# Quality by Design for Ciprofloxacin Encapsulation in PLGA

## Factors assessment followed by screening and optimization

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*The objective of this study is to formulate and characterize ciprofloxacin-PLGA nanoparticles in laboratory. Ciprofloxacin-PLGA nanoparticles were obtained with Ciprofloxacin and PLGA as raw materials at the room temperature. The optimum set of process parameters were determined using Design of Experiments (DoE) with the factors: PLGA concentration, Ciprofloxacin concentration, Agitation Rate and the responses: Particles Size, Ciprofloxacin Encapsulation. The optimized formulation had 36.83% Ciprofloxacin Encapsulation and 87.58nm Particles Size in the conditions of 10% PLGA Concentration, 24.8 mg Ciprofloxacin Concentration and 1500 Rpm.*

*Keywords: Design of Experiments, Ciprofloxacin, PLGA*

Osteomyelitis is an infection of the bone that occurs as a complication after surgical treatment or after a trauma. The current treatment of osteomyelitis implies the debridement of the infected tissue followed by prolonged antibiotic systemic treatment. The systemic treatment may lead to a low concentration of the antibiotic at the infected site due to poor vascularization [1-4].

A biofilm may develop on the surface of the surgically implanted prosthesis. Bacteria in the biofilm is usually more resistant to antibiotic treatment due to the low grow rate, resistant subpopulations and the presence of a microenvironment that interferes with the antibacterial activity. Therefore a local delivery of the antibiotic might be a better choice [4-5].

Poly (methyl methacrylate) beads impregnated with an antibiotic are used for the local delivery of the antibiotic, but this option requires a second surgical intervention because the poly (methyl methacrylate) is a polymer that it is not biodegradable [6-8]. Ciprofloxacin was researched in different studies for delivery systems that could be used in osteomyelitis [9-10].

Currently, microencapsulation research in the pharmaceutical field is focused on the formulation of drug delivery systems (DDS) to obtain new products with fewer adverse reactions and a better posology, which leads to a higher compliancy. Microencapsulation can be used to protect the active component from degradation and to ensure its release in a proper concentration at a specific site. Micro/ nanoparticles contain an active component and a biomaterial [11-12].

Ciprofloxacin (CIP) is a second generation quinolone. Furthermore it is a broad spectrum antibiotic. It is considered that ciprofloxacin is highly effective against a number of pathogens that cause infections like chronic osteomyelitis. CIP is used in the treatment of orthopedic infections due to its low minimum inhibitory concentration on a series of pathogens such as *Staphylococcus aureus, Staphylococcus epidermidis* and *Pseudomonas aeruginosa* [13-14].

Poly (lactic-co-glycolic acid) (PLGA) is a copolymer that combines the advantages of both polylactic acid and polyglicolide. It has the advantage of being both biodegradable and biocompatible. Furthermore, PLGA is approved by FDA for clinical applications. Moreover, PLGA may be easily processed to obtain different formulations for orthopedic implants [15-18].

Polyvinyl alcohol (PVA) is a surfactant that ensures the stability of the emulsion by reducing the surface tension of the continuous phase. Furthermore PVA ensures that the particles will not aggregate during the formulation process [19].

In this experiment, statistical experimental design MODDE 9.1 was used to optimize the process parameters for the synthesis of ciprofloxacin-PLGA. Applying Design of Experiments as a Quality by Design (QbD) tool enables the evaluation of all potential factors in a systematic manner, highlighting their effects and possible interactions.

## Experimental part

### *Synthesis of Nanoparticles*

There are a few parameters such as disperse phase viscosity, amount of drug added to the disperse phase, agitation rate and temperature that may have an influence over microspheres dimensions, encapsulation efficiency and particle morphology [19].

The disperse phase viscosity may be modified by either raising the polymer concentration or its molecular weight. The solvent type determines the viscosity of the disperse phase but changing the solvent is not a valid option because this solvent type ensures a fast evaporation and an optimal fabrication. This is the reason we chose to modify the viscosity by raising the polymer concentration [19].

Solvent evaporation rate may be raised by lifting the temperature of the continuous phase. A high temperature is usually associated with several disadvantages such as lower encapsulation efficiency, higher particle distribution and lower yield. Furthermore a high temperature could affect the active component or reach the boiling point of the solvent [19].

In the present study we chose to vary the disperse phase viscosity, amount of drug added to the disperse phase and agitation rate.

### *Materials*

Poly (lactic-co-glycolic) acid (PLGA) with a 65:35 lactide: glycolide ratio was purchased from Sigma-Aldrich, polyvinyl alcohol (PVA) and ciprofloxacin were purchased from Merck. All the other chemicals used were of analytical grade.

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*Nanoparticles formulation*

The method used for the ciprofloxacin encapsulation was the solid/oil/water (s/o/w) dispersion solvent evaporation technique following the steps listed below:

-Obtaining the oil phase by dissolving the PLGA in the organic solvent (methylene chloride) to render the pre-established concentrations

-Dispersing the ciprofloxacin in the oil phase

-Obtaining the aqueous phase by dissolving the polyvinyl alcohol in water at 80-90 degrees Celsius; the solution is afterwards cooled

-Obtaining the suspension-emulsion (s/u/a) at 30000 rpm

-Solvent evaporation at the pre-established agitation rate

-Separation followed by drying

*Particle size determination*

Dynamic light scattering (DLS) was used to determine the microspheres size with a Brookhaven 90 Plus. DLS is used to determine the sizes of the nanoparticles in Brownian motion in colloidal suspensions within the nano and micro range. DLS is a very useful technique to apply in the determination of nanoparticles' size in Design of Experiments [20].

The dried nanoparticles tend to aggregate in water. Therefore, ammonia hydroxide was added to the water and the nanoparticle suspension was ultrasonicated for 20 minutes.

All experiments were carried at 25°C.

*The measurement of Encapsulation efficiency*

To determine the encapsulation efficiency, the ciprofloxacin was firstly extracted from the microspheres. Therefore, 3 ml of methylene chloride were used to dissolve 10 mg microspheres. Then 2 mL of water were added to the solution. Furthermore the *p*H was set to 11 using ammonia hydroxide. Moreover the samples were ultrasonicated for 20 min and centrifuged afterwards at 10000 rpm for 10 min. The supernatant was extracted and completed to a volume of 5 mL with mobile phase. Then the samples were analyzed by high-performance liquid chromatography (HPLC) using a Thermo Finnigan Surveyor HPLC System. The mobile phase was obtained by mixing a 20 mM citrate solution (sodium citrate dihydrate 3.3 mM and citric acid hydrate 16.7 mM) and acetonitrile (40:60). This same treatment was applied to each one of the formulations. All experiments were carried at 280 nm.

The ciprofloxacin encapsulation efficiency was calculated with the following equation:

$$\text{Encapsulation efficiency (\%)} = \frac{\text{actual drug encapsulated}}{\text{theoretical drug encapsulated}} \times 100 \quad (1)$$

**Results and discussions**

The main steps in the Design of Experiments consist in the selection of the experimental objective, definition of factors and responses that are relevant to the experimental aims, selection of the regression model and the generation of proper experimental design. (21) The Design of Experiments assessment was done with MOODE 9.1 software. A two-level full factorial design, interaction model, was used to evaluate the significance of the experiment variables and the interactions between them in the formulation process of Ciprofloxacin-PLGA. The MODDE software selects the best subset of runs through an automatic search algorithm. We evaluated 3 factors (PLGA Concentration, Ciprofloxacin Concentration, Agitation rate) and 2 responses (Encapsulation efficiency, Size). The concentration of PLGA was chosen 1% as the low level and 10% as the high level. The other two factors and their two levels that are investigated are: Ciprofloxacin Concentration (low level=5mg, high level=35mg) and Agitation rate (low level=500 rpm, high level=1500 rpm). The evaluated responses included encapsulation efficiency and size. For each response a number of 3 samples per formulation were tested and their mean was put in the table 1.

The condition number shows the sphericity and symmetry of a design. For a good screening design a condition number lower than 3 is needed. Our design used for screening had a condition number of 1.173.

The primary evaluation of the experimental data consists of a general appreciation of the homogeneity and particularities of the experimental data using statistical parameters. The statistical parameters that can be used for the primary evaluation of experimental data are presented below.

**Table 1**

EXPERIMENTAL RESULTS FROM DESIGN MATRIX FOR SCREENING FULL FACTORIAL DESIGN

| Experiment Name | Run order | $X_1$ Factor Concentration of PLGA(%) | $X_2$ Factor Concentration of CIP(mg) | $X_3$ Factor Agitation rate (rpm*) | $Y_1$ Response Encapsulation efficiency (%) | $Y_2$ Response Size (nm) |
|---|---|---|---|---|---|---|
| **N1** | 4 | 1 | 5 | 500 | 5.7 | 206 |
| **N2** | 5 | 10 | 5 | 500 | 17.7 | 226 |
| **N3** | 7 | 1 | 35 | 500 | 12.45 | 209 |
| **N4** | 11 | 10 | 35 | 500 | 18 | 293 |
| **N5** | 8 | 1 | 5 | 1500 | 1 | 63 |
| **N6** | 10 | 10 | 5 | 1500 | 29.25 | 92.5 |
| **N7** | 2 | 1 | 35 | 1500 | 2.7 | 75.9 |
| **N8** | 9 | 10 | 35 | 1500 | 37.65 | 99.5 |
| **N9** | 3 | 5.5 | 20 | 1000 | 25.2 | 103 |
| **N10** | 1 | 5.5 | 20 | 1000 | 26.3 | 98 |
| **N11** | 6 | 5.5 | 20 | 1000 | 24.81 | 101 |

*rpm=rates per minute

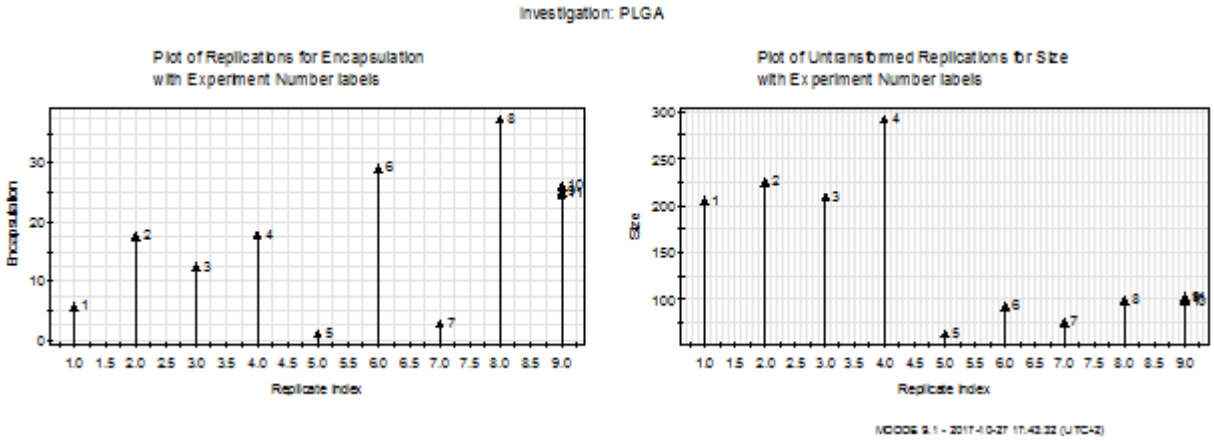


Fig. 1. Plot of Replications for Encapsulation efficiency and Size

Replicate plot is a graphical method for evaluating experimental data that consists of plotting the response value based on the experiment's number. The graphical representation of this parameter is shown in the figure 1. In this graph the value of experimental responses with the same level of independent variables (the experiment in the center of the experimental field that is repeated for three times) is represented on the same bar (experiments with numbers 9, 10 and 11). The variation of the three experimental experiments under the same experimental conditions (the same level of the value of the dependent variables) is very small, so the variation of the whole experiment is very small and the interpretation of the experimental data is made easier, the replicate errors will not complicate the obtained data analysis.

Before performing the regression analysis, we evaluated the experimental data as a histogram using descriptive statistics. In order to perform a correct regression analysis, it is advantageous for the experimental data to show a normal or approximately normal distribution. A normal distribution improves the efficiency of the analysis and increases the validity of the model and confidence in the conclusions drawn. In general, normally distributed responses will give a better model [21].

The graphical evaluation of the normal distribution can be done by graphically presenting histogram responses. The histogram is a powerful graphical tool that helps us determine whether a response transformation is needed. In our responses, only the particle size did not have a normal distribution, so this response was logarithmized, obtaining the histogram from the figure 2.

For regression analysis the goodness of fit, capacity of prediction, model validity and reproducibility were considered. The goodness of fit of a model is given by the value of $R^2$ and represents the validation of the response explained by the model. $Q^2$ represents the goodness of prediction and reveals how well the model can predict new experiments. The values of statistical parameters ($R^2$, $Q^2$) showed very good fitting of data with the proposed model, so that a good correlation exists between the determined and predicted values of encapsulation efficiency and size of particles.

For a model to pass this diagnostic test, both $R^2$ and $Q^2$ should be high and preferably not separated by more than titutes a

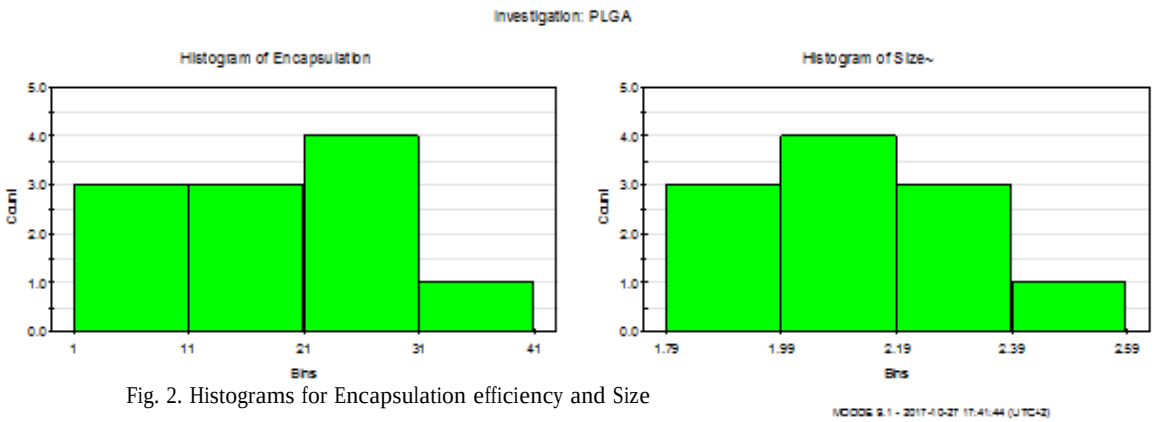


Fig. 2. Histograms for Encapsulation efficiency and Size

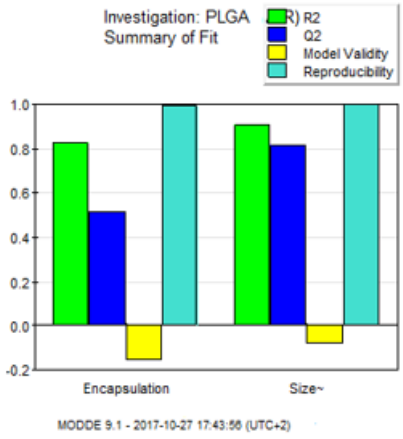


Fig. 3. Summary of Fit for responses Encapsulation Efficiency and Size

| | $R^2$ | $Q^2$ | $R^2$-$Q^2$ | Model Validity | Reproductibility |
|---|---|---|---|---|---|
| **Encapsulation efficiency** | 0.829 | 0.514 | 0.315 | -0.151637 | 0.995687 |
| **Size** | 0.909 | 0.818 | 0.091 | -0.081420 | 0.99757 |

**Table 2**
SUMMARY LIST FOR THE RESPONSES EVALUATED IN THE PROPOSED MODEL

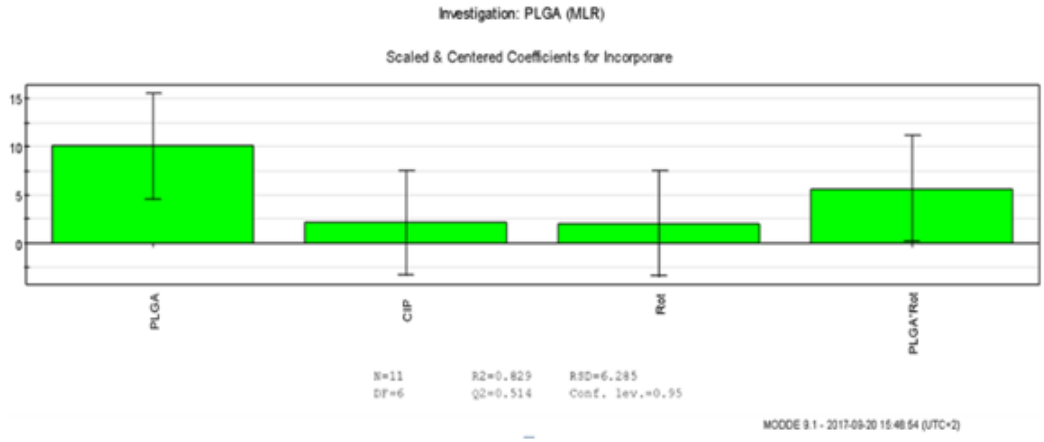


Fig. 4. Coefficients for Encapsulation efficiency

**Table 3**
COEFFICIENTS FOR ENCAPSULATION EFFICIENCY

| Encapsulation efficiency | Coeff. SC | P |
|---|---|---|
| **Constant** | 18.2509 | 7.17084e-005 |
| **PLGA %** | 10.0938 | 0.00392167 |
| **CIP (mg)** | 2.14375 | 0.371898 |
| **Agitation Rate** | 2.09375 | 0.38241 |
| **PLGA*Agitation rate** | 5.70625 | 0.0424405 |

**Table 4**
COEFFICIENTS FOR SIZE

| Size~ | Coeff. SC | P |
|---|---|---|
| **Constant** | 2.10063 | 6.69201e-012 |
| **PLGA** | 0.058918 | 0.0745755 |
| **CIP** | 0.0289526 | 0.337808 |
| **Agitation rate** | -0.22643 | 8.78784e-005 |

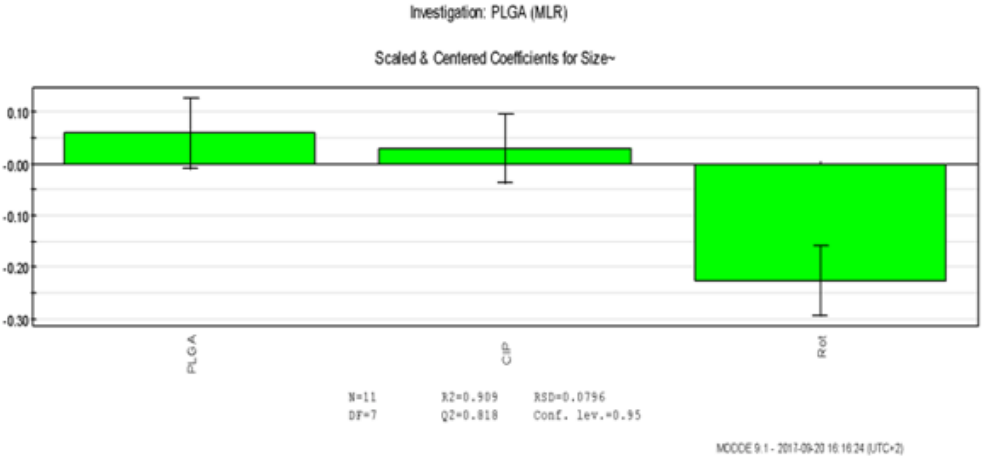


Fig.5. Coefficients for Size

warning of an inappropriate model [22]. The two $Q^2 > 0.5$ demonstrated both good models for both responses.

The model obtained is characterized by $R^2 = 0.83$ for Encapsulation efficiency and 0.91 for Size, respectively, so the prediction of the results will be very good. The value of the Model Validity is negative in both responses, less than 0.25, indicates the presence of extreme values (outliers). The value obtained for Reproducibility is greater than 0.5, which guarantees a good reproduction of the results obtained.

Scaled and centered coefficients for Encapsulation efficiency demonstrated that its important factors are: PLGA Concentration and interaction between PLGA Concentration and Agitation rate.

The model obtained is described by the equation (2), all these factors have positive influence on Encapsulation efficiency. The biggest influence on this response is done by PLGA Concentration.

$$\text{Encapsulation efficiency} = 18.25 + 10.09*\text{PLGA} + 5.71*\text{PLGA}*\text{Rot} \quad (2)$$

where PLGA is PLGA Concentration and Rot are the Agitation rate.

Moreover, a significant interaction between PLGA and Rot has been noted ($p < 0.05$). Thus, Encapsulation efficiency increases with PLGA Concentration, in a greater extent for higher number of Agitation rate.

In equation (3), Size is negatively influenced the most by the factor Agitation rate (small Size at big Agitation rate).

$$\text{Size} = 2.1 - 0.23*\text{Agitation rate} \quad (3)$$

The influence of the factors on the responses of the model is better viewed in the figure 6.

Further, ANOVA analysis of the model was performed. The ANOVA plot has three columns: SD Regression – shows the variation of response for the created model, RSD (Residual Standard Deviation) – shows the variation

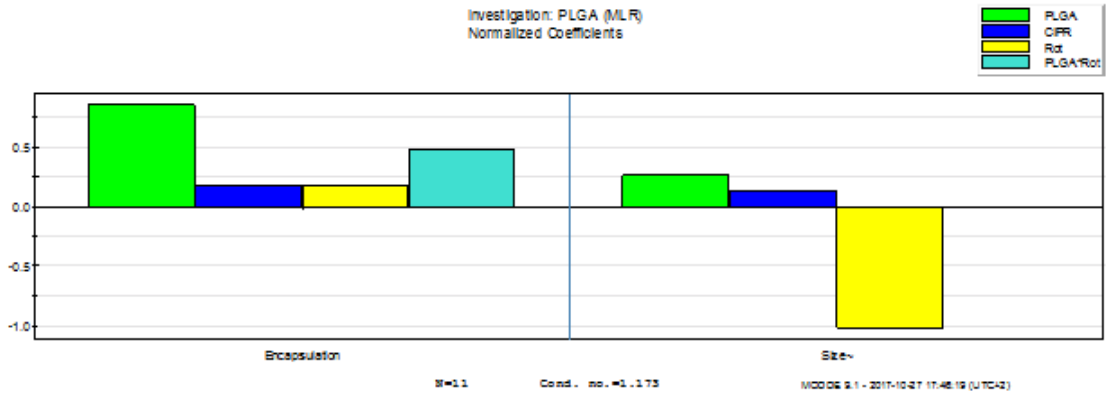


Fig. 6 Normalized coefficients for the regression in the design model

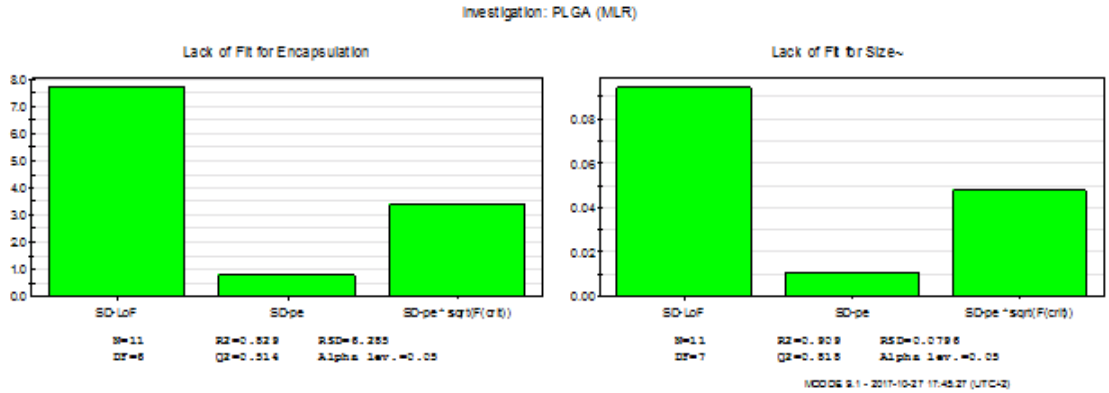


Fig. 7. ANOVA plots for the responses and Encapsulation efficiency and Size

of the response that isn't explained by the model and RSD*sqrt(F(crit)). If the third column is smaller than the first, the model is significant at a confidence interval of 95%. The results are listed in table 5.

In the table 5 are summarized the results of the 2 F-tests made in ANOVA (Analysis of Variance) - the regression analysis. The first test estimates the significance of the regression model (this test is satisfied when $p < 0.05$) we can see that the Encapsulation

| Encapsulation efficiency | DF | SS | MS (variance) | F | p | SD |
|---|---|---|---|---|---|---|
| Total | 11 | 5048.42 | 458.948 | | | |
| Constant | 1 | 3664.05 | 3664.05 | | | |
| | | | | | | |
| Total Corrected | 10 | 1384.37 | 138.437 | | | 11.766 |
| Regression | 4 | 1147.4 | 286.849 | 7.263 | **0.017** | 16.937 |
| Residual | 6 | 236.975 | 39.4958 | | | 6.285 |
| | | | | | | |
| Lack of Fit | 4 | 235.781 | 58.9452 | 98.73 | **0.010** | 7.678 |
| (Model Error) | | | | | | |
| Pure Error | 2 | 1.19406 | 0.597032 | | | 0.773 |
| (Replicate Error) | | | | | | |
| | | | | | | |
| | N = 11 | Q2 = | 0.514 | Cond. no. = | 1.173 | |
| | DF = 6 | R2 = | 0.829 | RSD = | 6.285 | |
| | | R2 Adj. = | 0.715 | | | |
| | | | | | | |
| **Size~** | **DF** | **SS** | **MS (variance)** | **F** | **p** | **SD** |
| Total | 11 | 49.0281 | 4.457 | | | |
| Constant | 1 | 48.5391 | 48.539 | | | |
| | | | | | | |
| Total Corrected | 10 | 0.489 | 0.0489 | | | 0.221 |
| Regression | 3 | 0.445 | 0.148 | 23.393 | **0.001** | 0.385 |
| Residual | 7 | 0.044 | 0.006 | | | 0.079 |
| | | | | | | |
| Lack of Fit | 5 | 0.044 | 0.008 | 74.45 | **0.013** | 0.094 |
| (Model Error) | | | | | | |
| Pure Error | 2 | 0.0002 | 0.0001 | | | 0.011 |
| (Replicate Error) | | | | | | |
| | | | | | | |
| | N = 11 | Q2 = | 0.818 | Cond. no. = | 1.173 | |
| | DF = 7 | R2 = | 0.909 | RSD = | 0.0796 | |
| | | R2 Adj. = | 0.870 | | | |

**Table 5**
ANOVA ANALYSIS

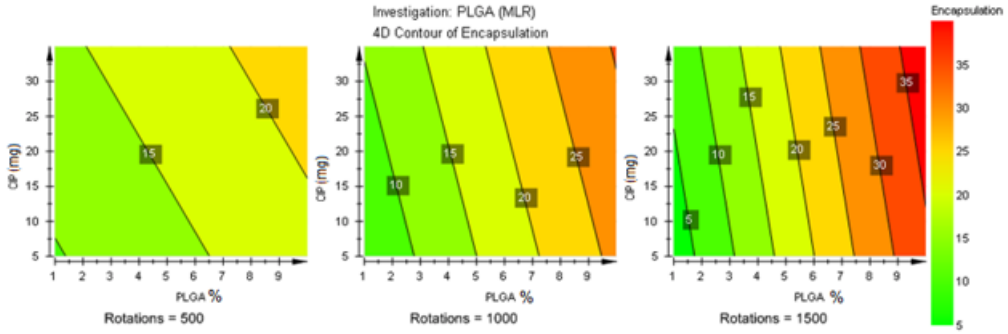


Fig.8. Contour plot for Encapsulation efficiency at 500rpm, 1000rpm and 1500rpm

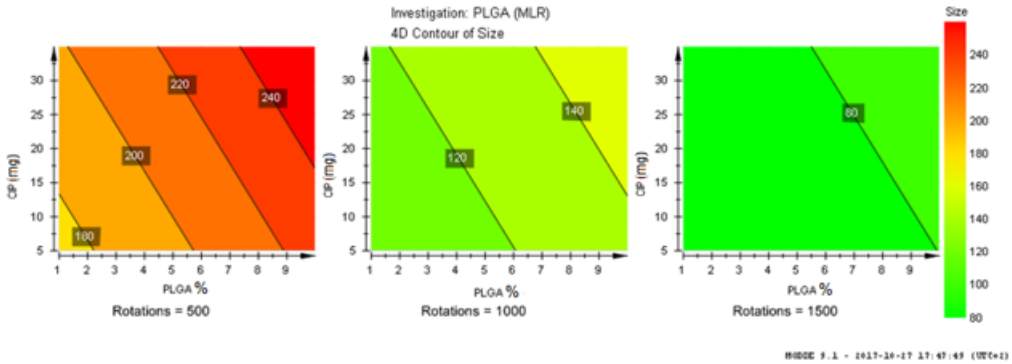


Fig. 9. Contour plot for Size at 500rpm, 1000rpm and 1500rpm

efficiency model is a statistically significant model because p = 0.017 <0.05. The second test compares model mistakes with replicated errors. When model errors are low enough we can assume that the model fits well with the data, in other words the model does not have *Lack of fit.* This second test is also known as *The lack of fit test* and is satisfied when p> 0.05. In the case of Encapsulation efficiency, p = 0.01 <0.05, and we may conclude that the model has some mismatches.

Regarding the Size Response we can say that it is a statistically significant model p = 0.001 <0.05 and that it has also certain mismatches p = 0.013 <0.05.

The regression model can be used now to predict the best conditions to obtain Ciprofloxacin-PLGA. Figures 8-9 show the response contours plot for different Agitation rate (500rpm, 1000rpm and 1500rpm).

The optimum conditions for preparation of Ciprofloxacin-PLGA were ultimately determined, to get the maximum Encapsulation efficiency and minimum Size of particles.

The formulation composition with 10% PLGA Concentration and 24.8mg Ciprofloxacin Concentration, at 1500 rpm, fulfilled the conditions of an optimum formulation.

For the agitation rate of 1500 rpm, our model predicted the values as in figure 10 for values greater than those proposed in the early steps of Design of Experiments. We

| PLGA % | CIP (mg) | Agitation rate | Encapsulation efficiency | Size | iter | log(D) | DPMO |
|---|---|---|---|---|---|---|---|
| 7.9016 | 34.9997 | 1499.99 | 30.9207 | 86.0197 | 178 | 0.6541 | |
| 10 | 16.6419 | 1499.95 | 35.6639 | 84.4608 | 168 | 0.2837 | |
| 5.5551 | 5.0003 | 1500 | 18.3944 | 70.1404 | 152 | 1.2723 | |
| 10 | 24.8004 | 1500 | 36.8306 | 87.5755 | 124 | 0.2427 | |
| 9.9996 | 21.0394 | 1500 | 36.2916 | 86.1228 | 149 | 0.2587 | |
| 7.5116 | 5.0008 | 1499.75 | 25.2615 | 74.4217 | 282 | 0.9627 | |

**Table 6**
OPTIMIZATION STEP

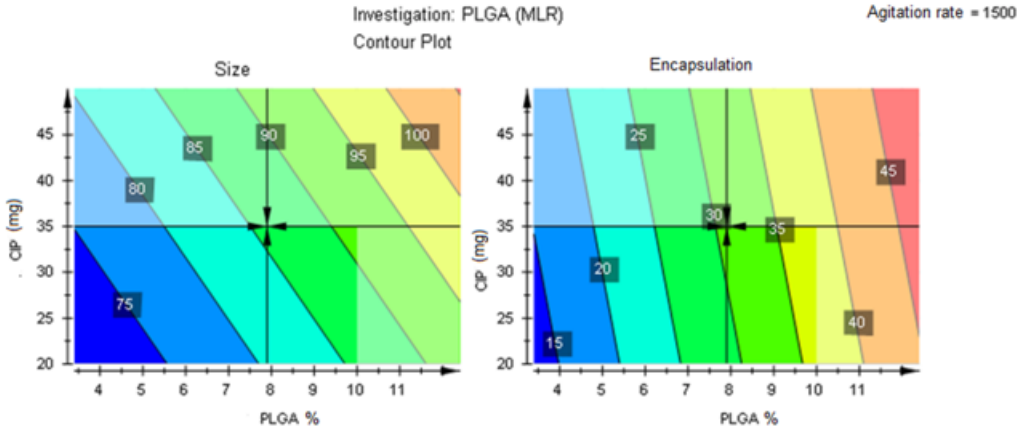


Fig. 10. Contour plot extrapolate for Encapsulation efficiency and Size at 1500 rpm

see we can obtain higher encapsulation efficiency and small size of particles by increasing the agitation rate and the concentration of PLGA. Optimum formulations are sugested by such designs [23, 24] as an economic way to produce efficient and stable formulations.

## Conclusions

The optimization of formulation is essential to achive the desired characteristics in short time, with a better precision and from an economical perspective. Our model demonstrated that the most influenced factors on Encapsulation efficiency are PLGA Concentration and its interaction with the Agitation Rate and for the size of the nanoparticles, the agitation rate: the higher agitation rate, the smaller Size. The quantitative variation level of PLGA Concentration in the screening design ranged from 1 to 10%, for Ciprofloxacin Concentration from 5mg to 35mg and from 500 rpm to 1500 rpm for the Agitation Rate. Our full factorial $2^2$ design showed that to obtain small particles and greater encapsulation efficiency, it is recommended to increase the agitation rate and the concentration of PLGA.

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