Research Article

Application of Factorial Design and Response Surface Methodology in the Optimization of Clindamycin Nanocomposites

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This study is aimed at achieving the optimized preparation of a new extended-release formulation of clindamycin (CLD) via loading of the CLD onto chitosan-chondroitin sulfate (CS-Chondro). The CS-Chondro-CLD nanocomposites were prepared by mixing different masses of CS (50, 100, and 200 mg) with Chondro (50, 100, and 200 mg) and different masses of CLD (75, 150, and 300 mg). The prepared nanocomposites were characterized by different techniques including loading efficiency (LE), X-ray powder diffraction (XRD), scanning electron microscopy (SEM), Fourier-transform infrared spectroscopy (FTIR), and release study. The XRD spectra of CS-Chondro-CLD nanocomposites showed two peaks at $2\theta = 22.5^\circ$ and $40.7^\circ$, indicating amorphous forms. The FTIR data shows incorporation of CLD into the CS-Chondro polymers. An in vitro release study of CLD from nanocomposites was carried out using PBS at pH 7.4. The result showed that the release rate was completed after 25 hours. This study showed that the CS-Chondro-CLD nanocomposites have promising applications in the delivery of CLD drug.

1. Introduction

A new advanced topic in pharmaceutical studies known as “pharmaceutical nanotechnology” focuses on pharmaceutical development. Nanoparticles are solid colloidal particles ranging in size from 10 nm to 1 μm [1]. The physical and chemical properties of materials change when prepared in nanoscale, leading to a noticeably different surface chemistry of nanoparticles compared to that of the original material [2, 3]. Major advantages of nanoparticles include increased rate of dissolution, solubility, oral bioavailability, therapeutic action, and lower amount of dose required [4].

Nanotechnology and nanoparticles are helping to considerably improve many technologies and industries such as information technology, photocatalytic applications, medicine, energy, food safety, and environmental sciences [5–8].

Polymeric nanoparticles are popular due to their ease in preparation with required characteristics for drug delivery systems. They provide an intelligent alternative for the conventional dosage form of therapeutic agents for chronic administration [9]. In addition, nanoparticles protect loaded bioactive substances against degeneration by enzymes [10]. Polymeric nanoparticles were used to overcome gastrointestinal challenge by interaction between the drug and epithelial cells in the gastrointestinal tract [11].

Chondroitin sulfate is a water-soluble component of cells, tissues, and organs. It is a major constituent of the extracellular structure [12, 13]. Chondroitin and other polymers such as albumin and chitosan have been a material of choice for the delivery of oligonucleotides, DNA, protein, and drugs [14, 15]. Chondroitin sulfate in tissues undergoes natural conversion in the physiological environment to provide controlled release of the drug [16]. The reaction of chondroitin with the drug to obtain the novel substances is carried out in a manner dependent on the functional groups involved. Chondroitin
is the most extensively studied encapsulating material for the controlled release of drugs [16].

Clindamycin (CLD) drug was approved by the US Food and Drug Administration for treatment of anaerobic infections. It is used alone or in combination with other therapies for treating several cases such as infections of the head and neck, respiratory system, babesiosis which causes flow out of red or blackish urine, acne vulgaris, vagina, bone and soft tissue, and abdomen. CLD can be bactericidal or bacteriostatic depending on its concentration and site of infection [17]. Common side effects of CLD use include rash and diarrhea which could be resolved through a strategy to prevent antibiotic-associated diarrhea and mainly probiotic administration for short time [18]. In spite of the comparatively short half-life of CLD, in which it is administered every 8 hours for 5-7 days, it is considered an appropriate treatment for most contagions. In patients with normal renal function, the half-life is 2.4 hours [19].

Clindamycin was used as a hydrophilic cationic drug loaded with dextran sulphate or sodium alginate as an anionic polymer. The results showed that these properties for polymers had a significant effect on drug loading, as the presence of dextran sulphate increased from 1.32 to 18.19%, as well as an effect on reducing the release for the drug. This improved performance of CLD was due to the novel properties of small particle size and increase surface area [20].

An attempt to study the effect of transfersomal (TRSS) on the delivery of CLD through the skin showed an enhanced penetration of the drug. The characteristics of the particles such as entrapment efficiency, particle size, surface charge, and morphology were evaluated. TRSS showed higher entrapment efficiency (EE) of 93.3%, which led to a higher in vitro release of CLD [21].

Rauta et al, focused on increasing the efficacy of CLD loaded on PLA/PLGA polymer as novel nanoparticle delivery system by oral administration. The results confirmed that CLD-PLA/PLGA polymers significantly increased the bioavailability and drug activity [22].

In another study that prepared CLD-alginate and chitosan composites as mucoadhesive drug delivery system for periodontal therapy, the results showed that concentration of sodium alginate and the molecular weight of chitosan affected the characteristics of the composites such as thickness, encapsulation, structure, tumescence, adhesion, and in vitro drug release [23].

The aim of the present study is to prepare a novel nanocomposite, which contains CLD loaded onto chitosan-chondroitin to enhance the release properties.

2. Experimental

2.1. Materials. The chemicals used in this study are clindamycin hydrochloride monohydrate (C_{16}H_{33}Cl_{2}N_{2}O_{5}S.H_{2}O, Sigma-Aldrich), low molecular weight chitosan (10-120 kDa, Sigma-Aldrich), chondroitin sulphate (C_{13}H_{21}NO_{13}S, Sigma-Aldrich), and phosphate-buffered saline tablets (PBS) (Sigma-Aldrich). The acetic acid was purchased from Chem CO (England).

| Table 1: Levels of CS, Chondro and CLD factors. |
|-----------------------------------------------|
| Factors | Unit | Low level | Middle level | High level |
| CS      | mg   | 50        | 100          | 200        |
| Chondro | mg   | 50        | 100          | 200        |
| CLD     | mg   | 75        | 150          | 300        |

2.2. Instrumentation. All the prepared samples (CS-Chondro and CS-Chondro-CLD) and standard CLD samples were studied using several instruments, which are detailed as follows:

1. X-ray diffraction (XRD) was studied using CuK radiation at 30 kV and 30 mA (GBC Scientific Equipment, USA) at the Institute of Functional Nanosystems, UIM University (Germany)
2. Fourier transform infrared spectroscopy (FTIR) data spectra were measured by a Thermo Nicolet Nexus, Smart Orbit spectrometer
3. The scanning electron microscope (SEM) images were obtained at 10 kV, Hitachi High-Tech Global
4. The UV-Vis spectra were measured using Shimadzu UV-1601
5. Zeta potential and particle size of formulations were analyzed through dynamic light scattering (DLS) with Zetasizer Nano S (Malvern UK)

3. Methods

3.1. Design of Experiment. Full factorial design was used to study the %LE, zeta potential, and particle size as responses of the CS, Chondro, and CLD preparations. The ranges of values used in the design are described in Table 1, and all the experimental run results are presented in Table 2.

3.2. Data Analysis of the Factorial Design. A plot of residuals versus corresponding predicted values, a response surface plot, a normal probability plot of the residuals, and interaction plots were used as graphical tools for the analysis of the factorial design.

A regression analysis was also used to study the relationship between the independent variables (CS, Chondro, and CLD) and the response (LE, size, and zeta potential). The experimental data were processed by using Minitab software version 18 [24].

3.3. Preparation of CS-Chondro Nanoparticles and CS-Chondro-CLD Nanocomposites. CS-Chondro nanoparticles were prepared by ionic gelation technique [25]. The CS (50, 100, 200 mg) was dissolved in 1% (w/v) acetic acid. Chondro solutions with masses of 50, 100, and 200 mg were added to CS solution, and the pH was controlled at 5, with stirring for 18 hours [26]. The product was collected at 11000 rpm.

CS-Chondro-CLD nanocomposites were formed by a drop-wise method of the aqueous solution of Chondro with
Table 2: Matrix for $3^3$ full factorial designs.

| Std. order | Sample code | CS | Chondro | CLD | %LE | Size | Potential |
|------------|-------------|----|---------|-----|-----|------|------------|
| 50         | 1           | 200| 100     | 150 | *   | +    | 10.8       |
| 67         | 2           | 100| 100     | 75  | *   | 130  | -14.3      |
| 69         | 3           | 100| 100     | 300 | 71.3| 181  | *          |
| 22         | 4           | 200| 100     | 75  | 31.3| *    | *          |
| 63         | 5           | 50 | 200     | 300 | 75.2| *    | -9.9       |
| 51         | 6           | 200| 100     | 300 | 75.7| 304  | 5.7        |
| 7          | 7           | 50 | 200     | 75  | 19.1| 150  | -7.6       |
| 47         | 8           | 200| 50      | 150 | 52.2| *    | 14.9       |
| 16         | 9           | 100| 200     | 75  | 6.3 | *    | -18.2      |
| 11         | 10          | 100| 50      | 150 | 66.0| 185  | -8.4       |
| 38         | 11          | 100| 50      | 150 | 62.7| *    | -6.3       |
| 74         | 12          | 200| 50      | 150 | 54.5| *    | 14.9       |
| 70         | 13          | 100| 200     | 75  | 5.5 | 171  | -18.6      |
| 42         | 14          | 100| 100     | 300 | 68.5| 244  | *          |
| 40         | 15          | 100| 100     | 75  | *   | 150  | -15.9      |
| 79         | 16          | 200| 200     | 75  | 33.6| 300  | 12.3       |
| 14         | 17          | 100| 100     | 150 | 50.8| 164  | -7.3       |
| 2          | 18          | 50 | 50      | 150 | *   | 176  | -2.0       |
| 3          | 19          | 50 | 50      | 300 | 86.9| 240  | -6.2       |
| 43         | 20          | 100| 200     | 75  | 7.8 | 190  | -19.1      |
| 77         | 21          | 200| 100     | 150 | *   | *    | 10.9       |
| 33         | 22          | 50 | 100     | 300 | *   | 182  | *          |
| 26         | 23          | 200| 200     | 150 | 26.8| 275  | 12.4       |
| 56         | 24          | 50 | 50      | 150 | *   | *    | -6.4       |
| 61         | 25          | 50 | 200     | 75  | 15.2| 111  | -7.4       |
| 8          | 26          | 50 | 200     | 150 | 23.3| 151  | -8.5       |
| 71         | 27          | 100| 200     | 150 | 22.2| 152  | -8.8       |
| 32         | 28          | 50 | 100     | 150 | 69.3| *    | *          |
| 19         | 29          | 200| 50      | 75  | 27.9| 460  | 19.2       |
| 5          | 30          | 50 | 100     | 150 | 52.3| *    | *          |
| 73         | 31          | 200| 50      | 75  | 28.5| 450  | 19.6       |
| 4          | 32          | 50 | 100     | 75  | 32.9| *    | *          |
| 46         | 33          | 200| 50      | 75  | 26.2| 445  | 20.0       |
| 20         | 34          | 200| 50      | 150 | 57.2| *    | 14.9       |
| 76         | 35          | 200| 100     | 75  | 33.2| 340  | *          |
| 34         | 36          | 50 | 200     | 75  | 14.3| 147  | -7.8       |
| 27         | 37          | 200| 200     | 300 | 71.1| 200  | 13.4       |
| 25         | 38          | 200| 200     | 75  | 23.8| 297  | 12.0       |
| 58         | 39          | 50 | 100     | 75  | 36.2| *    | *          |
| 60         | 40          | 50 | 100     | 300 | *   | 180  | *          |
| 75         | 41          | 200| 50      | 300 | 71.5| 308  | 8.0        |
| 53         | 42          | 200| 200     | 150 | 30.5| 252  | 12.4       |
| 24         | 43          | 200| 100     | 300 | 78.0| *    | 3.6        |
| 72         | 44          | 100| 200     | 300 | 56.0| 261  | -12.6      |
| 31         | 45          | 50 | 100     | 75  | 24.6| *    | *          |
50, 100, and 200 mg into 50, 100, and 200 mg solution of CS, containing 75, 150, and 300 mg of CLD, with constant stirring. The resulting nanocomposites were collected by centrifugation at 11000 rpm [26].

3.4. The CLD Loading Efficiency. The procedure for calculating loading efficiency (%LE) was as follows: 2.0 ml of suspension was centrifuged at 11000 rpm; then the free drug was measured by UV-Vis at $\lambda_{\text{max}}$ of 300 nm.

$\% \text{LE} = \frac{Q_T - Q_{un}}{\text{mass of nanocomposite}} \times 100$, (1)

where $Q_T$ is the total CLD used during preparation and $Q_{un}$ is the free CLD in the supernatant.

3.5. In Vitro Release Study of CLD from CS-Chondro-CLD Nanocomposites. An in vitro release study of CLD from CS-Chondro-CLD nanocomposites was carried out in PBS

| Std. order | Sample code | CS | Chondro | CLD | %LE | Size | Potential |
|------------|-------------|----|---------|-----|-----|------|-----------|
| 12         | 46          | 100| 50      | 300 | 64.8| *    | -19.4     |
| 23         | 47          | 200| 100     | 150 | *   | 328  | 11.0      |
| 59         | 48          | 50 | 100     | 150 | 62.3| *    | *         |
| 62         | 49          | 50 | 200     | 150 | 26.5| 201  | -7.8      |
| 6          | 50          | 50 | 100     | 300 | *   | 185  | *         |
| 64         | 51          | 100| 50      | 75  | *   | 177  | -10.6     |
| 17         | 52          | 100| 200     | 150 | 23.0| 203  | -18.7     |
| 55         | 53          | 50 | 50      | 75  | 60.5| *    | -6.8      |
| 49         | 54          | 200| 100     | 75  | 31.7| 379  | *         |
| 57         | 55          | 50 | 50      | 300 | 86.4| 230  | -6.1      |
| 45         | 56          | 100| 200     | 300 | 58.0| 225  | -12.6     |
| 66         | 57          | 100| 50      | 300 | 61.7| 238  | -18.9     |
| 80         | 58          | 200| 200     | 150 | 34.9| 302  | 12.3      |
| 1          | 59          | 50 | 50      | 75  | 61.4| *    | -8.7      |
| 36         | 60          | 50 | 200     | 300 | 76.2| *    | -7.9      |
| 44         | 61          | 100| 200     | 150 | 23.6| 206  | -13.8     |
| 9          | 62          | 50 | 200     | 300 | 87.7| *    | -5.9      |
| 30         | 63          | 50 | 50      | 300 | 88.4| 244  | -6.3      |
| 52         | 64          | 200| 200     | 75  | 27.7| 302  | 12.7      |
| 41         | 65          | 100| 100     | 150 | 62.8| 185  | -10.3     |
| 78         | 66          | 200| 100     | 300 | 79.9| 298  | 7.8       |
| 15         | 67          | 100| 100     | 300 | 72.2| *    | *         |
| 68         | 68          | 100| 100     | 150 | 60.1| 150  | -8.8      |
| 54         | 69          | 200| 200     | 300 | 75.0| 192  | 14.2      |
| 28         | 70          | 50 | 50      | 75  | 61.0| *    | -7.7      |
| 37         | 71          | 100| 50      | 75  | *   | 158  | -11.4     |
| 81         | 72          | 200| 200     | 300 | 74.0| 191  | 12.6      |
| 29         | 73          | 50 | 50      | 150 | *   | *    | -7.2      |
| 48         | 74          | 200| 50      | 300 | 71.0| *    | 8.6       |
| 13         | 75          | 100| 100     | 75  | *   | 132  | -17.5     |
| 21         | 76          | 200| 50      | 300 | 76.3| 303  | 7.5       |
| 65         | 77          | 100| 50      | 150 | *   | 150  | -10.5     |
| 39         | 78          | 100| 50      | 300 | 55.0| 229  | -18.4     |
| 10         | 79          | 100| 50      | 75  | *   | 149  | -9.8      |
| 35         | 80          | 50 | 200     | 150 | 29.6| 190  | -9.1      |
| 18         | 81          | 100| 200     | 300 | 55.5| 250  | -12.6     |

*The deleted sample during processing with data.
at pH 7.4, at $\lambda_{\text{max}}$ of 300 nm. A suitable amount of nanocomposite was added to the release media. The cumulative amount of CLD released into the media was measured at different time intervals [27]. The percentage release of CLD was calculated using the following equation:

$$\% \text{Release} = \frac{\text{Concentration of CLD time } t}{\text{Concentration of CLD in the nano Formulations}} \times 100.$$  

(2)

4. Results and Discussion

4.1. Detection Outlier Reading. An outlier is a reading which deviates from other data. For univariate data, familiar Euclidean distance and rarity are used in the data analysis, but for multivariate data, the Mahalanobis distance method was used. It enables detecting multivariate outliers that need to be eliminated from future analysis to enhance a model.

Mahalanobis distance (Dj) can be defined as the distance between a data point and overall mean. It is based on the vector of data ($x$) and the vector of mean values of independent variables ($m$) [28].

The formula used for Mahalanobis distance [29] is as follows:

$$Dj = (x - m)^T C^{-1} (x - m),$$  

(3)

where $T$ indicates the vector should be transposed and $C^{-1}$ is the inverse covariance matrix of independent variables.

From the Dj standard formula, the values of the outliers are identified for the independent variables values which are dependent on the concentration of CS, Chondro, and CLD.

In the Dj value, threshold values are assigned to the multivariate data. The list of the threshold values assigned for different variants is given in Figure 1.

As in the current work, the critical value is for three independent variables (threshold is 2.858) (Figure 1(a)); the Dj values found above 2.858 are outliers. Fortunately, there are no outlier values suspected as spam to all responses. With regard to %LE, particle size, and zeta potential data analysis, the amount of data was deleted to increase the $R^2$ value. Therefore, the Dj values become 2.870 (%LE) (Figure 1(b)), 2.891 (particle size) (Figure 1(c)), and 2.874 (zeta potential) (Figure 1(d)), without any outlier values for all experiments being recorded.
| Model         | LE model | Particle size model | Zeta potential model |
|--------------|----------|---------------------|----------------------|
|              | t value  | Coef                | F value | p value | VIF | t value | Coef | F value | p value | VIF | t value | Coef | F value | p value | VIF |
| Linear       | 19.83    | 44.23               | 72.61   | 0.00    | —    | 23.24   | 86.72 | 0.00    | —      | —    | 23.33   | 9.873 | 544.36 | 0.00    | 1.18 |
| CS           | -2.57    | -3.26               | 6.63    | 0.00    | 1.06 | CS      | 16.54   | 71.43 | 273.56 | 0.00    | 1.16 | CS     | 23.33   | 9.873 | 544.36 | 0.00    | 1.18 |
| Chondro      | -7.95    | -9.68               | 63.28   | 0.00    | 1.02 | Chondro | -4.39   | -17.42 | 19.26  | 0.00    | 1.15 | Chondro | -1.87  | -0.666 | 3.48    | 0.00    | 1.04 |
| CLD          | 17.35    | 21.82               | 300.85  | 0.00    | 1.05 | CLD     | 2.87    | 11.27  | 8.23   | 0.00    | 1.12 | CLD     | -2.71  | -1.099 | 7.33    | 0.00    | 1.06 |
| Square       | 14.46    | 0.00                | —       | —       | —    | 14.46   | 16.36  | 0.00    | —       | —    | 14.46   | 16.36  | 0.00    | —       | —    |
| Chondro*     | 3.80     | 10.22               | 14.46   | 0.00    | 1.11 | Chondro* | 3.52   | 27.91  | 12.38  | 0.00    | 1.20 | Chondro* | 17.94  | 14.371 | 321.76  | 0.00    | 1.14 |
| Chondro**    | —        | —                   | 17.34   | 0.00    | —    | 2-way interaction | 97.75 | 0.00    | 18.45  | 0.00    | — |
| CLD**        | —        | —                   | 17.34   | 0.00    | —    | 2-way interaction | 97.75 | 0.00    | 18.45  | 0.00    | — |
| CS*          | 4.13     | 5.84                | 17.07   | 0.00    | 1.00 | CS*     | -9.74  | -48.29 | 94.77  | 0.00    | 1.18 | CS*     | 5.17   | -1.467 | 26.71   | 0.00    | 1.02 |
| Chondro*     | 4.34     | 6.18                | 18.83   | 0.00    | 1.02 | Chondro* | 4.08   | 33.78  | 16.67  | 0.00    | 1.10 | Chondro* | 2.34   | 2.37   | 5.49    | 0.00    | 1.17 |
| CLD          | 4.34     | 6.18                | 18.83   | 0.00    | 1.02 | CLD     | -12.5  | -62.33 | 155.92 | 0.00    | 1.19 | CLD     | -3.03  | 2.187  | 9.16    | 0.00    | 1.05 |

Table 3: ANOVA data for %LE, particle size, and zeta potential models.
4.2. Statistical Data Analysis. The variance inflation factor (VIF) is one of the methods used to assess the multicollinearity properties, i.e., if the predictors are correlated. If no factors are correlated, the VIFs will all be < 5 [30]. Due to absence of multicollinearity in this work, the data was analyzed using the multivariate regression model.

Multiple regressions is a method used to predict the value of a variable based on the value of two or more other variables [31].

4.2.1. ANOVA Data. From ANOVA data for the %LE model in Table 3, we estimated the individual factors (CS, Chondro and CLD) and their combinations on the response (CS * CS, CS * Chondro, and Chondro * CLD). The significant effect on the %LE can be seen with a p value less than 0.05 and a high F value. From Table 3, we noted the following important characteristics: (i) the average p value for the model, individual factors, and their combinations are much less than 0.05, which means that the conditions in the model have a high significance [32]; (ii) the F values for individual factors (CLD, Chondro, and CS) were 300.85, 63.28, and 6.63, respectively, whereas the F value for combinations (Chondro * CLD, CS * Chondro, and CS * CS) was 18.83, 17.07, and 14.46, respectively.

Table 3 shows ANOVA data for the particle size model. The results of the statistical evaluation and variance analysis of the data show that all of the variables and their interactions had significant effects except CLD * CLD and CLD * Chondro. Also, the F value of the model was 86.72; this large value could occur due to noise [33, 34].

Table 3 also shows the F value and p value for zeta potential model. From the data in Table 3, the F values for individual factors (CLD, Chondro, and CS) were 7.33, 3.48, and 544.36, respectively, whereas the F value for combinations

| Term | %LE model | Particle size model | Zeta potential model |
|------|------------|---------------------|---------------------|
|       | Dn (calculated value for K-S test) | 0.143 | 0.156 | 0.107 |
|       | Dcrit (critical value for K-S test) | 0.170 | 0.183 | 0.167 |
|       | DAgostino-Pearson (A-P test) | 0.084 | 0.006 | 0.072 |

Figure 2: Pareto chart of the %LE (a), particle size (b), and zeta potential (c).
(CS * CS, Chondro * Chondro, CLD * CLD, Chondro * CLD, and CS * CLD) was 321.76, 5.49, 7.95, 26.71, and 9.16, respectively.

In statistics, the coefficient of determination (\(R^2\)) is the proportion of the variation in the dependent variable which is predictable from the independent variable(s). The \(R^2\) for the %LE model was 88.25%, and the \(R^2_{adj}\) is 87.04%. In addition, the value of \(R^2\) for particle size model was 92.81%, and the \(R^2_{adj}\) is 91.74%. Finally, the \(R^2\) for the zeta potential model was 95.84%, and the \(R^2_{adj}\) is 95.26%. There is no difference between the \(R^2\) and \(R^2_{adj}\) values, which indicate the significant conditions in the three models.

4.3. Goodness-Of-Fit Test for the Normal Distribution for Three Models

4.3.1. The Kolmogorov-Smirnov (K-S) Test for Goodness-Of-Fit. The K-S test is a nonparametric test of the equality of continuity, and it contains one and two samples. At the one-sample test, one-dimensional probability distributions are used to compare a sample with a reference probability distribution (hypothesized distribution), whereas a two-sample test is one of the most useful and general nonparametric methods for comparing two samples [35, 36].

The K-S test null hypothesis is defined as [37] follows: $H_0$: the sample data are not significantly different than a normal population.

$H_a$: the sample data are significantly different than a normal population.

The $D_n$ calculated value can be determined by the following equation:

\[
D_n = \max_{1 \leq i \leq N} \left( F(Y_i) - \frac{i - 0.5}{N}, \frac{i}{N} - F(Y_i) \right),
\]

where $F$ is the theoretical cumulative distribution.

The critical value of the K-S test can be calculated by the following equation [38]:

\[
D_{crit,0.05} = \frac{1.36}{\sqrt{n}}.
\]

4.3.2. The D'Agostino-Pearson (A-P) Test. The D’Agostino-Pearson test is a very powerful test for assessing departures from normality [39]. The null hypothesis is defined as follows:

$H_0$: the sample data are not significantly different than a normal population.
$H_0$: the sample data are significantly different than a normal population.

The $D$ is a test that can statistically calculate the value by the following equation:

$$D = \frac{T}{\sqrt{n^3SS}},$$

where "SS" is the sum of squares of the data and "n" is the sample size.

$$T = \sum \left( i - \frac{n + 1}{2} \right) X_i,$$

where "i" is the order or rank of observation.

From the results in Table 4, $D_{crit}$ (critical value) for %LE, particle size, and zeta potential are bigger than $D_n$ (calculated value); therefore, the null hypothesis is not rejected (for the normally distributed data). In addition, the $D_{Agostino-Pearson}$ value for %LE and zeta potential is bigger than 0.05 ($D > p$), indicating that the model set is not significantly different than normal.

4.4. Pareto Chart of the Standardized Effects of the Three Models. A Pareto chart is normally used as a graphical representation for evaluating the effects of the main variables and their interactions on the response. Figure 2 shows the bars of the main $f$ variables and their interactions, arranged in a descending order according to the $t$ values, the vertical line is the statistical threshold for a level of significance.

From the results in Figure 2(a), it can be seen that three main factors have a statistically significant effect on the %LE. In a descending order, according to their effect estimates, the three factors are CLD, Chondro, and CS, and the interactions Chondro $\times$ CLD (BC), CS $\times$ Chondro (AB), and CS $\times$ CS (AA).

In addition, in the Pareto chart in Figure 2(b) which is related to particle size model, bars that cross the reference line (2.01) are statistically significant with the following order: A$>$AC$>$AB$>$B$>$BB$>$AA$>$C.

Figure 2(c) plots the effects in the decreasing order of their absolute values with reference line at 2 values calculated by Lenth’s method [40]. In these results, the two main effects (CS and CLD) are statistically significant ($\alpha = 0.05$). In addition, the two square effects (CS $\times$ CS (AA), Chondro $\times$ Chondro (BB), and CLD $\times$ CLD (CC)) are statistically significant ($\alpha = 0.05$). The two-way interaction (Chondro $\times$ CLD (BC) and CS $\times$ Chondro (AB)) can also be seen, which also shows significant results.

4.5. Residual Plot. Figure 3 shows the correlation between residuals with predicted responses of the three models. The

Figure 4: Histogram of the standardized residuals for %LE (a), particle size (b), and zeta potential (c).

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residuals of three models are scattered randomly about zero. These results indicate that the errors have a constant variance and the experiment does not contain any possibility of systematic errors.

4.6. Histogram Plot. The histogram plot of residuals are used to determine whether the data are skewed or outliers exist in the data. Figures 4(a)–4(c) show the %LE, particle size, and zeta potential histogram residual plots, respectively. Results show symmetric bell-shaped curves, indicating that the data are complete residual normality assumption.

4.7. Normal Probability Plot of the Standard Effects. Figures 5(a)–5(c) shows the normal probability plot of the effects for %LE, particle size, and zeta potential, respectively.

From Figure 5(a), it could be seen that there are no factors or combinations on a line which lies close to zero. Three of the individual factors show deviation of varying degrees—A and B (negative direction) and C (positive direction). In addition, three of the combinations at level two—AA, AB, and BC—showed a significant result toward %LE response with positive direction. From Figure 5(b), it can be seen that three of the factors show negative direction (B, AB, and AC) and four of the factors show positive direction (A, C, AA, and BB). In addition, Figure 5(c) shows significant positive direction for A, AA, BB, and AB factors, whereas the factors C, CC, and AC show significant negative direction.

4.8. Contour Plot and Surface Plot of Responses against Different Factors. Figure 6(a) shows the contour plot of %LE. The %LE with a value higher than 60% corresponds with high values of CLD and Chondro, but varies from 50 mg to 200 mg, and the surface is maximum (Figure 6(b)). Figure 6(c) shows the dependence of %LE on CS and CLD. When CS concentrations are at their minimum and maximum levels and at same time the CLD is at maximum level, the %LE was found to be maximum surface (Figure 6(d)). However, the contour of CS against Chondro concentrations (Figure 6(e)) showed the highest %LE in the lower left of the plot, which corresponds with low values of CS and Chondro, and the surface is maximum (Figure 6(f)).
Figure 6: Contour plots and surface plots of %LE.
Figure 7: The contour plots and surface plots of size against different factors.
Figure 8: Continued.
Figure 7(a) shows the contour plot of size against the Chondro and CLD. The smallest values of particle size are at the bottom of the plot, which corresponds with 50 mg of CLD, and Chondro varies from 115 mg to 175 mg; the surface is minimum (Figure 7(b)).

In Figure 7(c), the contour plot shows that the minimum particle size occurs at low concentrations of CS and CLD; the surface is minimum (Figure 7(d)). In addition, Figure 7(e) shows the contour plot of size against the Chondro and CS. The smallest values of particle size are at the bottom left of the plot, which corresponds with 50 mg of CS, and Chondro and varies from 50 mg to 175 mg; the surface is minimum (Figure 7(f)).

Figures 8(a) and 8(b) shows the contour plot of zeta potential against the Chondro and CLD. The -15 mV zeta potential can be collected at Chondro concentration below 60 mg and CLD ranging between 50 and 160 mg. The -9 mV zeta potential can be collected at Chondro concentration below 60 mg and CLD ranging between 50 and 160 mg. The -15 mV zeta potential can be collected at Chondro concentration below 60 mg and CLD ranging between 50 and 160 mg.

Figure 8(c) shows that the zeta potential was dependent on CS and Chondro. When CS was more than 190 mg and at the same time the Chondro was between 50 and 200 mg, the zeta potential was found to be +10 mV (Figure 8(d)). In addition, when CS variables were in the range 75-125 mg and the Chondro was between 50 and 200 mg, the zeta potential was found to be -10 mV (Figure 8(d)). The contour of CS vs. CLD concentrations is shown in Figure 8(e). When CS variables were between 50 and 190 mg and at same time the CLD was between 50 and 300 mg, the zeta potential was found to be -15 mV (Figures 8(e) and 8(f)). Finally, when the CS concentrations were more than 160 mg, they produced a positive value of zeta potential.

Table 5: Comparison of the observed and predicted values of the response variables of optimized formulation.

| Concentrations | Experimental response | Predicted values | Observed values | Bias (%) |
|----------------|-----------------------|-----------------|----------------|---------|
| CS = 200 mg    | LE (%)                | 64.1            | 61.2           | -4.5    |
| Chondro = 138 mg | Particle size (nm) | 271.0           | 253.4          | -6.5    |
| CLD = 254 mg   | Zeta potential (mV)  | 11.7            | 11.3           | -3.4    |
| CS = 128 mg    | LE (%)                | 56.0            | 52.4           | -6.4    |
| Chondro = 138 mg | Particle size (nm) | 220.0           | 220.7          | 0.3     |
| CLD = 254 mg   | Zeta potential (mV)  | -11.3           | -10.9          | -3.5    |
| CS = 160 mg    | LE (%)                | 35.0            | 39.0           | 11.4    |
| Chondro = 112 mg | Particle size (nm) | 263.4           | 259.1          | -1.6    |
| CLD = 149 mg   | Zeta potential (mV)  | -13.9           | -14.5          | 5.4     |

Bias was calculated as \( \frac{\text{observed value} - \text{predicted value}}{\text{predicted value}} \times 100\% \).
4.9. Prediction, Optimization, and Validation of Three Models. Taking into account the main factors in our work (A, B, and C) and their combinations (A * A, B * B, C * C, A * B, A * C, and B * C), a general equation can be obtained with regard to the real values by a response surface method. For predicting %LE, particle size, and zeta potential, the following equations were derived:

\[
\%\text{LE} = 91.21 - 0.627\, \text{CS} - 0.3962\, \text{Chondro} \\
+ 0.1023\, \text{CLD} + 0.001816\, \text{CS} \times \text{CS} \\
+ 0.001038\, \text{CS} \times \text{Chondro} \\
+ 0.000733\, \text{Chondro} \times \text{CLD}
\]

Particle size = \[-56.3 + 2.170\, \text{CS} - 0.661\, \text{Chondro}
+ 1.0236\, \text{CLD} + 0.00496\, \text{CS} \times \text{CS}
+ 0.00601\, \text{Chondro} \times \text{Chondro}
- 0.008586\, \text{CS} \times \text{Chondro} \times \text{CLD}\]

Zeta potential = \[16.44 - 0.4745\, \text{CS} - 0.1626\, \text{Chondro}
+ 0.0443\, \text{CLD} + 0.002555\, \text{CS} \times \text{CS}
+ 0.000421\, \text{Chondro} \times \text{Chondro}
- 0.000173\, \text{CLD} \times \text{CLD} - 0.000174\, \text{CS} \times \text{CLD}
+ 0.000259\, \text{Chondro} \times \text{CLD}\]

(8)

According to our criteria for higher %LE (88.86%), the concentrations under canonical analysis were selected at 50 mg, 50 mg, and 300 mg of CS, Chondro, and CLD, respectively. In addition, to prepare nanoparticles with a size of 64 nm, 50 mg CS, 91 mg Chondro, and 75 mg CLD were used. Furthermore, the zeta potential with -17 mV value can be obtained by using 103.3 mg CS, 101.5 mg Chondro, and 300 mg CLD.

A bias formula under optimized factors was applied to compare with the predicted values. As shown in Table 5, the bias was around -4.5%, -6.5%, and 3.4% for the first formula (CS = 200 mg, Chondro = 138 mg, and CLD = 254 mg). In addition, the bias was around -6.4%, 0.3%, and -3.5% for the second formula (CS = 128 mg, Chondro = 138 mg, and CLD = 254 mg) and around 11.4%, -1.6%, and 15.4% for the third formula (CS = 160 mg, Chondro = 112 mg, and CLD = 149 mg), respectively. These results indicate the validity of the generated models with no statistically significant difference and good correlation between predicted and experimental values.

The zeta potential (mV) of nanocarriers represents the surface charge. It depends on the dispersing medium as well as the composition of the particles. The nanoparticles that possess zeta potentials between +30 mV and -30 mV are considered a stable system that prevents nanoparticle aggregation. From Table 5, the observed zeta potential values of nanocomposites are 11.3, -10.9, and -14.5 mV. These values indicate that the nanocomposites were stable.
5. Characterization of Formulations

5.1. X-Ray Diffraction (XRD). Figures 9 (a), (b), (c), (d), and (e) shows the XRD patterns of CS, Chondro, CLD, CS-Chondro, and CS-Chondro-CLD nanocomposites, respectively. From Figure 9(a), CS showed strong diffraction peaks at $2\theta = 10.7$ and $20.2^\circ$, indicating a high degree of crystallinity [41, 42]. Diffraction broad bands similar to Chondro were seen for CS-Chondro and CS-Chondro-CLD due to amorphous behavior of the polymer. The diffraction peaks of Chondro were observed at 24.7 and $40.1^\circ$ (Figure 9(b)), whereas the diffraction peaks with maximum intensity were observed at 22.5 for CS-Chondro and CS-Chondro-CLD as seen in Figures 9(d) and (e). These diffraction peaks in Chondro, CS-Chondro, and CS-Chondro-CLD were very broad which is indicative of their highly amorphous nature with low crystallinity [43].

5.2. Infrared Spectroscopy (FTIR). The IR spectra of CS (a), Chondro (b), CLD (c), CS-Chondro (d), and CS-Chondro-CLD nanocomposites (e), respectively, are shown in Figure 10. The IR spectrum of CS showed vibration at $3300$ cm$^{-1}$ due to overlapped $–\text{OH}$ and $\text{N–H}$ stretching; the $\text{C–H}$ stretching of $\text{CH}_3$ or $\text{CH}_2$ groups of CS is at $2890$ cm$^{-1}$. The peaks at 1671 and $1597$ cm$^{-1}$ correspond to the amide bonds and $\text{NH}_3$ group, respectively. The peak at $1385$ cm$^{-1}$ due to stretching of $\text{C=O}$ bond and the stretching of $\text{C–O}$ bond are shown at 1.079 and 1.030 cm$^{-1}$ [44].

The IR spectrum of Chondro in Figure 10(b) showed the $\text{–OH}$ and $\text{N–H}$ stretching at 3359 cm$^{-1}$. The peak at 2904 cm$^{-1}$ was due to $\text{C–H}$ stretching of $\text{CH}_3$ or $\text{CH}_2$ groups. The peak at 1614 cm$^{-1}$ was due to the amide bands, whereas the peaks at 1413 and 1380 cm$^{-1}$ are due to the $\text{C–O}$ stretch vibration, indicating the presence of a free carboxyl group. The peak at 1220 cm$^{-1}$ was due to the stretching of $\text{S=O}$ bond of $\text{SO}_4^{2–}$ [44].

The IR spectrum of CS-Chondro is shown in Figure 10(c). The peak at 1671 cm$^{-1}$ for amide in CS was shifted to 1600 cm$^{-1}$, whereas the peak of $\text{NH}_3$ group disappeared, and this leads to cross-linking of $\text{NH}_3$ of CS with Chondro [44]. The peak at 1220 cm$^{-1}$ of $\text{S=O}$ turned weaker and shifted to 1230 cm$^{-1}$, which indicates cross-linking of $\text{SO}_4^{2–}$ group of CS with Chondro.

The FT-IR spectrum of CLD in Figure 10(c) showed characteristics of peaks at 1683 cm$^{-1}$ (C=O stretching), 1080 cm$^{-1}$ (C-O stretching), 1553 cm$^{-1}$ (C=C stretching), and 2935 cm$^{-1}$ (C-H stretching) [45]. Comparing the FT-IR spectrum of CS-Chondro-CLD in Figure 10(e) with the FT-IR spectrum of the CS-Chondro in Figure 10(d), two specific peaks of CLD can be seen to have appeared at 1683 cm$^{-1}$ and 1553 cm$^{-1}$, indicating the incorporation of CLD into the CS-Chondro-CLD.

5.3. Scanning Electron Microscopy. SEM images are widely used to examine the surface morphology of polysaccharides [46]. The morphological analysis of Chondro-CS and Chondro-CS-CLD in this study was carried out using SEM.
Figure 11 shows the SEM images of Chondro-CS and Chondro-CS-CLD with magnifications of 10000x and 25000x. It could be seen that the morphological properties of both Chondro-CS and Chondro-CS-CLD were similar (Figure 11(a)–11(c)). The Chondro-CS and Chondro-CS-CLD had a smooth homogeneous surface with lots of particle layers stacked together.

5.4. In Vitro Release Study. The release profiles of CLD from the optimized sample at pH 7.4 are illustrated in Figure 12.

It was noticed that the release rate of CLD at pH 7.4 was significantly very slow, where it started after 1 hour. This result can be explained based on the swelling term [47, 48]. The last results suggest that the CLD release profile was prominently prolonged by the encapsulation.

6. Conclusion

The aim of this work was to study the factors affecting the %LE, zeta potential, and particle size using response surface methodology model. The results indicated that for %LE, the square (Chondro * Chondro and CLD * CLD) and two-way interaction (CS * CLD) was excluded from the final equation, whereas at size response, two-way interaction (Chondro * CLD) only was excluded from the final equation. In addition, at zeta potential response, two-way interaction (CS * Chondro) was excluded from the final equation. The developed multiple linear regression models provided the best fits to the data set. The XRD spectra of nanocomposites showed amorphous forms. The FT-IR data shows the functional group of CLD in nanocomposites, indicating incorporation of CLD into the nanocomposites. In addition, the prepared formulation shows sustained release, which may increase the chance for the patient to respond to the medication, since formulations are generally characterized by one daily given dose.

Data Availability

The original data can collected from Prof Samer AlAli by contact in his email sameralali72@yahoo.com or samer.alali@iu.edu.jo.

Conflicts of Interest

The authors report no conflicts of interest in this work.

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