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Original Article

# Validation of Transmittance as Tool to Measure Particle Size Distribution using Response Surface Methodology Approach

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ABSTRACT

In the study, Transmittance has been used a model for determining the size of nanoparticles. Nanoparticles are prepared using Lipid, Dextran and Tween 80 using Bath-sonicator. The modified Solvent-evaporation method has been used for nanoparticle formation. Central Composite Design has been used for optimising our variables and for finding SLN with highest T%. Our resolves have been proved with a Particle Size analyser. The Quadratic model from response surfaces for T%-200 nm was found significant. However, from several variable compositions best SLN is made with 0.5-1 % Tween 80, 12 mg Dextran, 8 mg LC and 18 min Sonication time. It has maximum T%-200 nm (36.27%) with Zeta potential as 35 mV. The proposed method is economic for determining an estimate of Particle size range.

**Keywords:** Solid Lipid Nanoparticles (SLN); Central Composite Design (CCD); Transmittance(T%); Lipid Concentration (LC); Sonication Time (ST).

## 1. INTRODUCTION

SLN are defined as an emulsifier stabilised lipid matrix in an aqueous phase [1]. Their suitability over other colloidal carriers has been well established by many researchers [2, 3, 4] .The differences in SLN arise due to their degradation rates. Several of the factors make SLN suitable for peptide delivery [5, 6]. The current emphasis lays stress onthe effect of variables like Sonication Time (ST), Lipid concentration (LC), Tween 80 & Dextran on Particle size below 200 nm. CCD has been used for creating response surfaces with

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Design Expert ver. 7. Our method uses Bath-Sonicator instead of Ultrasonicator as the latter destroys native state of protein. In addition, Simple sonication improves stability of nanosuspension [7, 8].

Spectral Transmission is our method of choice for analysing Particle size distribution estimateina deflocculated medium as Tyndall scattering reduces due to larger particles at smaller wavelength [9, 10]. Our study focuses on use of Transmittance (T%) for judging Particle size variation. Hence an increase in T% indicates percentage of particles at particular wavelength as particle size, which links to Mie theory. The Particles size of developed nanoparticles have also been validated using Zetasizer. The impact made by independent variables on Particles below 200 nm is an important aspect of our study.

Response surface methodology using CCD is popular in many phases of drug delivery. The greater number of designs in CCD makes it more applicable then other designs. Hence, more designs in CCD makes it better than other designs. In our study Lysozyme was selected as model protein due to its commercial availability and proper characterization.

## 2. MATERIAL AND METHODS

*2.1. Materials*

Lysozyme (Himedia Laboratories - USA). Stearic Acid (CDH, New Delhi – India). Tween 80 (M/s SD Fine Chemicals Ltd, Maharashtra - India). Dextran was a gift from IGL, India. All other reagents were of analytical grade and were used as received.

*2.2. Preperation of Stearic Acid – Lysozyme SLNs*

Dextran and Tween 80 were dissolved in aqueous phase, premixed with Lysozyme. The hydrophobic phase was made by dissolving Lipid in Chloroform. For Solvent emulsification evaporation method [12], both the phases were mixed at uniform temperature of 20$^{O}$C. The newly mixed phase is sonicated in Sonicator (150 W, Time is as per Optimisation Process; Zexter (GG Technologies), New Delhi-India). The Sonicator was operated for every 6 minutes, and again restarted. Several SLN formulations were prepared by following our design. The range for variables was fixed after several preliminary experiments.

*2.3. Experimental Design*

The independent variables for optimisation were (X1) Tween 80, (X2) Dextran, (X3) Sonication cycles and (X4) - LC. The experimental methodology used was CCD (Design Expert ver 7.0 (Stat-Ease, Minneapolis, MN, USA) with α = 2 and 6 centre points. Our dependent variable was T% at 200 nm. Hence, T% was selected as an appropriate measure of particle size. The independent variables of experiment are kept at -2,-1,0,+1,+2 factor levels as per Table 1. The design in Table 2 represents 30 runs.

*2.4.Transmittance Measurement*

SLN formulations were diluted, by 100 times with double distilled water. Transmittance % usingUV Spectrophotometer (UV-1800 Series, Shimadzu Corporation, Japan) was selected for representing Particle distribution below 200 nm..Light through colloidal particles, results in declining of Absorbance. Hence, in our study selected formulation particles shows reduced Absorbance and highest Transmittance (T%) at 200 nm [13].

*2.5.Statistical Analysis*

The study was based on enumerating effect of significant factors on Particle Size using T%. Hence it was necessary to develop the mathematical model. The model will be useful in determining response within design limits set by the variables as (X1) Tween 80 (%), (X2) Dextran, (X3) Sonication Cycles and (X4) Lipid. All the factors were studied through second order polynomial *Equation-1*:-

$$\text{Response} = a^0 + a^1(X1) + a^2(X2) + a^3(X3) + a^4(X4) + a^{11}(X1)^2 + a^{22}(X2)^2 + a^{33}(X3)^2 + a^{44}(X4)^2 + a^{12}(X1)(X2) + a^{13}(X1)(X3) + a^{14}(X1)(X4) + a^{23}(X2)(X3) + a^{24}(X2)(X4) + a^{34}(X3)(X4)$$

***Equation 1***

In *Equation-1* $a^0$ = Intercept, a = 0, 1, 2, 3, 4 while ($a^1$, $a^2$, $a^3$, $a^4$) are linear coefficients, ($a^{11}$, $a^{22}$, $a^{33}$, $a^{44}$) are quadratic coefficients and ($a^{12}$, $a^{13}$, $a^{14}$, $a^{23}$, $a^{24}$, $a^{34}$) are interaction effects. Within the equation a positive sign indicates increase of response, while reverse is found with negative sign. The contour & its 3 Dimensional Plots demonstrate effect of variables over responses. The appropriateness of Quadratic model is established using coefficient of determination ($R^2$) and Adjusted $R^2$, lying in range of 20 %. Our optimised formulations was also located in Overlay plots obtained using Overlay option of Design Expert software, while balancing off the responses.

*2.6. Particle size and Zeta potential determination*

The SLN were observed for particle size distribution using Zetasizer (Malvern Instruments Ltd, Worcestershire, UK). The operating conditions were at 25°C with clear disposable zeta cell and measurement position at 2 mm. The determination was performed for some formulations, with extremity in variable concentrations.These results were compared with measured Transmittance % and tabulated in *Table 3*

## 3. RESULTS AND DISCUSSION

*3.1.Design Statistics and its feasibility*

The values of Variance Inflation Model near to 1 proves significance of our model. It also shows non-correlation with the predicted value. The Polynomial equation representing T % at 200 nm is as follows:-

$$\text{T\%-200 nm (R1)} = 30.52 - 3.39(X1) + 1.88(X2) + 2.68(X3) - 2.55(X4) - 1.58(X1)(X2) + 0.40(X1)(X3) + 1.49(X1)(X4) - 0.63(X2)(X3) + 1.68(X2)(X4) + 0.76(X3)(X4) - 1.23(X1)^2 + 0.43(X2)^2 - 2.39(X3)^2 - 0.62(X4)^2$$

***Equation 2***

The F Value (17.53 ($p < 0.0001$) ) of Quadratic plot from Table 4indicates significance of our response R1. All

quadratic regression terms at T%-200 nm are negative except X2. The high $R^2$ value (R1) 94.24 % is an inference of response variability around mean [11]. The model represented reduced Coefficient of Variation of (R1) 7.46 % presenting a better dispersibility around mean. The significance of responses is defined by ANOVA, and is found to be significant [14]. ANOVA also provides details about factors with greater impact on design in comparison to factors with least impact.

*3.2. Influence of Variables at Transmittance 200 nm (T%-200nm)*

It's well observed from quadratic *Equation-2*, the linear regression coefficients $a^1$, $a^4$ are negative while $a^2$, $a^3$ are positive. All linear regression coefficients have a significant effect at $p<0.05$. The cross term reaction coefficients $a^{13}$, $a^{23}$ and $a^{34}$ were insignificant, hence they were removed from our investigation. The model has been not been reduced by removing insignificant terms as it lost its hierarchy. The significant negative quadratic coefficients $a^{11}$, $a^{33}$, follows a curved line below x axis. It seems with greater significance of cross-term coefficients with Dextran that it has greater impact on our SLN with T% - 200 nm.The "Predicted R-Squared value" of 72.34 is rationally agreed with "Adjusted R-Squared value" of 88.87 as they are within 20 % of each other. The adequate Precision is 14.651 which is greater than 4, proves the significance of our model.

The significant effect of Fig 1(a), was observed at highest Dextran concentration where our response reduces with increasing Tween 80 concentration (0.5 – 2%). Hence at 12 mg dextran smaller particles are formed as compared to 10 mg dextran. Hence, protein folding is enhanced by Dextran which makes it easier to pack in smaller cavity. This protein folding reduces its burden over the lipid coating and effecting the release rate. Hence, T % reaches maximum at higher Dextran and lowest Tween 80 concentration. The plots from Fig 1(b), 1(c) represents Lipid as an important component effecting T%-200 nm (Smaller particle size). It is understood from Fig 1(b)that increasing Tween 80 shows declining of response. Hence maximum T% is achieved at 0.5 % Tween 80 and 8 mg LC. Similarly, In Fig 1(c) highest T%-200 nm is at maximum at 12 mg Dextran / 8 mg Lipid concentration, followed by sharp reduction.

Infact with low ST and high LC, lipid fails to separate from its phase and encapsulate in aqueous phase. It remains partitioned in its organic solvent, which later on evaporates causing its aggregation. Moreover, lower ST forces membrane instability and brings lack of incomplete cavitation due to excess of lipids. In contrast, greater smaller particles in range of 200 nm are obtained at higher ST (18 min) and lower LC (8 mg), as more space for cavitation was available causing effective size reduction.

*3.4. Comparison of T% with Particle size Analysis*

The T%-200 nm of SLN formulations has been statistically compared with their Particle Size distribution measurements using Zetasizer. A 2-sample t-test was performed between T% - 200 nm, of our selected runs and cumulative percent of Particles below 200 nm. The analysis was performed with $p = 0.05$.Statistically, at 0.05 level, the difference of means was found to be insignificantly dissimilar, proving that similar results are obtained from different experimental results.

*3.5. Comparison of variables over Transmittance (T%)*

The response of T%-200 nm determines effect of variables over Particle size. Thefactors analysed through our responses are important for attaining an economic method. The effect of selected and optimised variables from our response have been compared graphically in Fig 2. The graphs have been drawn using maxima and minima of selected variables.

*3.5.1.Effect of Lipid concentration*

Lipid directly and independently effects encapsulation efficiency, pore size and release from SLN. As among several graphs Fig 2(a) signifies effects of changing LC over T% along with maxima and minima of variables under study. Its very clear that T% increases with declining Lipid levels at varying levels of maxima and minima. Thus, highest T% - 200 nm levels is achieved at LC of 8 mg ie smallest SLN are obtained. This has been reported by many other researchers in their studies.In our study usage of high lipid concentration has increased particle size as seen from response curves also. In our study Stearic acid was used as lipid. It is 18 C saturated fatty acid, with slower conversion rate from stable α form to unstable β form. Lipids like these have minor imperfections and stable configuration [1]. It can be concluded that higher concentration of lipids forms greater complexes with higher imperfections. This creates a hurdle in release of folded proteins. Such lipids would not allow protein release at desired site. The other added variableslike Tween 80 and Dextran show their effects along with lipids on encapsulation, core formation, lipid stability, firmness of protein, its aggregation, precipitation and particle size.

*3.5.2. Effect of Sonication Time*

In our method Sonication is adopted to persuade size-reduction phenomenon. The effect of Sonication for forming smaller particles is seen in Fig. 2(c).It's evident that T% at 200 nm surges up with increasing decreasing ST. Hence, smaller particles are formed at higher and optimum ST, but bigger particles are made at lower ST causing aggregate formation. Our results from Fig. 2(c) report max T% 200 nm at 12 min of ST, with 8 mg lipid and at 0.5 % Tween 80. Hence, the bulk dispersal of bigger and smaller nanoparticles is dependent on sonication [15]. At lower ST the particles exist as loose aggregates along with remains of solvent, which hampers creation of proper cavitation for encapsulation of drug. The solvent content is reduced with increasing ST, during formation of lipid layer over hydrophilic compound. Higher sonication time reduces α form of lipids, which promotes aggregate formation [16].

*3.5.3. Effect of Dextran*

Dextran being a protein stabiliser effects T% to great extent. Dextran along with Lysozyme is incorporated in Stearic acid-SLNs. The effect of Dextran on T% can very well be determined from Fig 2(d). This graphical comparison details that effect of Dextran in presence of decreasing LC with varying Tween 80 concentrations. In the Fig 2(d) T% 200 nm increases with rising Dextran concentration along with changing LC, which supports our concept of using higher Dextran for stabilising effect. Hence, highest T % 200 nm effect is achieved at 12 mg Dextran. Therefore, ST and LC along with Dextran plays a major role in determining Particle size. Dextran as a stabiliser helps in maintaining the folded state of protein, by protecting it from Sonication.

*3.5.4. Effect of Tween 80*

Tween 80, a non-ionic surfactant regulates particle size at suitable concentrations. Higher concentrations may be detrimental for proteins and may increase particle size below limits by causing aggregation. The effect of changing T% due to Tween 80 is observed with fluctuating variables used in our study. In Fig 2(b) at LC of 8 mg and12 mg Dextran highest T % 200 nm is achieved at lowest Tween 80 concentration of 0.5 %. decreasing ST, T% - 300 nm increases with Tween 80 from 0.5 to 1.5 % at 10 mg Dextran. Our study clearly states that smaller particles are easily formed at lower Tween 80 concentrations. Dhawan et al reported that increasing Tween 80 concentration coats SLNs surface, increases its size and made particle coalesce even at low lipid concentrations [17]. Ginnavola et al also has reported that hydrophilic Tween 80 forms a thicker surfactant film over surface of Nanoparticles, thus reducing zeta potential and promoting instability at lower lipid concentration. These results support our study [17, 18] as increase in Tween 80 at lowest LC of 8 mg reduces T% - 200 nm. Hence it's reflected that higher Tween 80 concentration with higher lipids increases viscosity, effects sonication and increases particle aggregation. Thus, optimised concentration of surfactant is required for formation of smaller nanoparticles [19]. Hence, it is determined that higher concentration of Tween 80 when assisted by Dextran increases viscosity, which promotes aggregates formation by increasing Particle size at lower lipid concentration.

**Table 1: Central Composite Design (CCD) - Independent Variables with Levels**

| Factor Levels as -2, -1, 0, +1, +2 | |
|---|---|
| (X1) Tween 80 in mg = (0, 0.5, 1, 1.5, 2) | (X2) Dextran in mg = (9, 10, 11, 12, 13) |
| (X3) Sonication Cycles (in min) = (0, 6, 12, 18, 24) | (X4) Lipid in mg = (1, 8, 15, 22, 29) |

**Table 2: Central Composite experimental design with Transmittance – 200 nm**

| Runs | A:Tween 80 X1 | B:Dextran X2 | C:Sonication Time X3 | D:Lipid Concentration X4 | Transmittance @200nm |
|---|---|---|---|---|---|
| | Percentage | mg | min | mg | Response 1 |
| 1 | 0.5 | 12 | 6 | 8 | 34.25 |
| **2** | 1 | 11 | 12 | 1 | 35.26 |
| 3 | 1 | 11 | 12 | 29 | 20.53 |
| 4 | 2 | 11 | 12 | 15 | 18.33 |
| 5 | 1 | 11 | 12 | 15 | 32.52 |
| 6 | 0.5 | 10 | 18 | 8 | 33.52 |
| 7 | 1.5 | 10 | 6 | 22 | 15.27 |
| 8 | 0.5 | 10 | 18 | 22 | 23.53 |
| 9 | 1 | 11 | 24 | 15 | 26.35 |
| 10 | 1.5 | 12 | 18 | 8 | 25.89 |
| 11 | 0.5 | 12 | 18 | 8 | 38.25 |
| 12 | 1 | 9 | 12 | 15 | 30.98 |
| 13 | 1 | 13 | 12 | 15 | 33.21 |
| 14 | 1.5 | 10 | 18 | 22 | 26.52 |
| **15** | 1 | 11 | 12 | 15 | 28.36 |
| 16 | 1 | 11 | 0 | 15 | 15.23 |
| 17 | 1.5 | 12 | 18 | 22 | 27.13 |
| 18 | 1 | 11 | 12 | 15 | 29.85 |
| 19 | 1 | 11 | 12 | 15 | 30.25 |
| 20 | 1.5 | 12 | 6 | 22 | 23.05 |
| 21 | 0.5 | 10 | 6 | 8 | 29.87 |
| 22 | 0.5 | 12 | 6 | 22 | 30.33 |
| 23 | 0.5 | 12 | 18 | 22 | 35.62 |
| 24 | 0.5 | 10 | 6 | 22 | 17.04 |
| 25 | 1.5 | 10 | 6 | 8 | 21.78 |
| 26 | 1 | 11 | 12 | 15 | 29.63 |
| 27 | 1 | 11 | 12 | 15 | 32.52 |
| 28 | 1.5 | 10 | 18 | 8 | 36.78 |
| 29 | 0 | 11 | 12 | 15 | 32.56 |
| 30 | 1.5 | 12 | 6 | 8 | 21.73 |

**Table 3: Comparison between T% -200 nm representing Particle Size, Cumulative Particle size distribution and Zeta Potential**

| Run Number | X1 | X2 | X3 | X4 | From UV Spectrophotometer | From Paricle Size Analyser | |
|---|---|---|---|---|---|---|---|
| | | | | | Transmittance (T%) -200 nm | Cumulative Percentageof Particles | Zeta Potential |
| 2 | 1 | 11 | 12 | 1 | 35.26 | 92.80 | -9.7 |
| 9 | 1 | 11 | 24 | 15 | 26.35 | 0.00 | -12.4 |
| 10 | 1.5 | 12 | 18 | 8 | 25.89 | 0.00 | -12.6 |
| 11 | 0.5 | 12 | 18 | 8 | 38.25 | 41.4 | -23.5 |
| 23 | 0.5 | 12 | 18 | 22 | 35.62 | 21.3 | -10.8 |

**Table 4: ANOVA Table for CCD ( T%-200 nm)**

| **Source** | **Sum Of Squares** | **df** | **Mean Squares** | **f Value** | **p-Value** |
|---|---|---|---|---|---|
| **Model** | 1032.29 | 14 | 73.73 | 17.53 | < 0.0001 |
| **X1 -Tween 80** | 276.45 | 1 | 276.45 | 65.73 | < 0.0001 |
| **X2 - Dextran** | 84.84 | 1 | 84.84 | 20.17 | 0.0004 |
| **X3 - Sonication Time** | 172.62 | 1 | 172.62 | 41.05 | < 0.0001 |
| **X4 - Lipid Concentration** | 156.32 | 1 | 156.32 | 37.17 | < 0.0001 |
| **X1X2** | 39.88 | 1 | 39.88 | 9.48 | 0.0076 |
| **X1X3** | 2.51 | 1 | 2.51 | 0.60 | 0.4517 |
| **X1X4** | 35.63 | 1 | 35.63 | 8.47 | 0.0108 |
| **X2X3** | 6.41 | 1 | 6.41 | 1.52 | 0.2359 |

| | | | | | |
|---|---|---|---|---|---|
| **X2X4** | 45.10 | 1 | 45.10 | 10.72 | 0.0051 |
| **X3X4** | 9.13 | 1 | 9.13 | 2.17 | 0.1613 |
| **$X1^2$** | 41.45 | 1 | 41.45 | 9.86 | 0.0068 |
| **$X2^2$** | 5.16 | 1 | 5.16 | 1.23 | 0.2855 |
| **$X3^2$** | 157.01 | 1 | 157.01 | 37.33 | < 0.0001 |
| **$X4^2$** | 10.42 | 1 | 10.42 | 2.48 | 0.1364 |
| **Residual** | 63.09 | 15 | 4.21 | | |
| **Lack of Fit** | 49.11 | 10 | 4.91 | 1.76 | 0.2775 |
| **Pure Error** | 13.98 | 5 | 2.80 | | |
| **Cor Total** | 1095.37 | 29 | | | |

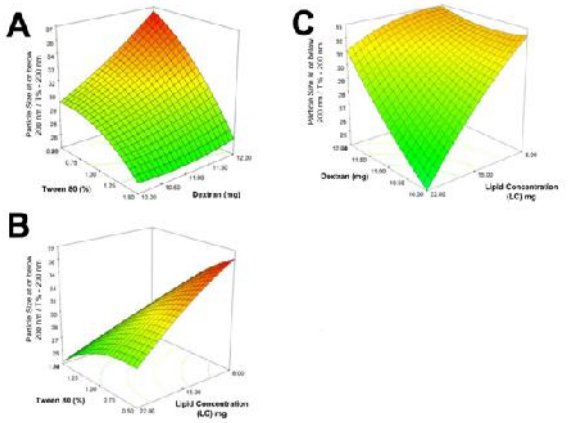


**Fig 1: Response surface plot of transmittance at 200 nm**
**A: Tween 80 and Dextran B: Tween 80 and Lipid concentration C: Dextran and lipid concentration**

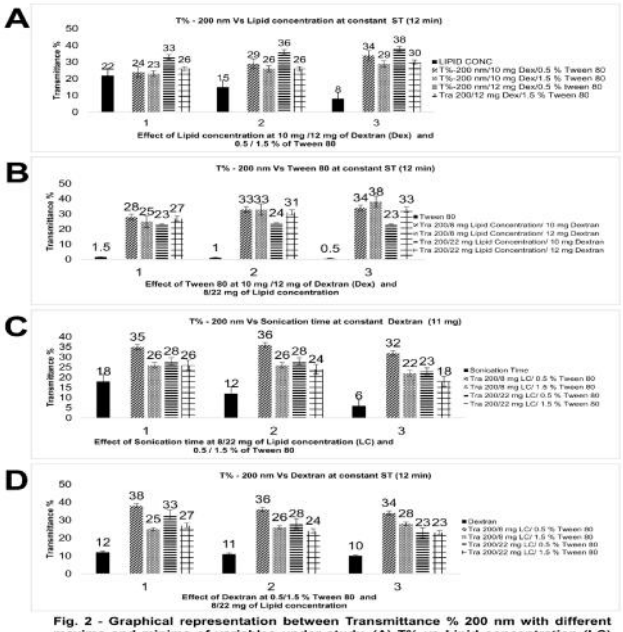


**Fig. 2 - Graphical representation between Transmittance % 200 nm with different maxima and minima of variables under study. (A) T% vs Lipid concentration (LC) (B) T% vs Tween 80 (C) T% vs Sonication time (ST) (D) T% vs Dextran**
Data were expressed as mean values (±SD, n = 3).

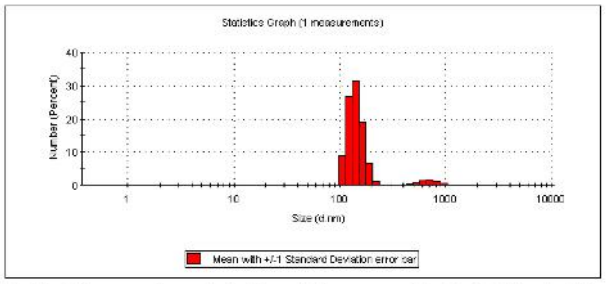


**Fig. 3 – Histogram of our selected Formulation representing Particle Size Variation**

## 4. CONCLUSIONS

It can be very well concluded using response surface designs that Transmittance can be used as an effective tool for analysing preliminary Particle size distribution analysis. Batch Sonicator can be very well used for preparation of Nanoparticles. Our results support usage of lower lipid levels and Tween 80 for obtaining SLNs. Development of such formulations may definitely promote prolonged release action of proteins at desired site.

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