall ST 8R, ThermoFisher Scientific) (for 30 min at 8,000 rpm and 4°C to obtain PLGA-TDF-NCs as pellet. This was washed twice with distilled water to take out any unentrapped TDF and the pellet was collected. The pellet was again dispersed in fresh distilled water and freeze-dried (FDB-5502, Operon) for 24hr to yield solid PLGA-TDF-NCs in powder.

Table 1: Description of the factors and their levels taken in this study.

Factor	Description	Level		
		-1	0	+1
A	Polymer concentration (%w/w) in final weight of nanocapsules	50	62.5	75
B	Surfactant concentration (%w/v) in secondary emulsion	0	0.25	0.5
C	Concentration of glycerol (%v/v) in external phase	0	25	50

Physical characterization studies

The prepared PLGA-TDF-NCs were studied for various physical characteristics. Differential Scanning

Table 2: Combinations of the factors and the obtained results* of PLGA-TDF-NCs.

S. No.	Formulation code	Run order	Factor level			EE (%)	LE (%)	Particle size (nm)	k (h^{-1})
			A	B	C				
1	F1	21	−1	−1	−1	20.95±2.12	10.48±1.06	326.9±8.3	0.184±0.02
2	F2	18	−1	0	−1	30.67±2.89	15.34±1.44	318.2±3.2	0.151±0.03
3	F3	17	−1	+1	−1	26.84±3.21	13.42±1.61	312.6±6.5	0.122±0.04
4	F4	16	−1	−1	0	29.36±4.11	14.68±2.06	300.2±4.8	0.145±0.05
5	F5	3	−1	0	0	45.76±2.16	22.88±1.08	290.7±8.3	0.103±0.04
6	F6	4	−1	+1	0	38.17±1.19	19.09±0.60	284.4±6.1	0.055±0.01
7	F7	13	−1	−1	+1	36.58±5.18	18.29±2.59	270.1±2.9	0.117±0.03
8	F8	11	−1	0	+1	58.15±4.81	29.08±2.40	258.6±5.3	0.082±0.02
9	F9	26	−1	+1	+1	50.75±7.12	25.38±3.56	249.3±5.1	0.05±0.02
10	F10	22	0	−1	−1	34.45±1.65	11.22±0.54	352.2±7.8	0.165±0.03
11	F11	9	0	0	−1	39.62±4.02	12.91±1.31	343.6±9.2	0.133±0.06
12	F12	25	0	+1	−1	37.91±3.52	12.35±1.14	337.2±3.4	0.096±0.02
13	F13	14	0	−1	0	40.25±6.13	13.11±1.99	338.5±7.6	0.119±0.01
14	F14	7	0	0	0	56.13±2.57	18.28±0.84	330.6±1.9	0.078±0.02
15	F14	8	0	0	0	55.26±3.14	18.00±1.02	325.9±2.5	0.078±0.02
16	F15	2	0	+1	0	45.65±4.52	14.87±1.47	318.7±10.5	0.059±0.01
17	F16	5	0	−1	+1	51.58±4.73	16.80±1.54	308.6±5.3	0.08±0.02
18	F17	1	0	0	+1	68.15±2.59	22.20±0.84	301.1±8.6	0.055±0.02
19	F18	19	0	+1	+1	56.62±1.89	18.44±0.61	292.6±5.4	0.041±0.01
20	F19	10	+1	−1	−1	29.55±3.15	7.39±0.79	376.3±11.8	0.147±0.05
21	F20	6	+1	0	−1	37.61±2.86	9.40±0.71	367.2±12.4	0.108±0.04
22	F21	15	+1	+1	−1	36.25±4.53	9.06±1.13	358.4±7.3	0.073±0.02
23	F22	23	+1	−1	0	43.56±2.96	10.89±0.74	349.8±6.4	0.101±0.03
24	F23	12	+1	0	0	60.43±7.15	15.11±1.79	346.7±8.9	0.059±0.02
25	F24	27	+1	+1	0	50.95±3.92	12.74±0.98	337.4±1.8	0.048±0.02
26	F25	24	+1	−1	+1	56.25±3.16	14.06±0.79	331.8±10.3	0.052±0.01
27	F26	28	+1	0	+1	71.35±4.55	17.84±1.14	317.5±12.1	0.046±0.01
28	F27	20	+1	+1	+1	60.35±3.67	15.09±0.92	307.9±6.3	0.039±0.02

*results expressed as average ± standard deviation for $n = 3$

Calorimetry (DSC) (Schimadzu DSC-60) was done in order to check the physical state (amorphous/crystalline) of the drug.[15] The surface morphology including particle shape was studied by using Transmission Electron Microscope (TEM) (Tecnai G2-30). Size and surface charge were determined by dynamic light scattering principle employing ZetaSizer Nano-ZS90.

Entrapment Efficiency and Drug Loading

The nano-suspension of PLGA-TDF-NCs obtained after preparation was kept for centrifugation for a period of 30 min at 8,000 rpm and at a temperature of 4°C. Supernatant and solid-pellet were separated. The pellet was carefully washed with water over a filtration medium to remove the unentrapped drug. The washings were mixed with the supernatant and spectrophotometrically (Evolution 201, ThermoFisher Scientific) analyzed at λ_{max} of 260 nm to know the unentrapped amount of TDF from which the entrapped amount of TDF was quantified. The following formulae were used to obtain drug entrapment efficiency and drug loading

$$\text{Entrapment Efficiency (\%)} = \frac{\text{amount of drug entrapped}}{\text{theoretical amount of drug in nanocapsules taken}} \times 100$$

$$\text{Drug loading (\%)} = \frac{\text{amount of drug entrapped}}{\text{total amount of drug and polymer taken}} \times 100$$

In vitro Drug Release Studies

In vitro drug release studies were performed employing dialysis bag method as reported by Bohrey S *et al.*[16] and Guptha N *et al.*[17] using dialysis membrane (Dialysis Membrane-110; Molecular weight cut off between 12,000 to 14,000; HiMedia Lab. Pvt. Ltd., Mumbai) and 0.1N HCl as buffer. The amount of TDF released was determined using UV-Visible spectrophotometer at 260 nm. The data obtained were treated using various kinetic models to determine the kinetics and mechanism of drug release.

Experimental Design Validation and ANOVA

According to the selected design, 3^3 factorial design under response surface methodology, all the possible 27 combinations of the factors were taken as a single block with one center point (so, 28 runs). The obtained values of response variables of all formulations of PLGA-TDF- were statistically treated using polynomial quadratic model of response surface methodology without need of transformation. Linear model represents the effect of factors on a response in linear regression only though the actual effect might be non-linear. But, the quadratic model expresses whether the effect of factors on a response is linear or nonlinear. The selected experimental design was validated for its fit into this polynomial quadratic model by employing ANOVA and also by plotting normal plot of residuals as well as predicted versus actual plot for every response.

Optimization by Desirability Functions Approach

Desirability function approach was adopted in this study to optimize the factors of this design to achieve the set goals of all the three response variables in a single formulation of PLGA-TDF-NCs of TDF. The goals were set as entrapment efficiency to its maximum and, particle size and release rate constant to their minimum. This optimization was performed through desirability function approach by using the Design Expert software. In this method, every response (y_i) is taken as individual desirability function (d_i) which fluctuates over the range of 0 to 1. The d_i is taken as either one when the y_i is at its goal or taken as zero when the y_i is outside the acceptable area, so that the independent variables (factors) are selected to maximize the desirability. If the target (T) for any response is to maximize it, then the desirability is calculated from

$$d_i = [(y - L)/(T - L)]^r$$

If the target (T) for any response is to minimize it, then the desirability is calculated from

$$d_i = [(U - y)/(U - T)]^r$$

where, U and L are upper and lower limits of the response variable respectively. In this study, linear desirability function approach was considered so that r was taken as one.[13,18]

In vitro Cytotoxicity Studies

The toxicity of PLGA-TDF-NCs was evaluated by MTT assay on HeLa cell lines, which were purchased from NCCS, Pune. Maintenance of the cells and procedure used for determining cytotoxicity were as reported by Venkanna A *et al.*[19] The treated cells were incubated with different concentrations of the optimized PLGA-TDF-NCs in wells in 96 plates for 48 hr. Then the test solution was replaced with fresh media of MTT solution (0.5 mg/mL) and incubated at 37°C for 3 hr. The color changed to purple due to the reduction of MTT into formazan by the active mitochondria of live cells. The color intensity/ optical density (O.D) was measured at 570 nm on a microplate reader (Varioskan™ LUX, ThermoFisher Scientific). The cells untreated with the

sample were considered as control. The below equation was used to calculate percentage inhibition

$$\% \text{ Inhibition} = \frac{\text{O.D of Control} - \text{O.D of Treatment}}{\text{O.D of Control}} \times 100$$

RESULTS AND DISCUSSION

Experimental Design

Three level full factorial design of response surface methodology was employed in this work to investigate the influence of the selected formulation parameters as independent factors on the characteristics of the PLGA-TDF-NCs as response variables. Full factorial designs are useful in elucidating quadratic effects of the factors on the responses. The results of responses of all the formulations were statistically treated using polynomial quadratic model. The obtained equations for the selected responses were

EE $= 55.43 + 6.06*A + 3.39*B + 12.00*C - 0.89*AB + 1.46*AC + 0.61*BC - 4.27*A^2 - 10.52*B^2 - 0.92*C^2$

Particle size $= +326.76 + 26.78*A - 8.66*B - 25.28*C - 0.27*AB + 2.92*AC - 1.12*BC - 7.80*A^2 + 0.35*B^2 - 3.45*C^2$

k $= 0.081 - 0.019*A - 0.029*B - 0.034*C + 0.007*AB + 0.001*AC + 0.007*BC + 0.002*A^2 + 0.004*B^2 + 0.012*C^2$

From the equations, it was understood that the entrapment efficiency was positively affected (increasing upon increasing the level of factor) by all the three selected factors; particle size was affected positively by factor A and negatively (decreasing upon increasing the level of factor) by both factors B and C; and drug release constant was negatively affected by by all the three selected factors.

Transmission Electron Microscopy (TEM)

The surface morphology of PLGA-TDF-NCs was studied by TEM and the images were shown in Figure 1. These images depicted that the PLGA-TDF-NCs were closely spherical and had uniform surface without any dents or protrusions.

Differential Scanning Calorimetry (DSC)

DSC spectra of pure TDF, pure polymer PLGA and PLGA-TDF-NCs were shown in Figure 2. The spectrum for pure TDF indicated a sharp endotherm at 118.1°C matching to its melting point which is near 115°C.[20] But this peak was not observed in the spectrum of PLGA-TDF-NCs, thus designated that the TDF was not in crystalline state in the PLGA-TDF-NCs instead

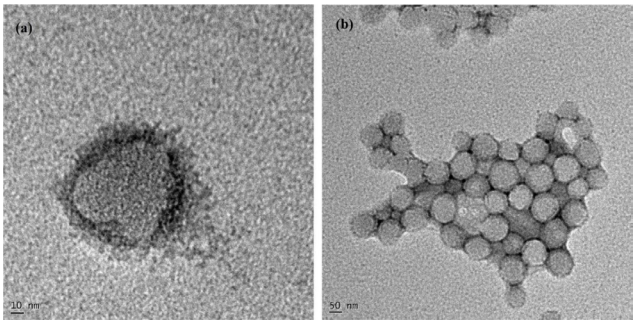

Figure 1: TEM images of PLGA-TDF-NCs of the optimized formulation.

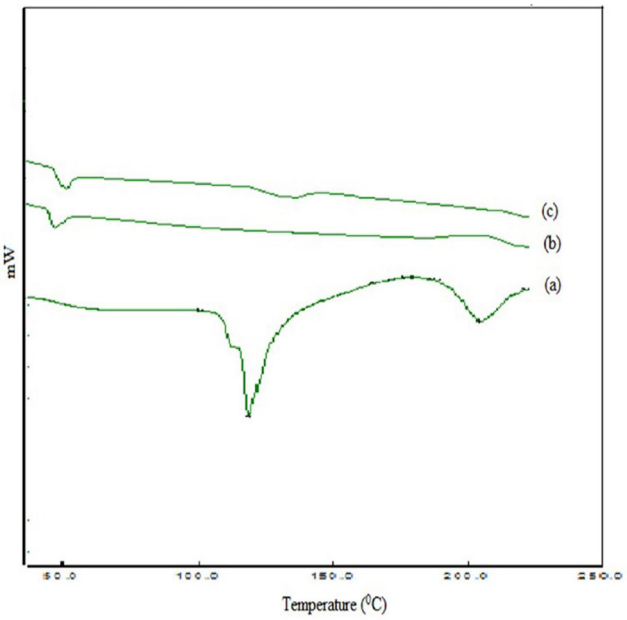

Figure 2: DSC spectra of (a) Pure TDF; (b) Pure PLGA RG503H; and (c) PLGA-TDF-NCs.

the TDF might be in amorphous state or in molecular dispersion form.[21] This could be due to the way of assimilation of drug i.e. in its solution form into the PLGA-TDF-NCs during formation. In case of PLGA, an endotherm at 48.1°C was observed in its pure spectrum agreeing to its glass transition temperature (*Tg*). But the spectrum of PLGA-TDF-NCs showed an endotherm at 52.3°C, thus indicating the *Tg* of PLGA was increased which could be due to its quick solidification upon evaporation of chloroform during the process.[22]

Particle Size

Particle size values of all formulations of PLGA-TDF-NCs were found to be in the range of 249.3 – 376.3 nm and were given in Table 2. Effect of the factors on particle size was showed in Figure 3(a). Increase in

Table 3: Results of ANOVA for response surface quadratic model for the response particle size.						
Source	**Sum of squares**	**Degrees of freedom**	**Mean sum of squares**	**F value**	***p*-Value**	**Inference[a]**
Model	26370.51	9	2930.06	269.01	<0.0001	Significant
A[b]	12906.89	1	12906.89	1184.97	<0.0001	Significant
B[c]	1350.27	1	1350.27	123.97	<0.0001	Significant
C[d]	11506.45	1	11506.45	1056.40	<0.0001	Significant
AB	0.91	1	0.91	0.083	0.7761	*Not significant*
AC	102.08	1	102.08	9.37	0.0067	Significant
BC	15.19	1	15.19	1.39	0.2530	*Not significant*
A^2	388.25	1	388.25	35.64	<0.0001	Significant
B^2	0.76	1	0.76	0.07	0.7942	*Not significant*
C^2	76.05	1	76.05	6.98	0.0166	Significant
Residual	196.06	18	10.89	--	--	--
Lack of Fit	185.01	17	10.88	0.99	0.6721	*Not significant*

Note: [a] *p*-Value less than 0.05 indicates model terms are significant; [b] Polymer concentration (%w/w); [c] Surfactant concentration in secondary emulsion (%w/v); [d] Concentration of glycerol in external phase (%v/v)

polymer concentration resulted in increase of particle size. This might be because of the fact that the polymer deposits on the surface of core material upon solvent removal[23] and hence at higher polymer levels more amount of polymer might deposit around the dispersed phase globules thus resulting in bigger particles. Shearing efficiency might also be diminished at higher viscosities that might lead to formation of large particles.[24] The particle size of nanoparticles containing surfactant was found to be less than those prepared without surfactant. This might be because of the potential of surfactant to decrease the interfacial tension henceforth the interfacial free energy, such that an emulsion with smaller particle size (or higher interfacial area) could be obtained with greater stability. These results were correlated with those reported by Y. Krishnamachari *et al.*[25] and Gupta *et al.*[26] Particle size was observed to be more in water as the external phase of secondary emulsion than that with aq. glycerol 25% and 50% v/v. The increased viscosity of the outer phase upon increase in concentration of glycerol, would resist the aggregation of globules from primary emulsion. The effect of all the factors on particle size was found to be significant at $p<0.05$ (Table 3).

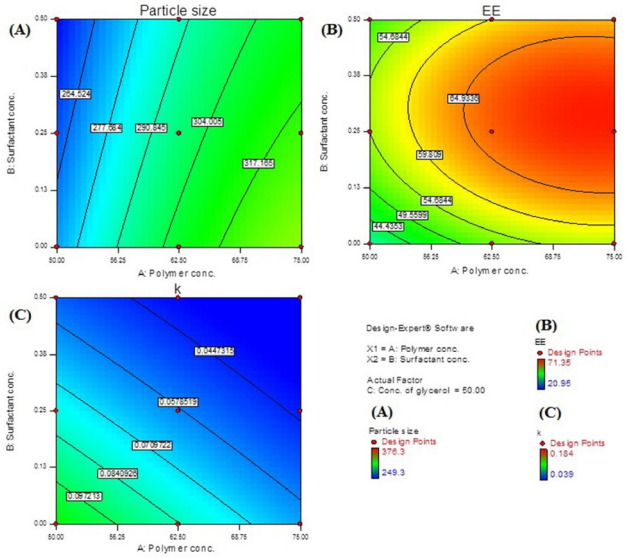

Figure 3: Contour plots showing influence of polymer concentration (factor A) and surfactant concentration (factor B) at 50% v/v glycerol in the external aqueous phase on the responses (A) Particle size; (B) Entrapment efficiency; (C) Drug release rate constant.

Zeta Potential

Zeta potential values of all formulations of PLGA-TDF-NCs were given in Table 2 and found to be varied from a minimum of −21.6 mV to −27.6 mV with the average zeta potential of −24.25 mV. These values showed that none of the three factors influenced as the zeta potential might depend majorly on the chemical nature of polymer that forms the outer surface of the nanocapsules. The polymer employed in all the formulations here was PLGA RG503H and the negative zeta potential of PLGA-TDF-NCs was due to the free carboxylic acid end groups on the polymer.[27]

Entrapment Efficiency

Entrapment efficiency values of all formulations of PLGA-TDF-NCs were given in Table 2 and were observed to be varied from 20.95% to 71.35%. Effect of the selected factors on entrapment efficiency was showed in Figure 3(b). The influence of concentration of polymer was clearly evident that the entrapment efficiency was observed to be increased as the polymer

concentration was increased. This might be because of the fact that high amount of polymer could bind the drug more tightly so that leakage of the drug would be minimized. Higher amounts of polymer also lead to increase in viscosity so that the diffusion of drug from innermost aqueous phase to organic phase could be minimized resulting in higher entrapment efficiency.[25] Also, at higher polymer levels, the increase in particle size minimized the escape of the drug by diffusion out of the nanocapsules through reduced surface area and increased path length and hence the entrapment efficiency could be increased[28] (Görner *et al.* 1999). The influence of surfactant concentration was interesting as upon its increase from 0.0% to 0.25%, the entrapment efficiency was increased but a further increase to 0.5% lead to decrease in the entrapment efficiency. The initial increase could be due to the stabilization of the emulsion because of the surfactant and thus abridged leakage of the drug. But, the drug would be diffused out of the nanocapsules and would be micellar solubilized in the external aqueous phase upon a further increase in surfactant concentration.[29] The increase in entrapment efficiency upon increase in glycerol concentration in the external phase of the secondary emulsion would be attributed to the increase in viscosity as well density the external phase. Since, the viscosity would reduce the diffusion according to Stokes – Einstein equation,[30] leakage of the drug into external phase would be reduced and lead to increase in the entrapment efficiency. Effect of all the three factors was found to be significant at $p<0.05$(shown in Table 4).

The maximum entrapment efficiency was found to be only 71.35%, which could be due to highly water soluble nature of the drug TDF that might leach to some extent into the external aqueous phase. However this entrapment efficiency value for the prepared PLGA-TDF-ACNs was found to be more than those reported by other authors[31,32] in the case of similar high water soluble drugs signifying that both PLGA polymer and ACNs technique were capable of loading water soluble drugs into nanocapsules.

In vitro Drug Release Studies

The drug release rate constant values of all formulations of PLGA-TDF-NCs were given in Table 2 and were observed to be varied from 0.039 hr^{-1} to 0.184 hr^{-1}. Effect of the three factors was showed in the Figure 3(c). The effect of amount of polymer was significant on the release of the drug from the prepared PLGA-TDF-NCs that upon increasing the amount of polymer, the drug release was found to be decreased. This might be due to the increase in particle size, which reduces the dissolution rate, upon increase in the amount of polymer. As the particle size was increased, the mean surface area would be decreased and the path length for diffusion would be increased, which might combinely result in decreased drug release rate. Effect of concentration of surfactant in secondary emulsion was significant that upon increase in its concentration, the drug release was observed to be decreased. As the amount of surfactant increases, the outer volatile organic phase (Chloroform) of primary emulsion might interact more with the external aqueous phase that lead to a decrease in the rate of evaporation which finally resulted in nanocapsules with a more rigid membrane[10,33] so that the drug release rate got decreased. The concentration of glycerol in the

Table 4: Results of ANOVA for response surface quadratic model for the response entrapment efficiency.

Source	Sum of squares	Degrees of freedom	Mean sum of squares	F value	*p*-Value	Inference[a]
Model	4381.61	9	486.85	56.00	<0.0001	Significant
A[b]	660.90	1	660.90	76.02	<0.0001	Significant
B[c]	206.45	1	206.45	23.75	0.0001	Significant
C[d]	2590.32	1	2590.32	297.95	<0.0001	Significant
AB	9.51	1	9.51	1.09	0.3096	*Not significant*
AC	25.58	1	25.58	2.94	0.1035	*Not significant*
BC	4.39	1	4.39	0.51	0.4863	*Not significant*
A^2	116.22	1	116.22	13.37	0.0018	Significant
B^2	705.72	1	705.72	81.18	<0.0001	Significant
C^2	5.39	1	5.39	0.62	0.4412	*Not significant*
Residual	156.49	18	8.69	--	--	--
Lack of Fit	156.11	17	9.18	24.26	0.1585	*Not significant*

Note: [a] *p*-Value less than 0.05 indicates model terms are significant; [b] Polymer concentration (%w/w); [c] Surfactant concentration in secondary emulsion (%w/v); [d] Concentration of glycerol in external phase (%v/v)

Table 5: Results of ANOVA for response surface quadratic model for the response drug release constant.

Source	Sum of squares	Degrees of freedom	Mean sum of squares	F value	*p*-Value	Inference[a]
Model	0.045	9	5.011×10^{-3}	78.09	<0.0001	Significant
A[b]	6.272×10^{-3}	1	6.272×10^{-3}	97.74	<0.0001	Significant
B[c]	0.015	1	0.015	240.45	<0.0001	Significant
C[d]	0.021	1	0.021	329.59	<0.0001	Significant
AB	5.201×10^{-4}	1	5.201×10^{-4}	8.10	0.0107	Significant
AC	2.408×10^{-5}	1	2.408×10^{-5}	0.38	0.5478	*Not significant*
BC	6.163×10^{-4}	1	6.163×10^{-4}	9.60	0.0062	Significant
A^2	2.550×10^{-5}	1	2.550×10^{-5}	0.40	0.5364	*Not significant*
B^2	9.368×10^{-5}	1	9.368×10^{-5}	1.46	0.2426	*Not significant*
C^2	8.927×10^{-4}	1	8.927×10^{-4}	13.91	0.0015	Significant
Residual	1.155×10^{-3}	18	6.417×10^{-5}	--	--	--
Lack of Fit	1.155×10^{-3}	17	6.794×10^{-5}	--	<0.0001	*Not significant*

Note: [a] *p*-Value less than 0.05 indicates model terms are significant; [b] Polymer concentration (%w/w); [c] Surfactant concentration in secondary emulsion (%w/v); [d] Concentration of glycerol in external phase (%v/v)

external phase significantly influenced the release rate of TDF from the PLGA-TDF-NCs as the drug release was observed to be decreased upon increase in the concentration of glycerol. This might be because of the increase in viscosity at higher concentrations of glycerol that might decrease the evaporation of chloroform. The nanocapsules obtained by slow evaporation rates might have the polymer membrane rigid with less porosity,[33] which could reduce the rate of drug release from them. Effects of all the three factors was found to be significant at $p<0.05$ (shown in Table 5). All the formulations showed first – order kinetics of drug release and non – Fickian diffusion type of mechanism of drug release.

Experimental Design Validation and ANOVA

The normal plot of residuals, shown in Figure 4 (a), was obtained as a straight line which indicated the residuals followed normal distribution and thus the response values did not require any transformation for further analysis. The predicted versus actual plot, shown in Figure 4(b), indicated that most of the actual values obtained were close to the values predicted by the design, which confirmed any transformation of responses was not necessary. The results of ANOVA were shown in Tables 3-5. The model F value for every response indicated that the model was significant designating that the response surface quadratic model selected was appropriate for the experimental design adapted in this work. This was further evidenced by insignificant lack-of-fit values. The lack-of-fit means the selected model does not fit to the experimental design which further suggests that change of model or experimental design. But, as the obtained lack-of-fit values were insignificant, the selected quadratic model was fit for the chosen experimental design.

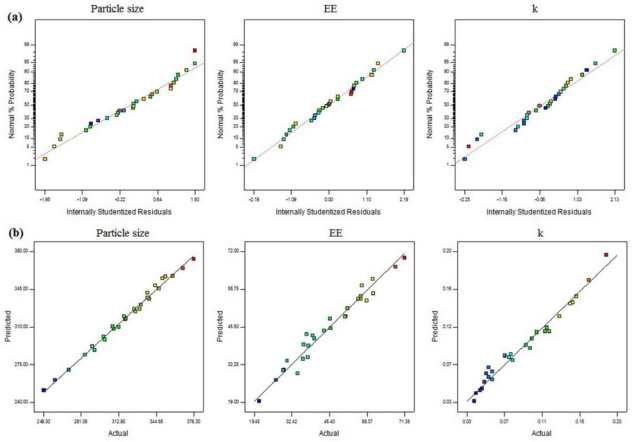

Figure 4: (a) Normal plot of residuals of all the three responses; (b) Predicted versus actual plots of all the three responses.

Optimization by Desirability Function Approach

High entrapment efficiency (decrease the quantity of formulation per dose), less particle size (increases tissue permeability) and low drug release rate constant (prolongs the duration of action) are always desirable for improved *in vitro* as well as *in vivo* performance of nanoparticles. The results so far indicated that influence of a factor on all the responses was not as desirable which could be evident from the effect of polymer concentration on the responses. The increase in polymer concentration resulted in increased in entrapment efficiency and decreased drug release rate constant which are desired but it also caused increase

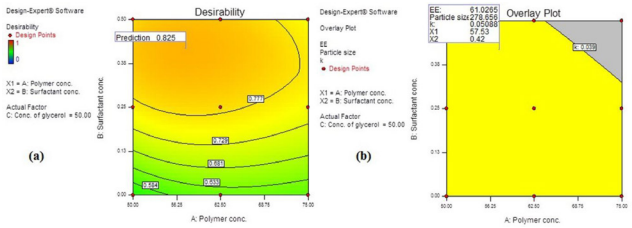

Figure 5: (a) Desirability plot; (b) Overlay plot showing the optimized values of the factors and predicted values of the responses.

in particle size which is not desirable. Hence, optimization of the factors should be done for desirable results of all the characteristics. Levels of the selected independent variables were optimized with the desirability of PLGA-TDF-NCs having less size, high entrapment and low drug release constant. Desirability was set as the entrapment efficiency to maximum; particle size to minimum; and drug release constant to minimum. Among the various solutions suggested by the software for the set desirability, one solution with maximum desirability of 0.825 (shown in Figure 5(a)) at a combination of the factors as Factor A at 57.69% w/w, Factor B at 0.42% w/v and Factor C at 50.00% v/v was considered where the response variables as shown by the software were 61.16% of entrapment efficiency, 279.12 nm of particle size and 0.051 h^{-1} of release rate constant as Figure 5(b). A formulation of PLGA-TDF-NCs was prepared by taking this optimized combination of independent variables and characterized for the response variables. The obtained results were found to be 63.08% of entrapment efficiency, 284.53 nm of particle size with zeta potential of −26.1 mV and 0.054 h^{-1} of release rate constant which were correlated to those given by the design. Hence, this combination was considered as the optimized levels of the studied independent variables.

In vitro Cytotoxicity Studies

In vitro cytotoxicity study was performed by MTT assay on HeLa cell lines for the PLGA-TDF-NCs of optimized formulation at five different concentrations ranging from 5 – 200 µg/mL. The pictures of cells after the treatment were shown in Figure 6(a) – 6(e). From the obtained % viability at these concentrations, the viability plot as shown in Figure 6(f), was made by taking concentration on x-axis and % viability on y-axis and obtained its linear regression equation. The half maximal inhibitory concentration (IC_{50}) is the concentration of the test compound required to inhibit the growth of 50% of cells.[34] The IC_{50} of PLGA-TDF-NCs was found to be 236.5±17.4 µg/mL. This high IC_{50} value indicated that either TDF or PLGA RG503H in the PLGA-TDF-NCs is not toxic and relatively safe.

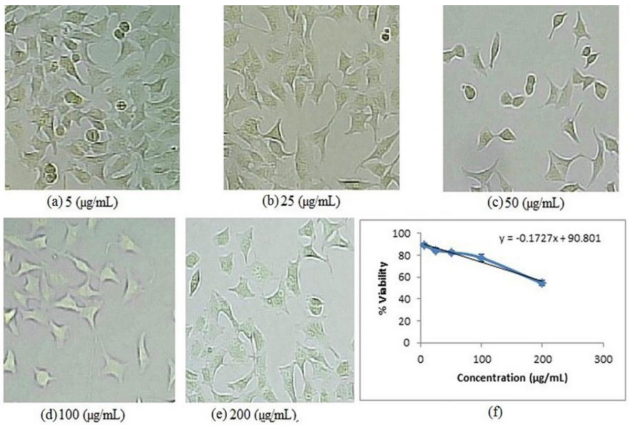

Figure 6: MTT assay images showing (a) – (e) The intensity of cells at different concentrations of PLGA-TDF-NCs; (f) Plot of concentration versus % viability.

CONCLUSION

The principle objective of this study was to optimize formulation of PLGA nanocapsules of TDF for improved *in vitro* as well as *in vivo* performance. PLGA-TDF-NCs were prepared by modified double emulsion (w/o/w) technique as a 3^3 full factorial design under response surface methodology. The influence of all the selected formulation parameters on all the response variables and also the selected response surface quadratic model were found to be significant at $p<0.05$ by ANOVA studies. Optimization was performed by desirability functions approach which resulted in PLGA-TDF-NCs with entrapment efficiency of 63.08%; particle size of 284.53 nm with zeta potential of −26.1 mV and 0.054 h^{-1} of release rate constant at polymer concentration of 57.69% w/w; at surfactant concentration in secondary emulsion of 0.42% w/v; and at 50.00% v/v of glycerol in water as external phase. Thus, PLGA nanocapsules of TDF with effective characteristic properties were developed by statistical optimization and the set objective was achieved.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

ABBREVIATIONS

ANOVA: Analysis of Variance; **DMEM:** Dulbecco's modified Eagles medium; **DMSO:** Dimethyl sulphoxide; **DoE:** Design of Experiments; **DSC:** Differential Scanning Calorimetry; **EE:** Entrapment efficiency; **FBS:** Fetal Bovine Serum; **IC_{50}:** Half maximal inhibitory concentration; **LE:** Loading efficiency; **MTT:** [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide]; **O.D.:** Optical Density; **PBS:** Phosphate Buffered Saline; **PLA:** Poly lactic acid; **PLGA:** Poly (lactide-co-glycolide); **PLGA-TDF-NCs:** PLGA Nanocapsules of TDF; **RSM:** Response Surface Methodology; **SLS:** Sodium lauryl sulphate; **TDF:** Tenofovir Disoproxil Fumarate; **TEM:** Transmission Electron Microscopy.

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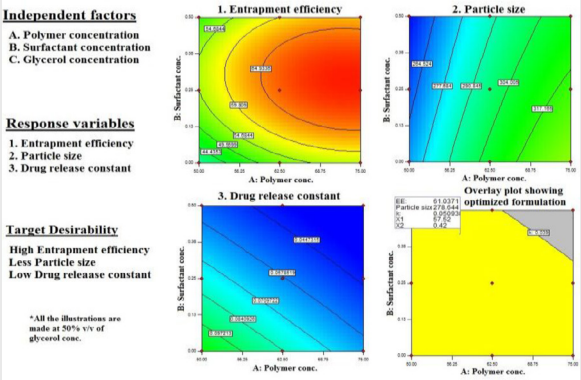
PICTORIAL ABSTRACT



SUMMARY

Optimization using statistical techniques and software for formulation of PLGA nanoparticles loaded with TDF was performed in this work. PLGA-TDF-NCs were prepared by modified multiple emulsion (w/o/w) technique. Polymer concentration, surfactant concentration in the secondary emulsion and composition of outermost phase were selected as the independent variables. Entrapment efficiency, particle size and drug release constant were taken as response variables. The experiment was designed as a 3^3 factorial design under RSM. The prepared formulations were evaluated for the response variables. The obtained results were statistically treated by response surface polynomial quadratic model using Stat Ease design expert software. The selected model and influences of all the factors on responses were found to be significant. Later, optimization was performed using desirability functions approach with the target of high entrapment efficiency, less particle size and low drug release constant. The optimized formulation was obtained with maximum entrapment efficiency of 63.08%; particle size of 284.53 nm and 0.054 h^{-1} of drug release constant, thus indicating the set objectives of the work were successfully achieved.

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