Desirability combined response surface methodology approach for optimization of prednisolone acetate loaded chitosan nanoparticles and in-vitro assessment.

Syed Yasir Iftikhar 1, Furqan Muhammad Iqbal 1, Waseem Hassan 2, Bushra Nasir 1 and Abdur Rehman Sarwar 1

1 Department of Pharmaceutics, Faculty of Pharmacy, Bahauddin Zakariya University, Multan, Pakistan
2 Department of Pharmacy, Comsats University Islamabad, Lahore campus, Lahore, Pakistan
E-mail: furqaniqbal@bzu.edu.pk

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Abstract
The objective of the current study was to design and optimize prednisolone acetate-loaded chitosan nanoparticles (NPs) through design experts for ophthalmic drug delivery. Chitosan NPs were prepared by ionic gelation using sodium tripolyphosphate (TPP). The effects of variables, such as chitosan concentration, chitosan to TPP mass ratio (ch:TPP), and prednisolone concentration on particle size, zeta potential (ZP), and polydispersity index (PDI), were studied using a three-factor three-level central composite design (CCD), and optimum experimental conditions were determined using the desirability function combined response surface methodology (RSM). Quadratic and reduced quadratic polynomial models were generated to predict and evaluate the independent variables with respect to the dependent variables. The composition of the optimal formulation was determined to be a chitosan concentration of 0.26%, chitosan to TPP mass ratio of 6:1, and drug concentration with respect to chitosan mass of 8.11%. The optimized formulation showed a percentage entrapment efficiency (% EE) of 78.32%, mean particle size of 193.5, PDI of 0.219, ZP of 10.3 mV, and 86.15% cumulative drug release. The morphology of the NPs was found to be nearly spherical in shape by scanning electron microscopy (SEM). Differential scanning calorimetry (DSC) revealed successful loading of the drug in NPs, and FTIR confirmed polymer and drug compatibility.

1. Introduction
In recent years, with the rapid advancement of modern nanotechnology, nanodrug carriers have emerged as a novel platform for delivering a wide range of therapeutic agents, including anti-cancer, antifungal, and antiviral drugs. Nanoparticles (NPs) can divulge many benefits to drug formulations, safety, efficacy, and pharmacokinetics [1]. Because of their large surface-to-volume ratio, they offer the opportunity to transfer major masses of drugs and targeting ensures the limited systemic distribution of drugs.

Nanotechnology has established a high capacity to target specific areas of the body, and it also exhibits control release of drugs. Different types of NPs are polymeric NPs, ceramic NPs, and metallic NPs [2]. Various polymers, such as alginate, xanthan gum, chitosan, PVA, PLGA, and PEG, have been used for the synthesis of polymeric NPs [3]. Several polymeric NPs have been developed in the last few decades to be used as drug delivery systems and in other medical applications. They have the advantage of controlled drug delivery, which improves stability and reduces side effects [4]. The NPs have easy penetration through epithelial tissue and capillaries, allowing for the proficient delivery of therapeutic agents to target sites in the body. Chitosan is a natural polyaminosaccharide that is cationic in nature. Owing to its biocompatibility, bioreducibility, and low toxicity, it is a good candidate for research and development in nanotechnology [5]. Chitosan NPs have wide pharmaceutical applications, primarily for drug and gene delivery [6]. Drug delivery to mucus-containing cavities of the body (e.g., the nose, trachea, eye, and mouth) is enhanced by polymers with some special
characteristics, such as cationic charges, robust hydrogen bonding groups, and necessary chain flexibility [7]. Chitosan possesses all of these properties, making it a suitable candidate for the mucoadhesive delivery of therapeutic agents, such as drugs and insulin. As it has a cationic polyelectrolyte, it can interact strongly with a negative charge containing the mucosal surface [8]. The mucosal delivery of drugs with pre-systemic effects provides distinct benefits over oral administration.

Various methods have been reported for the preparation of NPs, such as emulsion droplet coalescence, reverse micelle synthesis, polyelectrolyte complexation, emulsion solvent diffusion, ionic gelation, and desolvation. All of these methods comprise bottom-up processes in which molecules are assembled in solution to form NPs [9]. Bottom-up and top-down methodologies are usually used in nanofabrication. In the bottom-up approach, molecules interact either by electrostatic or covalent interaction to form small particles, i.e., NPs. It can be viewed as a synthesis tactic where the building blocks combine to form a nanostructure and most often results in polydisperse formulations [10]. Other processes involve the top-down approach, in which larger particles are reduced to smaller particles by mechanical stress or other processes. We can say that in the top-down preparation strategy, building blocks are detached from the substrate to achieve nanostructure.

In the current study, the ionic gelation method, which belongs to the bottom-up approach, was used to characterize the mean particle size, zeta potential (ZP), polydispersity index (PDI), and percentage entrapment efficiency (% EE). The prepared nanodrug delivery system was optimized using response surface methodology (RSM). The ionic gelation method was selected to prepare chitosan-based biodegradable NPs because the physicochemical properties of chitosan are suitable for this method. Chitosan gelsates when it comes into contact with crosslinking agents, permitting the formation of beads under very mild conditions. Crosslinking agents could be glutaraldehyde or polyanions, e.g., sodium tripolyphosphate. Glutaraldehyde has antigenic behavior, which is why a hydrophilic polyanion is selected for the preparation of NPs [11]. The solutions of the polymer and polyanion were mixed and formed NPs because of electrostatic interactions between chitosan and polyanions such as sodium tripolyphosphate (TPP) [12].

The ZP of NPs represents their charge relative to the surrounding conditions. The surface charge of individual molecules cannot be determined by ZP; rather, it measures the electrical double layer, which is produced by ions surrounding the molecule in a solution. In a suspension, it measures the charge stability and controls particle–particle interactions. The ZP is the measure of the electrical potential at the slip plane between the bound layer of the diluent molecules surrounding the particle, and the bulk solution. In a simple solution, the particle surface charge determines the ZP, but its dependence is relatively larger for the diluent solution. When ZP is high, electrostatic repulsion is also high between the particles so flocculation is minimum. Samples with zeta potentials between $-30$ mV and $+30$ mV tended to aggregate. When the zeta potential of nanoparticles is above $+30$ mV, particles will have minimum aggregation and high size stability, which is suitable for pharmaceutical applications [13]. Nanoparticles with low zeta potential have applications in water purification, where very small particles that are difficult to remove are flocculated and filtered out. All particle systems in aqueous media carry an electric charge that may be negative, positive, or neutral. If an acidic group containing a moiety such as acetic acid dissociates on the surface of the nanoparticles, a negatively charged surface will be produced, while the basic group dissociates to produce a positive charge on the surface of the surface-modified nanoparticles. When nanoparticles are unmodified, the charge depends upon the individual atoms that comprise the surface [14].

RSM is a set of statistical and mathematical techniques that describe the conduct of a dataset with the aim of making statistical foresight. This technique centered on the fit of a polynomial equation to the data obtained from the experiment. It has wide application specifically when several variables affect the responses. The aim is to optimize these variables for accomplishing the finest system performance [15]. The optimized model is helpful in obtaining multi-variant correlation and also reduces trial and error in experimental methodology [16]. Optimization refers to enhancing the performance of a process for obtaining the maximum advantage from it. In analytical approach optimization, the conditions at which a certain procedure when applied yields the best possible outcomes [17]. Before applying the RSM, an experimental design is selected that defines the limited experiments out of the experimental region that should be performed [15].

Among the various glucocorticoids, prednisolone acetate is promising for providing immunosuppressive and anti-inflammatory effects. It is equally effective compared to prednisolone sodium phosphate [18]. It belongs to class II of the biopharmaceutical classification system [19]. Therefore, to increase its solubility and delivery, it is loaded into chitosan NPs. Treatment with corticosteroids has been increasing in ocular and topical infections to provide symptomatic relief over the past few decades; their use has been enhanced by the formulation of sustained drug delivery systems. They provide therapeutic effects with minimal side effects. Currently, corticosteroids are also used in providing palliative care to cancer patients [20].
2. Materials and methods

2.1. Materials

The prednisolone acetate was a kind gift from Remington Pharmaceuticals. Low-molecular-weight chitosan and sodium tripolyphosphate (technical grade) were purchased from Sigma Aldrich, deionized distilled water. All reagents and chemicals were of analytical grade [21].

2.2. Calibration curve of prednisolone acetate

First, 100 mg of prednisolone acetate was dissolved in a minimum amount of alcohol in a 100 ml volumetric flask to obtain a final volume of 100 ml and obtain a 1000 μg ml⁻¹ concentration. The prepared stock solution was diluted with alcohol to obtain final concentrations of 2, 4, 6, 8, and 10 μg ml⁻¹ of the drug. UV spectrophotometry was performed on these solutions and the absorbance was measured against a blank solvent at 243 nm [11, 22].

2.3. Preparation of prednisolone acetate (PA)-loaded central composite-designed nanoparticles

Twenty formulations are outlined in table 1. To prepare these formulations, a stock solution of chitosan (3 mg gm⁻¹) was prepared. For this purpose, 300 mg of chitosan was dissolved in 80 ml of 1% glacial acetic acid solution in a 100 ml volumetric flask, and the final volume was made up to 100 ml. The pH was adjusted to 4.8 with 20 M NaOH solution. The prepared solution was vacuum filtrated and diluted to obtain concentrations of 1 and 2 mg ml⁻¹. A stock solution of TPP 2.5 mg ml⁻¹ was prepared. The drug was dissolved in a minimum amount of ethanol and added to the chitosan solution. TPP was then added dropwise in magnetically stirred chitosan and drug solution over 20–30 min. After addition, the solution was stirred for 30–40 min at 25 °C [23].

2.4. Differential scanning calorimetry (DSC)

Differential scanning thermograms of chitosan, pure drug, blank nanoparticles, and drug-loaded nanoparticles were obtained using the SDT Q600 V8.3 Build 101. Ten milligrams of the samples were accurately weighed in aluminum pans and heated from 25 to 500 °C at a heating rate of 20 °C min⁻¹ under a continuous nitrogen flow.

2.5. Fourier transform infrared spectroscopy (FTIR)

The FTIR spectra of PA and loaded NPs were recorded with an ATR-FTIR spectrophotometer (Bruker IR Affinity 1 Model, Japan).

2.6. Scanning electron microscopy (SEM)

A scanning electron microscope was used to determine the surface morphology. It produces images by scanning the surface with a focused beam of electrons. The electrons interact with atoms in the sample, producing various
signals that contain information about the surface topography and composition of the samples. Characteristics such as the surface morphology and shape of PA-loaded NPs were observed under a ZEISS electron microscope.

2.7. Evaluation of formulations
The primed formulations were further evaluated for different parameters as cited.

2.8. Determination of particle size, ZP, and PDI
The particle size, zeta potential, and PDI of the samples were determined using dynamic light scattering with the Zetasizer Nano ZS 90 (Malvern Instruments, Worcestershire, UK), equipped with Nano DTS software (version 6.34) and a He–Ne laser at a wavelength of 635 nm and static scattering angle of 90°. Finally, 10 μl of formulation was taken and diluted to 1 ml and evaluated [24].

2.9. Measurement of pH
The pH of ophthalmic formulations should vary from 5 to 7.4. The pH of the formulations was determined using an electronic pH meter.

2.10. Evaluation of % EE
The formulations were centrifuged for 40 min at 14,000 rpm. The supernatant was taken from the formulations and evaluated using a UV spectrophotometer at 243 nm to determine drug concentration. Drug concentration in the supernatant indicates free drug. Entrapment was found by subtracting free drug from the total amount of drug initially added to the formulation. The % EE was determined using the following formula [25]:

\[
\% EE = \frac{W_{\text{initial}} - W_{\text{free drug}}}{W_{\text{initial}}} \times 100
\]

2.11. Release study
In-vitro release studies of polymeric nanoparticles are most widely studied by the membrane diffusion method [26, 27]. First 5 ml of formulation was placed in a dialysis membrane bag with a molecular cut-off of 12 kDa, tied, and placed into 100 ml of phosphate-buffered saline (PBS) with pH 7.4. The temperature of the system was maintained at 37 °C with continuous magnetic stirring. After a predetermined time, five ml of the medium was removed and the concentration of PA was determined by UV measurement. The original volume was retained by adding an equal volume of PBS solution. The release of PA from nanoparticles was determined using a calibration curve [28]. A similar method was adopted to determine the release of PA from its formulations already available in market.

3. Results and discussion

3.1. Mechanism of ionic gelation method to produce NPs
Chitosan is a natural polymer with a positive charge and has the potential to form nanoparticles when a polyanion such as sodium tripolyphosphate (TPP) interacts with it by electrostatic attraction [29]. The TPP solution is alkaline in nature and contains tripolyphosphoric ions and hydroxyl ions (P_3O_{10}^{5-,}O\text{H}^\text{+}). Both ions compete to bind to the –NH_3\text{+} sites of chitosan. Charge neutralization occurs when these ions bind to chitosan, resulting in the formation of NPs. PA is a synthetic glucocorticoid and has stable ester linkage, it has no possible intramolecular interaction with cationic polyaminosaccharide chitosan. The mechanism of ionic gelation and PA structure is shown in figure 1 [30].

3.2. Surface morphology
The microphotographs were taken by SEM to estimate the morphology of PA-loaded NPs. The micrographs of the optimized formulation OP3 are shown in figures 2(a) and (b). These images indicate the polydispersity of the formulation as particles of various sizes can be seen in them. The particles are discrete with a rough surface and variable morphology [31]. In figure 2(a), the area under focus is 3 μm, with 10,000 × magnification clearly depicting NPs.

3.3. Differential scanning calorimetry (DSC) analyses
DSC thermograms of PA showed an endothermic peak at 237 °C, indicating its melting point. Low molecular chitosan showed a characteristic peak at 305 °C. The dispersion of the drug in the polymer matrix led to the disappearance of the endothermic peak of PA in chitosan nanoparticles, as shown in figure 3. From the results
obtained, it can be concluded that the formulation ingredients were compatible and that the drug was successfully loaded into chitosan nanoparticles [32].

3.4. FTIR

IR spectrum of chitosan is shown in figure 4(a) depicting N-H bend vibrations and characteristic amide band at 1524 cm$^{-1}$ and 1636 cm$^{-1}$ respectively. When compared with TPP cross-linked blank NPs in figure 4(c) these peaks disappear suggesting that the amino group of chitosan is cross-linked with TPP [33]. IR analysis of the drug is shown in figure 4(b), which indicates the transmission characteristic peaks at 2010.84 cm$^{-1}$ (multiple bonded C–O groups), 1524.72 cm$^{-1}$ (aromatic ring bands), 3022.21 cm$^{-1}$ (aromatic C–H stretch), 896.47 cm$^{-1}$.
The combination of the last two peaks shows unsaturation. The FTIR of the drug loaded formulation is shown in figure 4(d), which retains all peaks of the drug and showed no interaction between the drug and polymer. Changes in vibrational frequency from 3022.21 to 3013.86 show that aromatic C–H stretching was reduced. The increase in vibrational frequency from 2010.84 to 2034.46 depicts that the carbonyl group interaction was enhanced by drug loading. Changes in frequency from 1524.72 to 1545.41 and 896.47 to 852.91 exhibited successful drug entrapment in NPs.

3.5. Statistical analysis of experimental data by design expert® software
The experimental design results showed that the particle size (PS), zeta potential (ZP), PDI, and % EE were influenced by the concentration of chitosan, chitosan to TPP mass ratio, and percentage drug concentration.
Different models were selected depending on the largest $r^2$ value. Table 2 presents various models for the response analysis.

### Table 2. Comparison of different models to analyze responses.

| Model                | $r^2$   | Adjusted $r^2$ | Predicted $r^2$ | Model                | $r^2$   | Adjusted $r^2$ | Predicted $r^2$ |
|----------------------|---------|----------------|-----------------|----------------------|---------|----------------|-----------------|
| Linear               | 0.2559  | 0.1164         | −0.3979         | Reduced Linear       | 0.2436  | 0.2016         | 0.0856          |
| 2 F                  | 0.4685  | 0.2232         | −2.0083         | 2 F                  | 0.4551  | 0.2036         | −0.0953         |
| Reduced Quadratic    | 0.6177  | 0.4811         | 0.0857          | Quadratic            | 0.5462  | 0.1377         | −1.0606         |

Table 3. ANOVA for reduced quadratic models.

| Parameters | Effect  | p-Value | Parameters | Effect  | p-Value |
|------------|---------|---------|------------|---------|---------|
| $X_1$      | 74.61   | 0.0118  | $X_1$      | −0.0495 | 0.1942  |
| $X_2$      | −20.44  | 0.4415  | $X_2$      | 0.0740  | 0.0608  |
| $X_3$      | 15.95   | 0.5464  | $X_3$      | 0.0325  | 0.3857  |
| $X_2 X_3$  | −76.70  | 0.0187  | $X_1 X_3$  | −0.0778 | 0.0760  |
| $X_1^2$    | 90.71   | 0.0262  | $X_1^2$    | 0.1689  | 0.0054  |

Table 4. ANOVA for quadratic models.

| Parameters | Effect  | p-Value | Parameters | Effect  | p-Value |
|------------|---------|---------|------------|---------|---------|
| $X_1$      | −1.51   | 0.2585  | $X_1$      | 3.50    | 0.0007  |
| $X_2$      | −1.32   | 0.3186  | $X_2$      | 2.43    | 0.0077  |
| $X_1^2$    | −2.92   | 0.0430  | $X_1^2$    | 6.03    | <0.0001 |
| $X_2$      | −1.61   | 0.2802  | $X_1 X_2$  | 1.99    | 0.0351  |
| $X_1$      | 1.25    | 0.3965  | $X_1 X_2$  | −1.72   | 0.0615  |
| $X_2$      | −0.2625 | 0.8558  | $X_1 X_3$  | −1.24   | 0.1587  |
| $X_1^2$    | −2.51   | 0.3212  | $X_1^2$    | 2.14    | 0.1543  |
| $X_2$      | −0.3018 | 0.9025  | $X_2^2$    | −0.5105 | 0.7214  |
| $X_1^2$    | 0.2982  | 0.9037  | $X_1^2$    | −2.36   | 0.1214  |

3.6. Analysis of variance (ANOVA)

ANOVA was carried out to estimate the significance of appropriate models on their responses and quantitative effects. Tables 3 and 4 summarize the effect of variables on responses, where the variables are $X_1 =$ chitosan concentration, $X_2 =$ ch:TPP mass ratio, and $X_3 =$ prednisolone concentration. The responses are $Y_1 =$ PS, $Y_2 =$ ZP, $Y_3 =$ PDI, and $Y_4 =$ % EE. A model was considered significant at the 95% confidence level ($p < 0.05$). The sign and value of the quantitative effect represent the tendency and magnitude of the variables influencing the response, respectively. The negative (−ve) value in the regression equation exhibits an antagonistic effect between the factor and response, while a positive value indicates a synergistic effect.

3.7. Responses

Different responses along with their graphical presentations are described below.

3.7.1. Particle size

By assessing the impact of various variables on PS, it becomes obvious that smaller particles will have more developed mucoadhesive strength for biological surfaces such as tissues [34, 35]. For the 20 formulations, combinations of different factors resulted in PSs varying from 161.5 to 604.9 nm. Without the addition of a
stabilizing agent, particles were relatively smaller on the nanoscale [36]. PS for all formulations are listed in table 1.

As shown in figure 5, the PS of PA-loaded NPs is polydisperse, which is because of the bottom-up methodology for NP preparation. Particles of less than 100 nm were also obtained, but the highest intensity between 100 and 200 nm indicated that maximum particles were obtained in this region. As shown in figure 6, the particles were in the range of 400 to 800 nm with a mean diameter of 604.9.

The ANOVA for the reduced quadratic model showed that the independent factors that affected the PS were the concentration of chitosan ($X_1$), chitosan/TPP weight ratio, and prednisolone concentration ($X_2X_3$), and quadratic term for chitosan/TPP weight ratio ($X_2^2$) with $p < 0.05$. A large positive value for $X_1$ and $X_2^2$ indicates that these two factors have a prime influence on the size of NPs [37]. The regression equation for the most fitted model fashioned for PS is shown below:

\[
Y_1 = 197.53 + 74.61X_1 - 20.44X_2 + 15.95X_3 - 76.70X_2X_3 + 90.71X_2^2
\]

Regarding PS, the reduced quadratic model was best fitted ($r^2 = 0.6177$). The PS was amplified by enhancing the concentration of chitosan solution, which could be related to the fact that more cation groups are available for the TPP to bind, resulting in larger particles [38]. Figures 7(a), (b) represent effect of variable change on PS.

3.7.2. PDI

PDI is a vital parameter for measuring particle size distribution in multimodal delivery. All formulations showed the PDI to be in the range of 0.257 to 0.827. As shown in table 3, only the polynomial term for chitosan concentration ($X_1^2$) had a significant effect on PDI ($p = 0.0054$). The regression equation in terms of factors for PDI is derived as follows:

\[
Y_2 = a_0 + a_1X_1 + a_2X_2 + a_3X_3 + a_4X_2X_3 + a_5X_1X_2 + a_6X_1X_3 + a_7X_2X_3 + a_8X_1^2 + a_9X_2^2 + a_{10}X_3^2
\]
The method adopted to prepare NPs is bottom-up, which always shows polydispersity in formulations. Thus, small coefficient values are obtained, which indicates the minimum effect of variable on response [39]. A reduced quadratic model with the largest $r^2$ value is fitted to this response. The positive coefficient of chitosan to TPP ratio and PA concentration showed a direct relationship between the concentration of chitosan and PDI; however, this relationship was weak and insignificant.

As shown in figures 7(c), (d), the blue area represents the combination of factors where the minimum PDI is obtained. It is clearly shown that only the variable that affects PDI is chitosan concentration, and it relates directly to PDI [40]. With the increase in the concentration of the chitosan solution, more molecules are available to interact with TPP ions; therefore, particles of different sizes are obtained.

3.7.3. Zeta potential
The zeta potential of NPs is an important parameter, as it shows the stability of the formulation. The positive zeta potential of all formulations showed the stability of the NPs. The zeta potential of the 20 formulations ranged from 5.72 to 21 mV. Positive values for ZP signifies that only a part of amino groups is neutralized in NPs synthesis [41]. Based on the $r^2$ value (0.5462), the quadratic model was fitted with the following regression equation:

$$Y_2 = 0.3812 - 0.0495X_1 + 0.0740X_2 + 0.0325X_3 - 0.0778X_1X_3 + 0.1689X_1^2$$

Figure 7. Contour graphs showing influence of variables on particle size and PDI. (Colored).

$$Y_2 = 0.3812 - 0.0495X_1 + 0.0740X_2 + 0.0325X_3 - 0.0778X_1X_3 + 0.1689X_1^2$$
All factors were weakly correlated with each other, and no factor had a significant effect on the zeta potential of the formulation, except for the prednisolone concentration. The negative coefficient with $X_3$ indicates that increasing the prednisolone concentration decreases the zeta potential.

As shown in figures 8(a), (b), the green area represents the combination of factors where a zeta potential of 10 to 16 mV is obtained. The chitosan concentration and ch:TPP mass ratio had a minimal effect on zeta potential, but the effect of prednisolone concentration was remarkable. The contour lines in figure 8(b) indicate that as prednisolone concentration increases, the zeta potential decreases.

![Figure 8](image)

**Figure 8.** Contour graph showing influence of variables on zeta potential and % EE (Colored).

$$Y_3 = 13.53 - 1.51X_1 - 1.32X_2 - 2.92X_3 - 1.61X_1X_2 + 1.25X_1X_3 - 0.2625X_2X_3$$
$$- 2.51X_1^2 - 0.3018X_2^2 + 0.2982X_3^2$$

(4)

All three factors were significant in the entrapment of PA in chitosan NPs. The highest positive coefficient with PA indicates that as the drug concentration increases, entrapment also increases [42]. Smaller particles possess a large surface and more drug is entrapped into the particles than in chitosan microparticles, which showed

3.7.4. % EE

For the 20 formulations, prednisolone % EE ranged from 59.48 to 81.26. The largest $r^2$ value obtained for the quadratic model is why it fitted the data with the following regression equation:

$$Y_4 = 72.04 + 3.50X_1 + 2.43X_2 + 6.03X_3 + 1.99X_1X_2 - 1.72X_1X_3$$
$$- 1.24X_2X_3 + 2.14X_1^2 - 0.5105X_2^2 - 2.36X_3^2$$

(5)

All three factors were significant in the entrapment of PA in chitosan NPs. The highest positive coefficient with PA indicates that as the drug concentration increases, entrapment also increases [42]. Smaller particles possess a large surface and more drug is entrapped into the particles than in chitosan microparticles, which showed
entrapment efficiencies of 4.6%–45.7% for PA [32]. Figures 8(c), (d) show the combination of the factors where highest %EE can be achieved.

3.8. Optimization
The deliberation of polynomial equations illustrates independent and dependent variables. An additional optimization by design expert software is commenced through the desirability function (DF), which is the established technique to determine optimal conditions. The DF differentiates and creates a function for each response and then determines the global function for desired responses by selecting the optimum values for variables and also considering the interaction between them [34]. Reporting the desirability of responses includes the identification of DF for each response (PS, ZP, PDI, and % EE). Desirability is scaled in the range of 0–1, with zero and one representing undesirable and very desirable, respectively. This scale is used to find a global function that should be amplified by effective selection and optimization of variables. The DF for different responses were selected as the minimum PS, maximum entrapment efficiency, zeta potential, and PDI in the range of the observed values. Optimization of the central composite design (CCD) is carried out by a design expert. Thirty-four solutions were obtained with desirability of 0.813–0.874.

3.8.1. Prediction of optimized formulation by design expert® software
The desirability ranges from zero to one, showing minimum to maximum desired results, respectively. In figure 9, the graph is plotted by keeping the ch:TPP mass ratio constant at 5.96. The blue region is the area of zero desirability, showing that in the combination of variables that lie in blue area cannot produce the desired results. The region that shades from green to orange includes the combination suitable for acquiring desired results, and as the concentration of prednisolone increases, the desirability also increases. A desirability of 0.87 can be achieved by combining the factors shown in figure 9.

In figure 10(a), a contour graph for optimized formulations is shown, which predicts the PS. The blue area in the graph represents the combination of chitosan and prednisolone concentration where a low PS up to 250 nm can be achieved while keeping the ch:TPP mass ratio constant. Similarly, the zeta potential, PDI, and % EE are predicted in figures 10(b)–(d), respectively. These figures provide a visual representation of the combination of all variables with predictions pointed on the graph. Three optimized formulations were prepared according to the prediction. The variables and responses of the optimized formulations are shown in table 5.

3.9. In-vitro drug release
The in-vitro release data illustrate the cumulative percentage release as a function of time, as shown in figure 11. Initially, a burst effect is seen: in the first hour, drug release was 12.57%, 11.89%, and 15.37% for OP1, OP2, and OP3, respectively. Subsequently, the drug release was steady. The initial burst release can be ascribed to the drug loaded on the surface of the chitosan nanoparticles.
The zero order, Higuchi, and Hixson–Crowell kinetic models were used to analyze the in-vitro release results. The in-vitro release data showed that the release of PA from all optimized nanoparticle formulations followed diffusion control and fitted to Higuchi release kinetics. During the NP release study, gel structures were formed. Drug molecules were deposited inside the gel structure or entrapped in NPs. When the release phase started, the drug molecules entrapped in the inner layer naturally diffused to the closest gel layer. The release of PA from nanoparticles was concordant with the release of PA from microparticles [32]. Erosion and degradation might be the possible mechanism for the release of PA from chitosan nanoparticles. Drug release from PA containing formulations available in the market (marketed products) followed first-order kinetics and diffusion control. These products were in solution form. A comparison of the formulation and marketed products is shown in figure 11. This reveals that the drug was released from nanoparticles for a much longer duration than

![Figure 10. Prediction by contour plot for optimized formulations and various responses (Colored).](image)

| Formulation code | Chitosan % w/v | Chitosan TPP mass ratio | % Drug concentration | Particle size Nm | Zeta potential mV | PDI | %EE |
|------------------|----------------|------------------------|---------------------|-----------------|------------------|-----|-----|
| OP1              | 0.19           | 6:1                    | 10                  | 179.8 ± 124.4   | 10.2             | 0.528 | 74.64 |
| OP2              | 0.18           | 6:1                    | 10                  | 166 ± 71.07     | 10.5             | 0.327 | 70.35 |
| OP3              | 0.26           | 6:1                    | 8.11                | 193.5 ± 47.80   | 10.3             | 0.219 | 78.32 |

The zero order, Higuchi, and Hixson–Crowell kinetic models were used to analyze the in-vitro release results. The in-vitro release data showed that the release of PA from all optimized nanoparticle formulations followed diffusion control and fitted to Higuchi release kinetics. During the NP release study, gel structures were formed. Drug molecules were deposited inside the gel structure or entrapped in NPs. When the release phase started, the drug molecules entrapped in the inner layer naturally diffused to the closest gel layer. The release of PA from nanoparticles was concordant with the release of PA from microparticles [32]. Erosion and degradation might be the possible mechanism for the release of PA from chitosan nanoparticles. Drug release from PA containing formulations available in the market (marketed products) followed first-order kinetics and diffusion control. These products were in solution form. A comparison of the formulation and marketed products is shown in figure 11. This reveals that the drug was released from nanoparticles for a much longer duration than...
from marketed products. Thus, PA-loaded chitosan NPs could provide a therapeutic effect for an extended period with less dosing frequency and cost effectiveness.

4. Conclusion

PA-loaded chitosan NPs were successfully prepared by the ionic gelation method using RSM as a tool for optimization. The mean PS ranged from 161.5 to 604.9. The optimization of drug-loaded NPs is a complex procedure requiring various variables. The optimized formulation OP3 showed a PS of 193.5 nm with a minimum PDI of 0.219 and a maximum entrapment efficiency of 78.32%. The study convincingly revealed that the optimum formulation may be efficaciously obtained using a CCD. Because of the simple and safe method of preparation with high drug entrapment, prepared NPs can be used as appropriate drug carriers for ophthalmic formulations. The optimized formulation showed controlled release, which is superior to conventional dosage form release patterns.

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ORCID iDs

Syed Yasir Iftikhar https://orcid.org/0000-0002-4813-2163
Furqan Muhammad Iqbal https://orcid.org/0000-0002-5483-2473

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