***Supplementary data***

***Influence of experimental variables***

***Influence of experimental variables on particle size***

In the manufacturing process of the RTV loaded NLC, the variables may affect the results and product outcome. Herein, we used CCRD to assess the relationship between the independent variables and the dependent variable. Table 2 shows independent variables investigated in CCRD and their responses obtained in each experiment run. The principal objective of this work was to formulate NLCs with smaller particle size which enhances solubility and bioavailability. At the same time, sustained release of drug can be achieved by the NLCs.

Particle size is a key characteristic of NLC and the average size was ranged from 159.3±0.06 to 247.4±1.13 nm. ANOVA (Table S2) for the particle size associated with the ultrasound amplitude (A), lipid concentration (B), and surfactant concentration (C) affected significantly (p<0.05).The linear experimental parameters A, B, and C has significant value (P<0.05).In the Lack of Fit test, the F-value 5.63 implies the Lack of Fit was not significant relative to the pure error. This reveals the quadratic model was a good fit with a significant regression coefficient (R2) was 0.982 (Table S3).

According to obtained results of regression analysis for the response particle size, the predictive second-order polynomial equation is deduced as

Particle size, Y1 = 191.10 -25.46 A+18.70 B-10.29C-9.41AB-15.37AC-25.33BC+5.02A2 +4.10 B2-6.90C2 ........................................................................(S1)

Where A indicates the ultrasound amplitude, B indicates the lipid concentration and C indicates the surfactant concentration.

As can be seen in Eq. (S1), it was found that the linear term for ultrasound amplitude (A) and surfactant concentration (C) showed a negative influence on particle size and a positive influence on the lipid concentration (B). Graphical representation of the significant effect of interrelation and interactions of independent variables on respective responses are presented as 3-dimensional (3D) response surface plot (Fig. 2). These responses seem like the curvilinear response of the independent variable.

Ultrasound amplitude, selected as a critical process parameter has an inverse relationship with particle size. It indicates ultrasound wave generates tiny vapor bubbles through the cavitation generated by cycles of compression and rarefaction in the liquid dispersion medium [1]. The collapse of these bubbles leads to the propagation of an intense shock wave in the liquid promotes speed of the suspended particle. Thus, an increase in the intensity of amplitude, gradually decreases the particle size as supported the Eq. (S1).

Due to the higher intensity of amplitude causes the reduction in particle size resulting in enhancement of dissolution and solubility. This was well in agreement with previous published article [2].

The influence of lipidic concentration on the particle size is depend on amount of the lipids used in the formulation. Thus, higher amount of lipid concentration increases the particle size. This was in agreement with previously reported literature [3].

The amount of surfactant concentration is a determining factor in the production of the NLCs. Tween 80 possesses the hydrophilic (ethylene oxide chain) and the lipophilic domain (hydrocarbon chain) enhances the stability of the formulation. Tween 80 possess the longer aliphatic chain to adsorb on the surface of drug and hydrophilic part move towards the aqueous phase [4]. Tween 80 provides complete coverage to particles so as to reduce the interfacial tension between hydrophilic drug particles.

Because of the non-toxic nature of Pluronic F127 and Tween 80 surfactants, the combination was used in the production of NLC. Moreover, amphiphilic nature of these (Pluronic F127 and Tween 80) surfactant, have relatively high solubilizing efficiency of a hydrophobic moiety [5].

***Influence of experimental variables on PDI***

The PDI is obtained in a range from 0.144±0.34 to 0.362±1.03 (Table 2) depending upon the particle size distribution in the dispersion. The drug is expected to target the tissues to achieve therapeutic activity and delivery of drug depends on their physicochemical characteristics along with uniform particle distribution. Thus, it was considered a critical attribute.

Table S2 shows the ANOVA for PDI evidenced that the independent variables affected insignificantly (P>0.05). All the terms of this model were insignificant. This was might be various factors such as human error, instrument error, or environmental error.

As summarized in Table S3, the ‘Lack of Fit’ F-value of 8.22 indicates the Lack of Fit was significant representing lack of fit was bad. Moreover, the fitted model for the PDI was quadratic with significant regression coefficient (R2) of 0.728.

The regression analysis showed the predictive second-order polynomial equation in terms of coded factors by adequate fitting the experimental values as

PDI, Y2 =0.20+0.02 × A+0.02 ×B-0.02×C-0.02×AB+0.03×AC+0.03×BC+0.04×A2-0.03×B2-0.01×C ...............................................................................................................(S2)

Where A indicates the ultrasound amplitude, B indicates the lipid concentration and C indicates the surfactant concentration.

From the above Eq. (S2), the linear term, ultrasound amplitude (A), lipid concentration (B) has a positive influence and a negative influence for surfactant concentration (C) was observed. The 3D response surface plot was estimated for significant effect, and interactions of independent variables on respective responses were depicted in Fig. 2. The graphical representation showed a curvilinear response of the independent variable.

Ultrasound amplitude has a slight positive effect on the PDI because it generates the cavitation energy as a result of which the uniform particle size produced indicating uniform dispersity index. Above the optimum level the particles could aggregate due to the generation of high energy and size of particle grows thereby increase in PDI.

The lipid concentration was directly proportional to PDI and 2-3 % of lipid concentration was adequate for the formulation. Surfactant decreases the interfacial tension between the two phases viz. oil and water phase to form a stable emulsion with uniform particle distribution. Thus, as the surfactant concentration increases, PDI of the formulation decreases gradually.

***Influence of experimental variables on EE***

The EE is varied from 68.04±0.23 to 94.30±3.23 % obtained from the 15 experimental runs in CCRD design (Table 2). The Model F-value of 5.11 implies the model was significant (P<0.05). There was only a 4.36 % chance to obtain an F-value due to noise. In this response, B², C² are significant model terms. As shown in Table S3, the ‘Lack of Fit’ F-value of 0.18 implies the Lack of Fit is not significant relative to the pure error. There was a 69.51% chance that a Lack of Fit F-value was obtained due to noise. This indicates the model is a good fit with quadratic Eq.

According to the results obtained using variance analysis, the predictive second-order polynomial equation in terms of coded factors is presented as

EE, Y3 = 81.11- 4.23×A+0.35×B+1.8×C+2.47×AB-5.44×AC -3.43×B C -3.27×A2+5.49×B2+4.5×C2 ................................................................................................(S3)

Where A indicates the ultrasound amplitude, B indicates the lipid concentration and C indicates the surfactant concentration.

As shown in Eq. (S3), it was revealed that the negative influence of linear term, ultrasound amplitude (A) is a more influencing factor because the percent of amplitude must be optimum and limited duration. The positive influence can be seen in the linear term, lipid concentration (B), and surfactant concentration (C).

The graphical representation of the significant influence of interrelation and interactions of independent variables on the respective response was depicted in Fig. 2.

As the increase in ultrasound amplitude decrease in particle size due to the break down of matrix lipid during the process of NLC prepartion. Beyond the optimum level of the amplitude ultrasound wave creates continuous acoustic cavitation which increases the temperature and pressure due to which the breakdown of lipid particles and affecting the EE.

Moreover, the combination of solid lipid and liquid lipid enhances the EE due to their unordered lattice structure. It provides additional space to accommodate the excessive drug and prevents drug leakage to improve the EE[6]. The adequate use of surfactant concentration, EE improves by the function of solubility and the nature of surfactants.

### Table S2 Analysis of variance (ANOVA) for experimental results obtained by particle size, PDI, and EE and fitted model summary statistics

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | **Particle size** | | | | | **PDI** | | | | | | **EE** | | | | | | | |
| **Source** | **SS** | **df** | **MS** | **F-value** | **p-value** | **SS** | **df** | **MS** | **F-value** | **p-value** | **SS** | | **df** | **MS** | **F-value** | | **p-value** | |
| Model | 10217.08 | 9 | 1135.23 | 31.16 | 0.0007 | 0.0358 | 9 | 0.0040 | 1.49 | 0.3443 | 699.71 | | 9 | 77.75 | 5.11 | 0.0436 | |
| A | 3444.50 | 1 | 3444.50 | 94.56 | 0.0002 | 0.0027 | 1 | 0.0027 | 1.03 | 0.3576 | 71.64 | | 1 | 71.64 | 4.71 | 0.0822 | |
| B | 856.98 | 1 | 856.98 | 23.53 | 0.0047 | 0.0024 | 1 | 0.0024 | 0.8919 | 0.3883 | 0.4900 | | 1 | 0.4900 | 0.0322 | 0.8646 | |
| C | 417.60 | 1 | 417.60 | 11.46 | 0.0195 | 0.0025 | 1 | 0.0025 | 0.9179 | 0.3820 | 12.95 | | 1 | 12.95 | 0.8512 | 0.3986 | |
| AB | 176.45 | 1 | 176.45 | 4.84 | 0.0790 | 0.0018 | 1 | 0.0018 | 0.6631 | 0.4525 | 12.20 | | 1 | 12.20 | 0.8015 | 0.4117 | |
| AC | 747.91 | 1 | 747.91 | 20.53 | 0.0062 | 0.0022 | 1 | 0.0022 | 0.8108 | 0.4091 | 59.30 | | 1 | 59.30 | 3.90 | 0.1054 | |
| BC | 1713.46 | 1 | 1713.46 | 47.04 | 0.0010 | 0.0023 | 1 | 0.0023 | 0.8745 | 0.3926 | 23.49 | | 1 | 23.49 | 1.54 | 0.2692 | |
| A2 | 494.40 | 1 | 494.40 | 13.57 | 0.0142 | 0.0152 | 1 | 0.0152 | 5.70 | 0.0626 | 82.46 | | 1 | 82.46 | 5.42 | 0.0674 | |
| B2 | 16.34 | 1 | 16.34 | 0.4487 | 0.5326 | 0.0086 | 1 | 0.0086 | 3.21 | 0.1331 | 232.13 | | 1 | 232.13 | 15.25 | 0.0113 | |
| C2 | 338.02 | 1 | 338.02 | 9.28 | 0.0285 | 0.0031 | 1 | 0.0031 | 1.15 | 0.3332 | 155.91 | | 1 | 155.91 | 10.24 | 0.0240 | |
| **Residual** | 182.13 | 5 | 36.43 |  |  | 0.0133 | 5 | 0.0027 |  |  | 76.09 | | 5 | 15.22 |  |  | |
| Lack of Fit | 5.63 | 1 | 5.63 | 0.1277 | 0.7389 | 0.0090 | 1 | 0.0090 | 8.22 | 0.0456 | 3.23 | | 1 | 3.23 | 0.1775 | 0.6951 | |
| Pure Error | 176.50 | 4 | 44.12 |  |  | 0.0044 | 4 | 0.0011 |  |  | 72.86 | | 4 | 18.22 |  |  | |
| **Cor Total** | 10399.21 | 14 |  |  |  | 0.0491 | 14 |  |  |  | 775.81 | | 14 |  |  |  | |

### SS- Sum of squares, MS-Mean square, df - Degree of freedom, F - Fishers ratio, and p- probability

### Table S3 Fitted model summary statistics

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Response** | **Source model** | **Std. Dev.** | **R²** | **Adjusted R²** | **Predicted R²** | **Adequate precision** | **Press** | **Suggested** |
| Particle size | Quadratic | 6.060 | 0.982 | 0.951 | 0.915 | 18.170 | 874.870 | ✓ |
| PDI | Quadratic | 0.051 | 0.728 | 0.239 | -18.884 | 5.381 | 0.977 | ✓ |
| EE | Quadratic | 3.900 | 0.901 | 0.725 | 0.404 | 7.900 | 459.380 | ✓ |

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