

FORMULATION AND CHARACTERIZATION OF PIREFENIDONE NANOPARTICLE DISPERSION

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ABSTRACT

Background: Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive interstitial lung disease characterized by excessive extracellular matrix deposition, irreversible pulmonary fibrosis, and progressive decline in lung function. Although pirfenidone is an approved antifibrotic agent for IPF, its clinical efficacy is limited by short half-life, frequent dosing, gastrointestinal adverse effects, photosensitivity, and variable bioavailability. Therefore, the development of a nanoparticle-based drug delivery system may enhance pulmonary targeting and improve therapeutic outcomes.

Methods: Pirfenidone-loaded chitosan nanoparticles were prepared using the solvent evaporation technique employing chitosan as the polymer and PEG 400 as the stabilizer. Formulation variables were optimized using a Box–Behnken Design (Design-Expert software Version 13). The optimized formulation was evaluated for particle size, polydispersity index (PDI), zeta potential, drug entrapment efficiency, Fourier-transform infrared spectroscopy (FTIR) and in vitro drug release. Stability studies were performed according to ICH guidelines.

Results: The optimized nanoparticle formulation exhibited a mean particle size of **91.7 nm**, a **PDI of 0.279**, and a **zeta potential of 26.5mV**, indicating good colloidal stability. The formulation demonstrated an entrapment efficiency of **90.12 %** and sustained drug release of **93.45 % over 12 hours**. FTIR analyses confirmed the compatibility of pirfenidone with formulation excipients. Stability studies demonstrated that the optimized formulation remained stable under accelerated and long-term storage conditions without significant changes in physicochemical properties.

Conclusion: Pirfenidone-loaded chitosan nanoparticles successfully provided controlled drug release and good stability, indicating their potential as an effective pulmonary drug delivery system for the treatment of idiopathic pulmonary fibrosis. The developed nanoparticle formulation may improve therapeutic efficacy while reducing systemic adverse effects associated with conventional oral pirfenidone therapy.

KEYWORDS: Idiopathic pulmonary fibrosis; Pirfenidone; Chitosan; PEG 400; nanoparticles; Pulmonary drug delivery; Solvent evaporation; Sustained drug release; Box–Behnken design.

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF), previously referred to as cryptogenic fibrosing alveolitis (CFA), is a rare and progressive interstitial lung disease marked by excessive fibrotic remodeling of the lung parenchyma, leading to irreversible impairment of respiratory^{1,2}. IPF is associated with an increasing burden of respiratory symptoms, particularly chronic cough and exertional dyspnea which profoundly impair quality of life and are linked to poor survival outcomes, with untreated patients exhibiting a median survival of nearly three years^{3,4}. Fibrosis is a pathological process that can involve almost all organs of the human body, characterized by abnormal and excessive deposition of extracellular matrix and scar tissue, ultimately leading to organ dysfunction and death in advanced fibrotic conditions such as pulmonary, cardiac, nephrotic, and hepatic fibrosis^{5,6}. Current treatment strategies include antifibrotic drugs, supportive care, management of comorbidities, pulmonary rehabilitation, and lung transplantation⁷.

Nanoparticle drug delivery systems are characterized by drug-loaded particles ranging from 1 to 1000 nm in diameter. Defined as solid submicron carriers, these systems are structurally differentiated into nanocapsules and nanospheres. In nanocapsules configurations, the drug is sequestered within a central liquid cavity surrounded by a protective polymer shell. In contrast, nanospheres are defined by a matrix-type structure; here, the active pharmaceutical ingredient (API) is either surface-adsorbed or distributed throughout the particle. Depending on the formulation, the drug is either solubilized into an amorphous state within the matrix or incorporated as crystalline dispersions⁸. The emulsion solvent evaporation technique is often favored over alternative methods, such as spray drying, sonication, or homogenization, due to its requirement for mild processing conditions. By utilizing ambient temperatures and constant stirring, the stability of the emulsion is maintained without compromising the pharmacological activity of the therapeutic agent⁹. A primary advantage of nanoparticulate systems is their capacity for site-specific drug delivery, which minimizes off-target toxicity by distinguishing between malignant and healthy tissues¹⁰.

Pirfenidone is an orally active antifibrotic and anti-inflammatory agent that inhibits transforming growth factor- β (TGF- β)-stimulated collagen synthesis and fibroblast proliferation. Clinical trials have demonstrated that pirfenidone significantly reduces the rate of decline in forced vital capacity (FVC) and may reduce mortality risk in IPF patients¹¹. Common adverse effects include gastrointestinal disturbances, photosensitivity, and liver enzyme elevation¹².

Therefore, pirfenidone-loaded chitosan nanoparticles represent a promising approach for targeted pulmonary delivery in idiopathic pulmonary fibrosis. The aim of the study is to formulate and characterize the nanoparticles using the solvent evaporation method and evaluate their potential for sustained pulmonary drug delivery.

Materials and Methods

Materials

Pirfenidone was kindly provided as a gift sample by Allastir private Ltd. Chitosan was gifted from Molychem India, LLP. Ethanol was obtained from Changshu song sheng fine chemicals, while glacial acetic acid was purchased from Molychem, Mumbai. Polyethylene glycol 400 (PEG 400) was supplied by R.H Marketing chemicals. Electronic balance

(Reminotars) for accurate weighing of materials. Fourier Transform Infrared (FTIR) Spectroscopy was obtained from Bomen FTIR MB II Instrument; UV Spectrophotometer was using a UV-2001 Tech comp Spectrophotometer. Particle size and zeta potential were measured using a Malvern Zeta sizer. Magnetic stirrer was purchased from Reminotars and Mechanical stirrer was purchased from Tarsons Products Pvt. Ltd.

Aqueous phase preparation method

Chitosan is completely soluble in acidic media; therefore, 1% glacial acetic acid was incorporated into the nanoparticle formulation to ensure its dissolution. A 1%v/v concentration was selected because acetic acid effectively dissolves both chitosan and PFD. The quantities of acetic acid and PEG-400 solution were fixed at 5mL and 15 mL, respectively^{13,14}. To prepare 20 mL of the aqueous phase, chitosan was first dissolved in 5 mL of 1% acetic acid, after which the resulting solution was gradually added to 15 mL of 1%PEG-400 solution under continuous magnetic stirring.

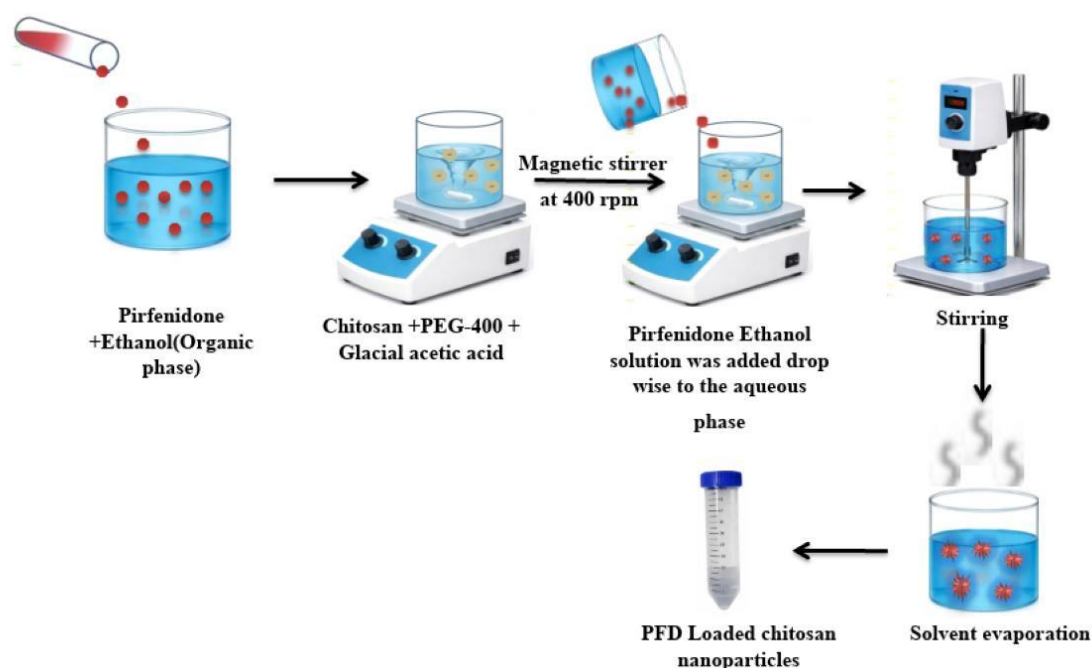


Figure 1 Preparation of PFD Loaded chitosan nanoparticles

PFD Loaded chitosan nanoparticles Formulation

PFD Loaded chitosan nanoparticles were prepared by quasi solvent evaporation method¹⁵. In the organic phase, 100 mg of PFD was dissolved in 5 mL of ethanol. The aqueous phase consisted of 20 mL of 1%w/v PEG-400 solution containing 1% acetic acid, in which chitosan was dissolved at varying concentration of 50, 175 and 300mg. The ethanolic PFD solution was added dropwise to the chitosan solution under continuous stirring at 400 rpm in a 100mL of flask. The resultant mixture was subjected to probe sonication for 1 min at 220W with

a 2s cycle. Following sonication, the solution was stirred in an uncovered beaker for 3 to 4 hrs to facilitate ethanol evaporation. The nanoparticles suspension obtained was subsequently washed twice to remove residual solvent and centrifuged at 15,000 rpm for 10 min using a refrigerated centrifuge. If necessary, the collected nanoparticles were lyophilized in the presence of mannitol as a cryoprotectant at -79°C for 24 hrs. After freeze drying, the solidified nanoparticles were collected and stored in vials for subsequent use¹⁶.

Selection of chitosan concentration in the organic phase

Using the polymer chitosan, PFD nanoparticles were formulated, with the volume ratio of the organic and water phases being 1:4 and the concentration of PEG 400 in the aqueous phase being 1%¹⁷. The range of the polymer concentration is between 50% (w/v) and 400% (w/v). Particle size was being used to evaluate this study. The particle size was developed to directly correlate with the CS concentration. As CS concentration increased and aqueous phase viscosity increases during emulsification, a compact polymer matrix with greater particle size was produced.

Selection of PEG 400 concentration in the aqueous phase

The polymer chitosan was used to form the PFD nanoparticles; the volume ratio of the organic and aqueous phases was 1:4; and the polymer quantity (100 mg) was dissolved in 1% acetic acid. The PFD particle size and entrapment effectiveness in the chitosan nanoparticles were affected by the PEG 400 concentrations. PEG 400 is employed as both a stabilizer and a surfactant. The amount of PEG 400 adsorbing at the polymer (chitosan)-organic solvent ethanol-water interface had an impact on the formulation. From 0.5% (w/v) to 2% (w/v), PEG 400 content in the aqueous phase is adjusted¹⁸.

Optimized of PFD Loaded nanoparticles using Box Behnken Design

Box Behnken experimental Design was employed to optimize the formulation variables and to investigate the linear, interaction, and quadratic effects of the independent factors on the selected responses. The design was generated by using design-expert version 13 and comprised 17 experimental runs for 3 independent variables at 3 levels. The Box Behnken design includes experimental points positioned at the midpoint of edge of the multidimensional cube, together with replicate centre points. The independent and dependent Variables used in the study are presented in table 1. The relationship between the response and the independent variables was described by the second order polynomial equation generated by this experimental design is as follows.

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2$$

Where Y represents the dependent Response, β_0 is the intercept, β_1 to β_{33} are the regression coefficients, and X_1 , X_2 and X_3 denote the independent variables selected from preliminary experiments. The complete experimental design matrix and corresponding Responses are summarized in table 1.

Table 1 Optimization of PFD Loaded nanoparticles using Box Behnken Design

INDEPENDENT VARIABLES	UNITS	LEVELS		
		LOW (-1)	MEDIUM(0)	HIGH(+)
A=Chitosan	mg	50	175	300
B=PEG 400	mL	5	15	25
C=Flow Rate	rpm	200	400	600
DEPENDENT VARIABLES	UNITS	CONSTRAINTS		
R1=Particle size	nm	Minimize		
R2=Zeta Potential	mV	Maximize		
R3=Entrapment Efficiency	%	Maximize		

Optimization validation and data analysis

The PFD loaded nanoparticles was optimized using the “Box -Behnken design” using the “design expert software-version 13” comprising the factor and three levels. The independent variables selected were chitosan concentration (A, -1=50mg and +1=300mg, PEG 400 concentration (B, -1=5mL and +1=25mg) and flow rate (C, -1=200rpm and +1=600rpm). The dependent variables were particle size (R1), % zeta potential(R2) and entrapment efficiency (R3). The experimental design is summarized in table 1

Statistical validation of the polynomial equation and ANOVA was calculated using design expert software. The resultant experimental values of the responses were quantitatively compared with the predicted values to calculate the prediction error¹⁹.

Characterization of the PFD NPs

Standard curve of PFD

The standard calibration curve of PFD were initially constructed in various solvents, such as distilled water, methanol, dichloromethane, ethyl acetate, and phosphate buffer saline (pH 7.4), within a concentration range of 10-50 µg/mL volumetric flask, dissolving it completely, and Making up volume with the mobile phase to obtain a final concentration of 1.0mg/mL . Subsequently dilutions of this stock solution were performed using the mobile phase to obtain working calibration standards.

The prepared standards were analyzed using a UV-spectrophotometer (UV-2001, Tech comp) at a wavelength of 311nm, corresponding to the absorption maximum (λ max) of PFD. All measurements were carried out in triplicate, and the average values were constructed by plotting absorbance versus concentration, as well as peak area versus concentration^{20,21}. The identified λ max was further utilized for the quantitative estimation of PFD in invitro dissolution studies and for determining drug entrapment within the chitosan-based polymeric matrix.

Drug-excipients compatibility studies

Fourier Transform Infrared (FTIR) spectroscopy studies

FTIR spectroscopy (Bomen FTIR MB II) was employed to evaluate the chemical stability of PFD and its compatibility with the selected excipients. Samples of pure PFD, a physical mixture of PFD and chitosan, and the optimized chitosan nanoparticle (CS NP) formulation were individually mixed with potassium bromide, compressed into pellets under a pressure of 1 ton and analyzed over a spectral range of 4000-400 cm^{-1} .

Particle size, zeta potential

The particle size distribution and polydispersity index (PDI) of PFD-loaded chitosan nanoparticles were determined using a zeta sizer (Malvern Instruments) nanoparticles size analyzer. For the measurement of mean particle diameter, freshly prepared nanoparticle dispersion (1%w/v) were approximately diluted with ethanol and subjected to sonication for 2 mins to ensure uniform dispersion. Subsequently, both particle size and zeta potential were analyzed using the same instrument. In this technique based on the principle that charged particles undergo oscillatory motion when exposed to a high- frequency alternating electric field. The modulated power spectrum (MPS) approach evaluates the Brownian motion-induced power spectrum of these oscillating particles to determine their physicochemical properties. Samples with appropriate obscuration levels were prepared, and all measurements were conducted in triplicate, with mean values recorded.

% Entrapment efficiency

The entrapment efficiency of PFD within the nanoparticles was evaluated by quantifying the amount of unencapsulated drug present in the supernatant following centrifugation of the nanosuspension. The nanoparticle formulation was accurately weighed, washed with distilled water, and centrifuged at 15,000 rpm for 10 mins at 10°C. The resulting supernatant was then analyzed for free (untrapped) drug content using a UV-Visible spectrophotometer at 311nm. The percentage entrapment efficiency was subsequently calculated based on the difference between the total drug content and the amount of free drug detected in the supernatant using the standard equation.

$$\%EE = \frac{\text{total amount of PFD added} - \text{Free PFD in supernatant}}{\text{total amount of PFD added}} \times 100$$

Yield of nanoparticles

The percentage yield of nanoparticles was determined by relating the total weight of the obtained nanoparticles to the polymer and drug used in the formulation. All measurements were carried out in triplicate, and the mean values were reported²².

$$\% \text{ Yield} = \frac{\text{Total amount of PFD NPs}}{\text{Amount of drug+polymer}} \times 100$$

In-vitro drug release study

In this technique, the dialysis membrane enables sampling of the released drug solution without interference from dispersed nanoparticles by acting as a barrier between the donor and receptor compartments. The volume of the dialysis bags and dissolution medium (phosphate buffer pH 7.4) is typically limited to 3-5 mL, and therefore minimal sampling is performed to help maintain sink conditions are difficult to achieve because the release drug cannot be directly withdrawn from the donor compartment, and its transfer to the receptor compartment is neither rapid nor strictly unidirectional. Moreover, the receptor compartment generally contains a larger volume of dissolution medium (100-200 mL), leading to significant dilution of the released drug, often reducing its concentration below the limit of quantification. Consequently, accurate estimation of burst release is not feasible using this technique²³.

Kinetic study

The invitro release profile of the nanoparticles was evaluated using various kinetic models, including zero-order (plotting Cumulative drug release against time), first-order (plotting the logarithm of the remaining drug percentage versus time), and the Higuchi model (plotting Cumulative releases versus the square root of time) to elucidated the release mechanism. Linear regression analysis of the corresponding plots was performed to determine the correlation coefficient (R^2) and the release rate constant (k). The best fitting model was identified based on the highest R^2 value along with the lowest Sum of Squared residuals (SSR), indicating the most appropriate description of the drug release kinetics.

Mechanism of Drug release

To analyze the mechanism of the drug release rate kinetics of the dosage form, the data obtained were graphed as:

Cumulative percentage drug release Vs Time (In-vitro drug release plots)

Cumulative percentage drug released Vs Square root of time (Higuchi's plots)

Log Cumulative percentage drug remaining Vs Time (First order plots)

Log percentage drug released Vs Log time (Peppas plots)

Zero-order release rate kinetics

To study the zero-order release rate kinetics the release rate data were fitted to the following equation

$$F=Kt$$

Where “F” is the fraction of drug release.

“K” is the release rate constant and

“t” is the release time

When the data is plotted as Cumulative percent drug release Vs Time, if the plot is linear equation the data obeys zero-order release kinetics, with a slope equal to K.

First order release rate kinetics

First order release was predicted by the following equation:

$$\text{Log } C = \text{log } C_0 - Kt/2.303$$

Where, “C” is the amount of drug remained at time “t”

“C₀” is the initial amount of drug

“K” is the first-order rate constant (‘hr⁻¹’) and

“t” is time in hours

When the data plotted as log Cumulative percentage remaining Vs time to yield straight line, indicating that the release followed the first order. The constant “K” was obtained by Multiplying 2.303 with slope value.

Higuchi's release model

To study the Higuchi release kinetics, the release rate data was fitted to the following equation

$$F = Kt^{1/2}$$

Where, “F” is the amount of drug release,

“K” is the release rate constant and

“t” is the release time.

When the data is plotted as Cumulative drug released Vs Square root of time, yields a Straight line, indicating that the drug was released by diffusion mechanism. The slope value is equal to K.

Korsmeyer and Peppas release model

The release rate data were fitted into the following equation,

$$\frac{M_t}{M_\infty} = K \cdot t^n$$

Where $\frac{M_t}{M_\infty}$ is the fraction of drug release

“K” is the release constant

“t” is the release time and

“n” is the diffusional exponent for the drug release that is dependent on the shape of the matrix dosage form.

When the data is plotted as Log of drug released Vs Log time, yields a Straight line with a slope equal to “n” and the “K” can be obtained from Y-intercept.

For non- Fickian release the “n” values falls between 0.5 and 1.0 while for Fickian (case II) diffusion, $n=0.5$ and zero order release, $n=1.0$ ²⁴

Accelerated stability study

The accelerated stability study was performed in accordance with International Council for Harmonisation (ICH) guidelines. The optimized PFD NPs formulation was subjected to storage in a stability chamber for a period of 45 days under the 25 $\pm$ 5% RH. At predetermined time intervals 15, 30 and 45 days. The samples were assessed for particle size, zeta potential, polydispersity index, entrapment efficiency and invitro drug release at 8 and 12 hrs, along with any visible changes in physical appearance ²⁵.

RESULTS

Solubility and standard curve of pirfenidone

Pirfenidone shows limited aqueous solubility, dissolving at about 19 mg/mL at 25 °C and 21 mg/mL at 37 °C. In contrast, it dissolves readily in organic solvents such as acetone, ethyl acetate, dichloromethane, chloroform, and is highly soluble in ethanol. The UV spectrum of pirfenidone in a maximum absorbance (λ_{max}) at 311 nm, which was selected for quantitative estimation . Beer–Lambert’s law was obeyed over the concentration range of 10–50 μ g/mL. The method showed Acceptable precision, and the calibration curve demonstrated excellent linearity ($R^2 \approx 0.997$ –0.999). A similar λ_{max} of 311 nm was also observed in pH 7.4 phosphate buffer(showed in figure 2).

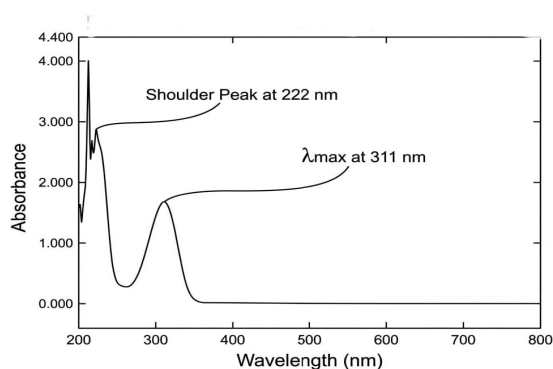


Figure 2 : λ_{max} Determination and Overlay UV spectra of Pure PFD in Phosphate buffer saline pH 7.4

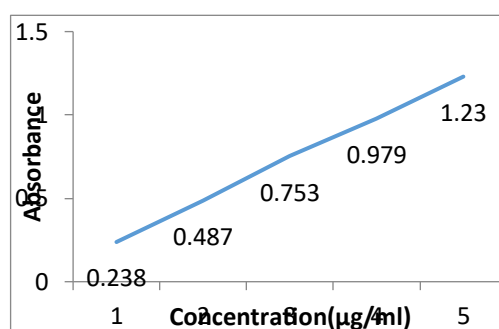


Figure 3 : Linearity plot of pirfenidone in phosphate buffer pH 7.4 at 311 nm

Table 2 :Standard curve of pure PFD at 311nm

S.NO	CONCENTRATION($\mu\text{g/mL}$)	ABSORBANCE(nm)
1	1mL	0.238
2	2mL	0.487
3	3mL	0.753
4	4mL	0.979
5	5mL	1.230

Compatibility study

FTIR spectroscopy

In the FTIR spectrum of CS , a broad peak at around 3400 cm^{-1} was attributed to $-\text{OH}$ stretching vibrations. The stretching bands of $-\text{CH}$, $-\text{CH}_2$, and $-\text{CH}_3$ appeared in the region of $2886\text{--}2974\text{ cm}^{-1}$. The characteristic amide I and amide II bands were observed at 1676 cm^{-1} and 1600 cm^{-1} , respectively. N–H stretching vibrations were detected within the range of $924\text{--}1169\text{ cm}^{-1}$.

In the FTIR spectrum of pirfenidone (Fig. 4A), the $\text{O}=\text{C}$ stretching vibration of the α,β -unsaturated ketone was observed at 1612 cm^{-1} , while the aromatic $\text{C}=\text{C}$ stretching band appeared at 1530 cm^{-1} . The aliphatic $\text{C}=\text{H}$ stretching peak was noted at 3054 cm^{-1} . Additionally, the aromatic $\text{C}=\text{N}$ stretching vibration (tertiary amine) was identified at 1274 cm^{-1} , and the phenyl $\text{C}=\text{C}$ stretching vibration was observed at 825 cm^{-1} .

FTIR spectroscopy was used to evaluate the surface chemical composition of CS nanoparticles and CS chains (Figs. 5 and 4A). The optimized pirfenidone nanoparticle formulation was analyzed by FTIR. A shift of the NH_2 bending peak to 1576 cm^{-1} was observed in the PFD nanoparticles, while a prominent band at 1412 cm^{-1} corresponded to $\text{C}=\text{H}$ bending of the alkyl group (Fig. 5). These spectral changes indicate ionic interactions between the components, confirming the conversion of chitosan into nanoparticulate form and the formation of cross-links with the incorporated molecules.

In the FTIR spectrum of PFD-loaded CS nanoparticles, the stretching vibrations of $-\text{CH}$, $-\text{CH}_2$, and $-\text{CH}_3$ were observed in the region of $2885\text{--}2910\text{ cm}^{-1}$, while the $-\text{OH}$ stretching band appeared at 3504 cm^{-1} . The aliphatic $\text{C}=\text{H}$ stretching peak was noted at 2970 cm^{-1} . The aromatic $\text{C}=\text{N}$ stretching vibration (tertiary amine) was detected at 1280 cm^{-1} , and the phenyl $\text{C}=\text{C}$ stretching band was observed at 842 cm^{-1} . The characteristic amide I and II bands were present at 1662 cm^{-1} and 1609 cm^{-1} , respectively, with N–H stretching vibrations appearing between 962 and 1147 cm^{-1} . The aromatic $\text{C}=\text{C}$ stretching peak of PFD-loaded nanoparticles was found at 1550 cm^{-1} . The retention of the characteristic peaks of both CS and PFD in the loaded nanoparticles indicates good compatibility between the drug and polymer, with no significant peak disappearance. (Table 3)

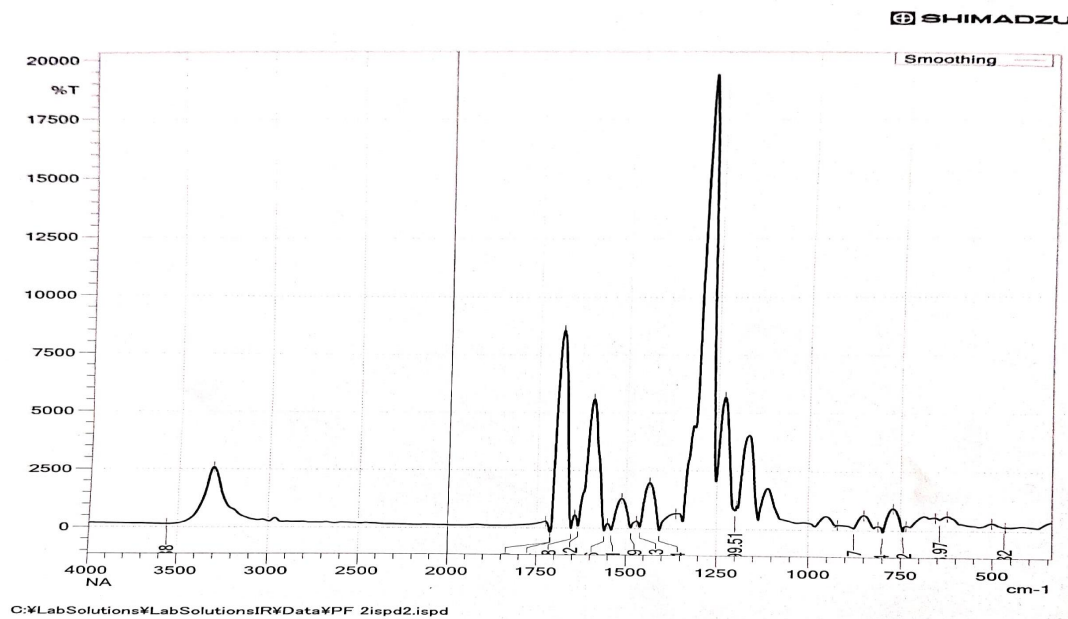


Figure 4A : FTIR Spectra of pirfenidone

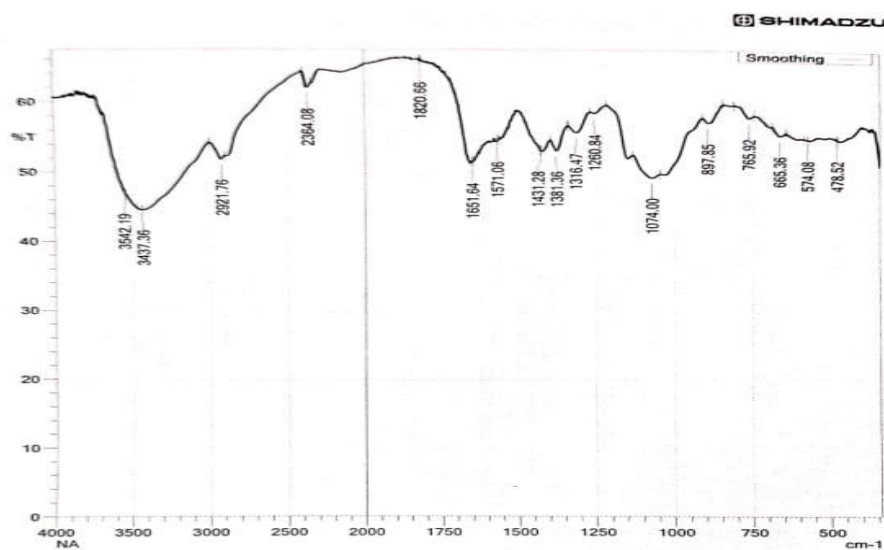


Figure 4 B : FTIR Spectra of chitosan

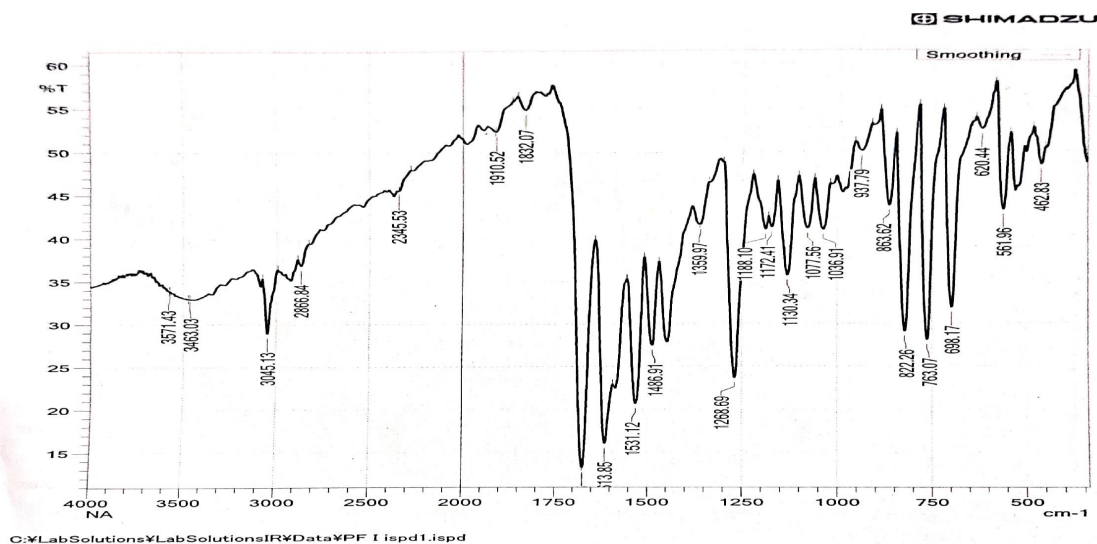


Figure 5: FTIR study of pirfenidone loaded nanoparticles

Table 3: FTIR spectrum of pirfenidone CS NPs

Sample	Functional Group / Vibration	Peak Position (cm ⁻¹)	Observation
Chitosan (CS)	–OH stretching	3400	Characteristic hydroxyl group vibration
	–CH, –CH ₂ , –CH ₃ stretching	2886 – 2974	Aliphatic C–H stretching vibrations
	Amide I vibration	1676	Protein/amide bond stretching
	Amide II vibration	1600	N–H bending vibration
	N–H stretching	924 – 1169	Amine group vibration
Pirfenidone	O=C stretch (α,β-unsaturated ketone)	1612	Carbonyl group vibration
	C–C aromatic stretching	1530	Aromatic ring vibration

(PFD)	C–H aliphatic stretching	3054	Aliphatic hydrocarbon vibration
	C–N aromatic stretching (3° amine)	1274	Aromatic amine vibration
	C=C phenyl stretching	825	Phenyl ring vibration
PFD Loaded CS Nanoparticles	NH ₂ bond shift	1576	Peak shift due to interaction with polymer
	C–H bending (alkyl group)	1412	Alkyl group vibration
	–CH, –CH ₂ , –CH ₃ stretching	2885 – 2910	Aliphatic stretching vibrations
	–OH stretching	3504	Hydroxyl group vibration
	C–H aliphatic stretching	2970	Aliphatic hydrocarbon vibration
	C–N aromatic stretching (3° amine)	1280	Aromatic amine vibration
	C=C phenyl stretching	842	Phenyl ring vibration

BOX-BEHNKEN DESIGN

In this study, a BBD was used to optimize the formulation variables of chitosan loaded PFD containing 3 factors and evaluated at 3 levels. Chitosan (A), PEG 400 (B) and flow rate (C) as independent variables and particle size (R1), entrapment efficiency (EE% ,R2) and Zeta potential (R3) were selected as dependent responses. The independent factors and their range levels used in the study . The experiments were designed by using Design Expert software (Version 13) and the layout of the design is shown in this table. A total of 17 formulations were designed by the software with 3 center points

Table 4 Actual Design Box Behnken Design for PFD-loaded chitosan nanoparticles

Std	Run	Factor 1	Factor 2	Factor 3	Response 1	Response 2	Response 3
		A:Chitosan mg	B:PEG400 ml	C:StirringSpeed rpm	ParticleSize nm	ZetaPotential mV	EE %
16	1	175	15	400	93	26	90.8
2	2	300	5	400	89	23	87
3	3	50	25	400	86	20	84
8	4	300	15	600	84	19	82
11	5	175	5	600	83	18	81
10	6	175	25	200	86	20	84
4	7	300	25	400	88	22	86
17	8	175	15	400	90.9	24.9	89.2
13	9	175	15	400	92.6	25	89.8
7	10	50	15	600	80	14	78
15	11	175	15	400	93	26.5	91
6	12	300	15	200	90	23.3	88
12	13	175	25	600	82	16	80
5	14	50	15	200	84	17.5	82
1	15	50	5	400	86	20.5	84.5
9	16	175	5	200	85	19.5	83
14	17	175	15	400	90.5	26	89.5

Response 1: Particle Size

Table 5 : Fit Summary for Response 1(Particle size)

Source	Sequential p-value	Lack of Fit p-value	Adjusted R ²	Predicted R ²	
Linear	0.3119	0.0099	0.0557	-0.1616	

2FI	0.9894	0.0051	-0.2137	-1.0495	
Quadratic	< 0.0001	0.4158	0.9023	0.6404	Suggested
Cubic	0.4158		0.9101		Aliased

Table 6: Sequential Model Sum of Squares [Type I] for Response 1(Particle size)

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Mean vs Total	1.294E+05	1	1.294E+05			
Linear vs Mean	60.25	3	20.08	1.31	0.3119	
2FI vs Linear	2.25	3	0.7500	0.0382	0.9894	
Quadratic vs 2FI	185.31	3	61.77	39.06	< 0.0001	Suggested
Cubic vs Quadratic	5.25	3	1.75	1.20	0.4158	Aliased
Residual	5.82	4	1.45			
Total	1.296E+05	17	7625.22			

Select the highest order polynomial where the additional terms are significant and the model is not aliased.

Table 7 :ANOVA for Quadratic model for Response 1(Particle size)

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	247.81	9	27.53	17.41	0.0005	significant
A-Chitosan	28.13	1	28.13	17.78	0.0040	
B-PEG 400	0.1250	1	0.1250	0.0790	0.7867	
C-Stirring Speed	32.00	1	32.00	20.23	0.0028	
AB	0.2500	1	0.2500	0.1581	0.7028	
AC	1.00	1	1.00	0.6323	0.4526	
BC	1.00	1	1.00	0.6323	0.4526	
A ²	19.01	1	19.01	12.02	0.0104	
B ²	29.01	1	29.01	18.35	0.0036	
C ²	121.64	1	121.64	76.92	< 0.0001	
Residual	11.07	7	1.58			

Lack of Fit	5.25	3	1.75	1.20	0.4158	not significant
Pure Error	5.82	4	1.45			
Cor Total	258.88	16				

Factor coding is **Coded**.

Sum of squares is **Type III - Partial**

The **Model F-value** of 17.41 implies the model is significant. There is only a 0.05% chance that an F-value this large could occur due to noise.

P-values less than 0.0500 indicate model terms are significant. In this case A, C, A², B², C² are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

The **Lack of Fit F-value** of 1.20 implies the Lack of Fit is not significant relative to the pure error. There is a 41.58% chance that a Lack of Fit F-value this large could occur due to noise. Non-significant lack of fit is good -- we want the model to fit.

Table 8 :Fit Statistics for Response 1(Particle size)

Std. Dev.	1.26	R²	0.9572
Mean	87.24	Adjusted R²	0.9023
C.V. %	1.44	Predicted R²	0.6404
		Adeq Precision	11.2753

The **Predicted R²** of 0.6404 is not as close to the **Adjusted R²** of 0.9023 as one might normally expect; i.e. the difference is more than 0.2. This may indicate a large block effect or a possible problem with your model and/or data. Things to consider are model reduction, response transformation, outliers, etc. All empirical models should be tested by doing confirmation runs.

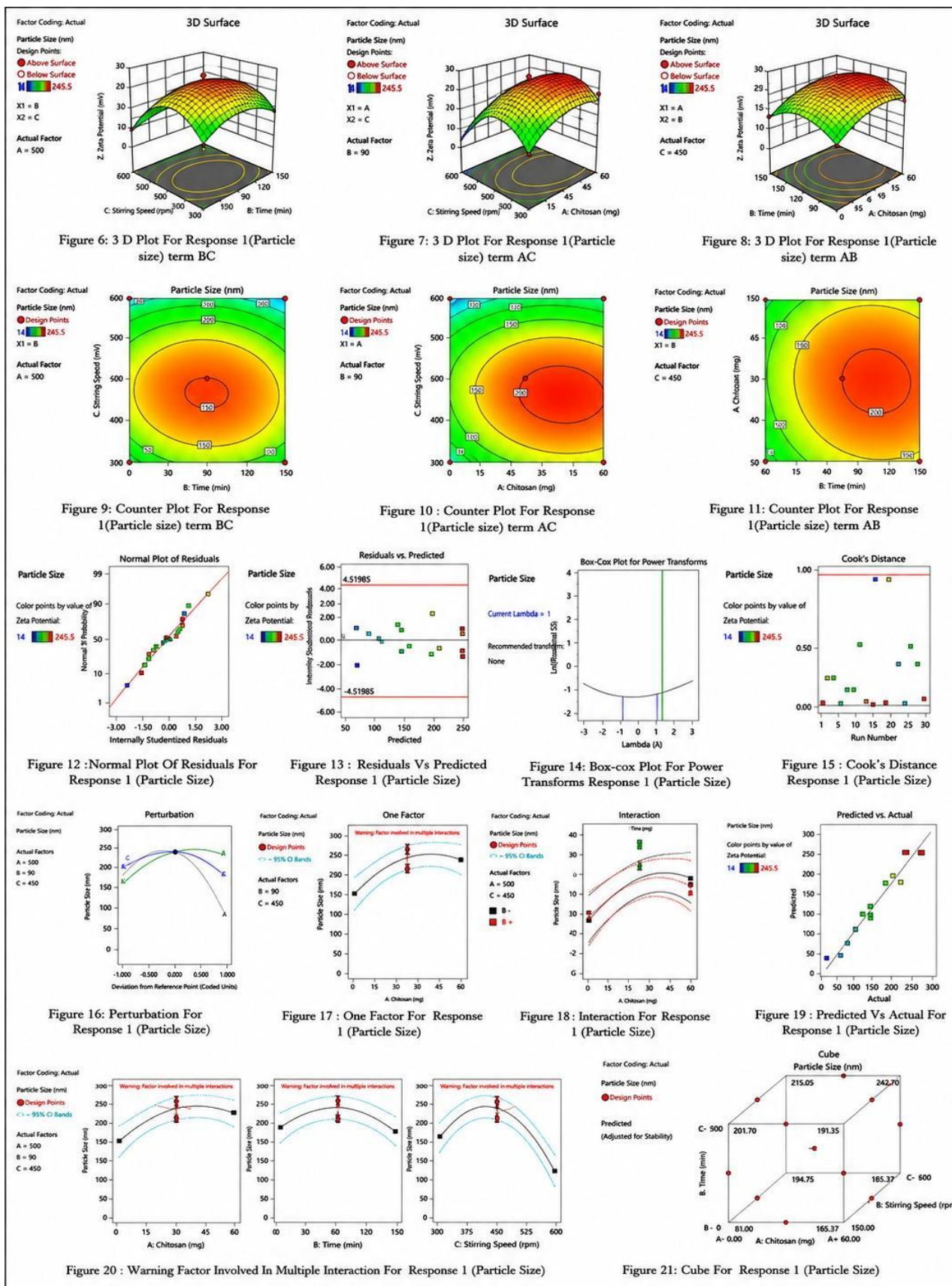
Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 11.275 indicates an adequate signal. This model can be used to navigate the design space.

Table 9:Coefficients in Terms of Coded Factors for Response 1 (Particle size)

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	92.00	1	0.5624	90.67	93.33	
A-Chitosan	1.88	1	0.4446	0.8237	2.93	1.0000

B-PEG 400	-0.1250	1	0.4446	-1.18	0.9263	1.0000
C-Stirring Speed	-2.00	1	0.4446	-3.05	-0.9487	1.0000
AB	-0.2500	1	0.6288	-1.74	1.24	1.0000
AC	-0.5000	1	0.6288	-1.99	0.9868	1.0000
BC	-0.5000	1	0.6288	-1.99	0.9868	1.0000
A ²	-2.13	1	0.6129	-3.57	-0.6758	1.01
B ²	-2.63	1	0.6129	-4.07	-1.18	1.01
C ²	-5.37	1	0.6129	-6.82	-3.93	1.01

The coefficient estimate represents the expected change in response per unit change in factor value when all remaining factors are held constant. The intercept in an orthogonal design is the overall average response of all the runs. The coefficients are adjustments around that average based on the factor settings. When the factors are orthogonal the VIFs are 1; VIFs greater than 1 indicate multi-collinearity, the higher the VIF the more severe the correlation of factors. As a rough rule, VIFs less than 10 are tolerable.



Response 2: Zeta Potential**Table10: Fit Summary for Response 2 (Zeta potential)**

Source	Sequential p-value	Lack of Fit p-value	Adjusted R ²	Predicted R ²	
Linear	0.3093	0.0015	0.0571	-0.1732	
2FI	0.9907	0.0008	-0.2131	-1.1057	
Quadratic	< 0.0001	0.1129	0.9229	0.5857	Suggested
Cubic	0.1129		0.9653		Aliased

Table 11: Sequential Model Sum of Squares [Type I] for Response 2 (Zeta potential)

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Mean vs Total	7674.44	1	7674.44			
Linear vs Mean	52.50	3	17.50	1.32	0.3093	
2FI vs Linear	1.78	3	0.5950	0.0350	0.9907	
Quadratic vs 2FI	162.61	3	54.20	50.12	< 0.0001	Suggested
Cubic vs Quadratic	5.62	3	1.87	3.85	0.1129	Aliased
Residual	1.95	4	0.4870			
Total	7898.90	17	464.64			

Select the highest order polynomial where the additional terms are significant and the model is not aliased.

Table 12 : ANOVA for Quadratic model for Response 2 (Zeta potential)

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	216.89	9	24.10	22.28	0.0002	significant
A-Chitosan	29.26	1	29.26	27.06	0.0013	
B-PEG 400	1.13	1	1.13	1.04	0.3417	
C-Stirring Speed	22.11	1	22.11	20.44	0.0027	

AB	0.0625	1	0.0625	0.0578	0.8169	
AC	0.1600	1	0.1600	0.1479	0.7119	
BC	1.56	1	1.56	1.44	0.2685	
A ²	18.83	1	18.83	17.42	0.0042	
B ²	20.19	1	20.19	18.67	0.0035	
C ²	110.16	1	110.16	101.86	< 0.0001	
Residual	7.57	7	1.08			
Lack of Fit	5.62	3	1.87	3.85	0.1129	not significant
Pure Error	1.95	4	0.4870			
Cor Total	224.46	16				

Factor coding is **Coded**.

Sum of squares is **Type III - Partial**

The **Model F-value** of 22.28 implies the model is significant. There is only a 0.02% chance that an F-value this large could occur due to noise.

P-values less than 0.0500 indicate model terms are significant. In this case A, C, A², B², C² are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

The **Lack of Fit F-value** of 3.85 implies the Lack of Fit is not significant relative to the pure error. There is a 11.29% chance that a Lack of Fit F-value this large could occur due to noise. Non-significant lack of fit is good -- we want the model to fit.

Table 13 :Fit Statistics for Response 2(Zeta Potential)

Std. Dev.	1.04	R²	0.9663
Mean	21.25	Adjusted R²	0.9229
C.V. %	4.89	Predicted R²	0.5857
		Adeq Precision	13.2960

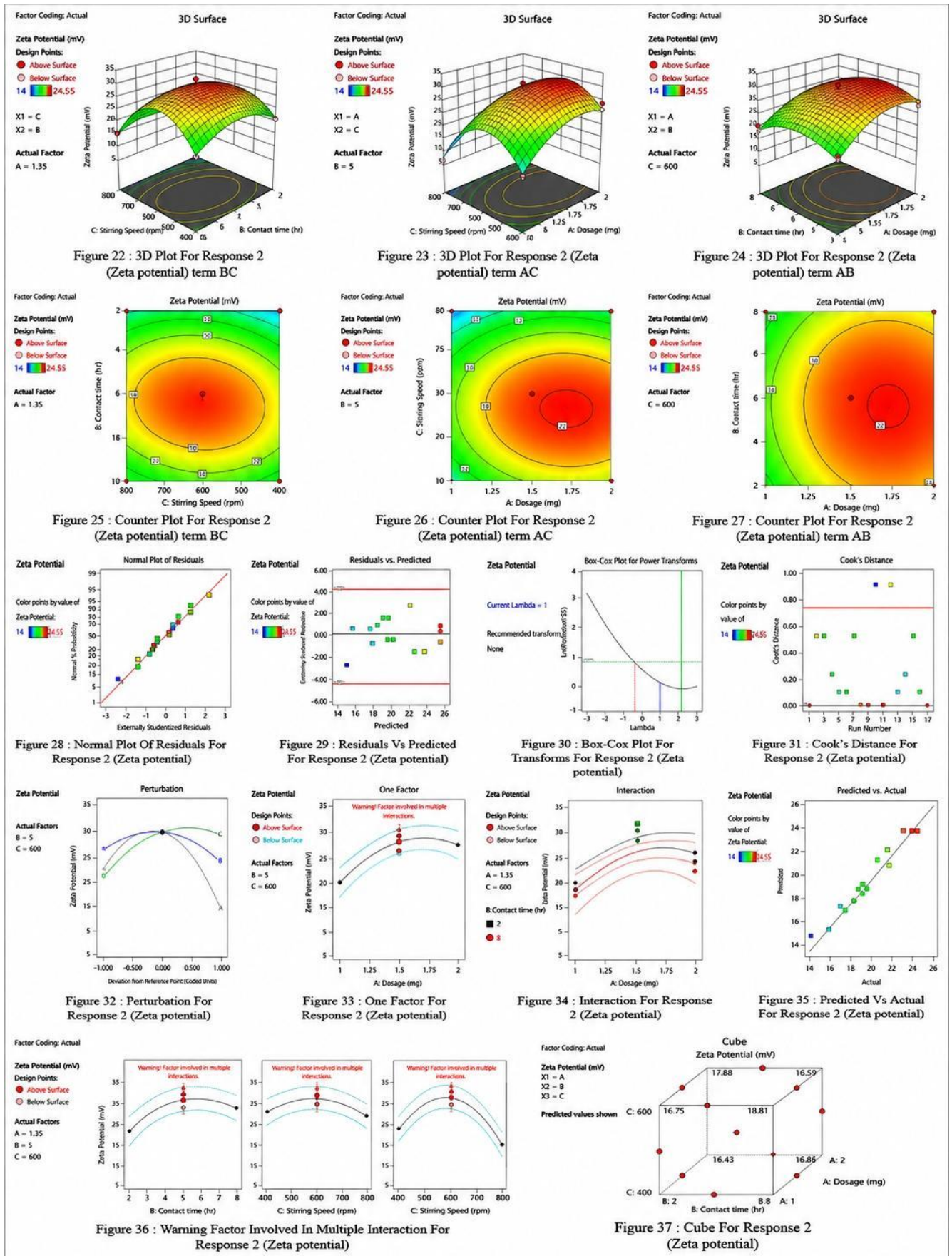
The **Predicted R²** of 0.5857 is not as close to the **Adjusted R²** of 0.9229 as one might normally expect; i.e. the difference is more than 0.2. This may indicate a large block effect or a possible problem with your model and/or data. Things to consider are model reduction, response transformation, outliers, etc. All empirical models should be tested by doing confirmation runs.

Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 13.296 indicates an adequate signal. This model can be used to navigate the design space.

Table 14 :Coefficients in Terms of Coded Factors

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	25.68	1	0.4651	24.58	26.78	
A-Chitosan	1.91	1	0.3677	1.04	2.78	1.0000
B-PEG 400	-0.3750	1	0.3677	-1.24	0.4944	1.0000
C-Stirring Speed	-1.66	1	0.3677	-2.53	-0.7931	1.0000
AB	-0.1250	1	0.5200	-1.35	1.10	1.0000
AC	-0.2000	1	0.5200	-1.43	1.03	1.0000
BC	-0.6250	1	0.5200	-1.85	0.6045	1.0000
A ²	-2.12	1	0.5068	-3.31	-0.9166	1.01
B ²	-2.19	1	0.5068	-3.39	-0.9916	1.01
C ²	-5.12	1	0.5068	-6.31	-3.92	1.01

The coefficient estimate represents the expected change in response per unit change in factor value when all remaining factors are held constant. The intercept in an orthogonal design is the overall average response of all the runs. The coefficients are adjustments around that average based on the factor settings. When the factors are orthogonal the VIFs are 1; VIFs greater than 1 indicate multi-collinearity, the higher the VIF the more severe the correlation of factors. As a rough rule, VIFs less than 10 are tolerable.



Response 3: EE**Table 15 : Fit Summary for Response 3(EE)**

Source	Sequential p-value	Lack of Fit p-value	Adjusted R ²	Predicted R ²	
Linear	0.3237	0.0020	0.0492	-0.1801	
2FI	0.9907	0.0010	-0.2232	-1.1112	
Quadratic	< 0.0001	0.1473	0.9235	0.6078	Suggested
Cubic	0.1473		0.9604		Aliased

Table 16 :Model Sum of Squares [Type I] for Response 3(EE)

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Mean vs Total	1.236E+05	1	1.236E+05			
Linear vs Mean	58.56	3	19.52	1.28	0.3237	
2FI vs Linear	2.06	3	0.6875	0.0349	0.9907	
Quadratic vs 2FI	188.23	3	62.74	50.98	< 0.0001	Suggested
Cubic vs Quadratic	6.06	3	2.02	3.17	0.1473	Aliased
Residual	2.55	4	0.6380			
Total	1.239E+05	17	7288.22			

Select the highest order polynomial where the additional terms are significant and the model is not aliased.

Table 17 :ANOVA for Quadratic model for Response 3(EE)

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	248.85	9	27.65	22.47	0.0002	significant
A-Chitosan	26.28	1	26.28	21.36	0.0024	
B-PEG 400	0.2813	1	0.2813	0.2285	0.6472	
C-Stirring Speed	32.00	1	32.00	26.00	0.0014	
AB	0.0625	1	0.0625	0.0508	0.8281	

AC	1.0000	1	1.0000	0.8126	0.3973	
BC	1.0000	1	1.0000	0.8126	0.3973	
A ²	18.44	1	18.44	14.98	0.0061	
B ²	28.30	1	28.30	23.00	0.0020	
C ²	125.87	1	125.87	102.28	< 0.0001	
Residual	8.61	7	1.23			
Lack of Fit	6.06	3	2.02	3.17	0.1473	not significant
Pure Error	2.55	4	0.6380			
Cor Total	257.46	16				

Factor coding is **Coded**.

Sum of squares is **Type III - Partial**

The **Model F-value** of 22.47 implies the model is significant. There is only a 0.02% chance that an F-value this large could occur due to noise.

P-values less than 0.0500 indicate model terms are significant. In this case A, C, A², B², C² are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

The **Lack of Fit F-value** of 3.17 implies the Lack of Fit is not significant relative to the pure error. There is a 14.73% chance that a Lack of Fit F-value this large could occur due to noise. Non-significant lack of fit is good -- we want the model to fit.

Table 18: Fit Statistics for Response 3(E)

Std. Dev.	1.11	R²	0.9665
Mean	85.28	Adjusted R²	0.9235
C.V. %	1.30	Predicted R²	0.6078
		Adeq Precision	12.7787

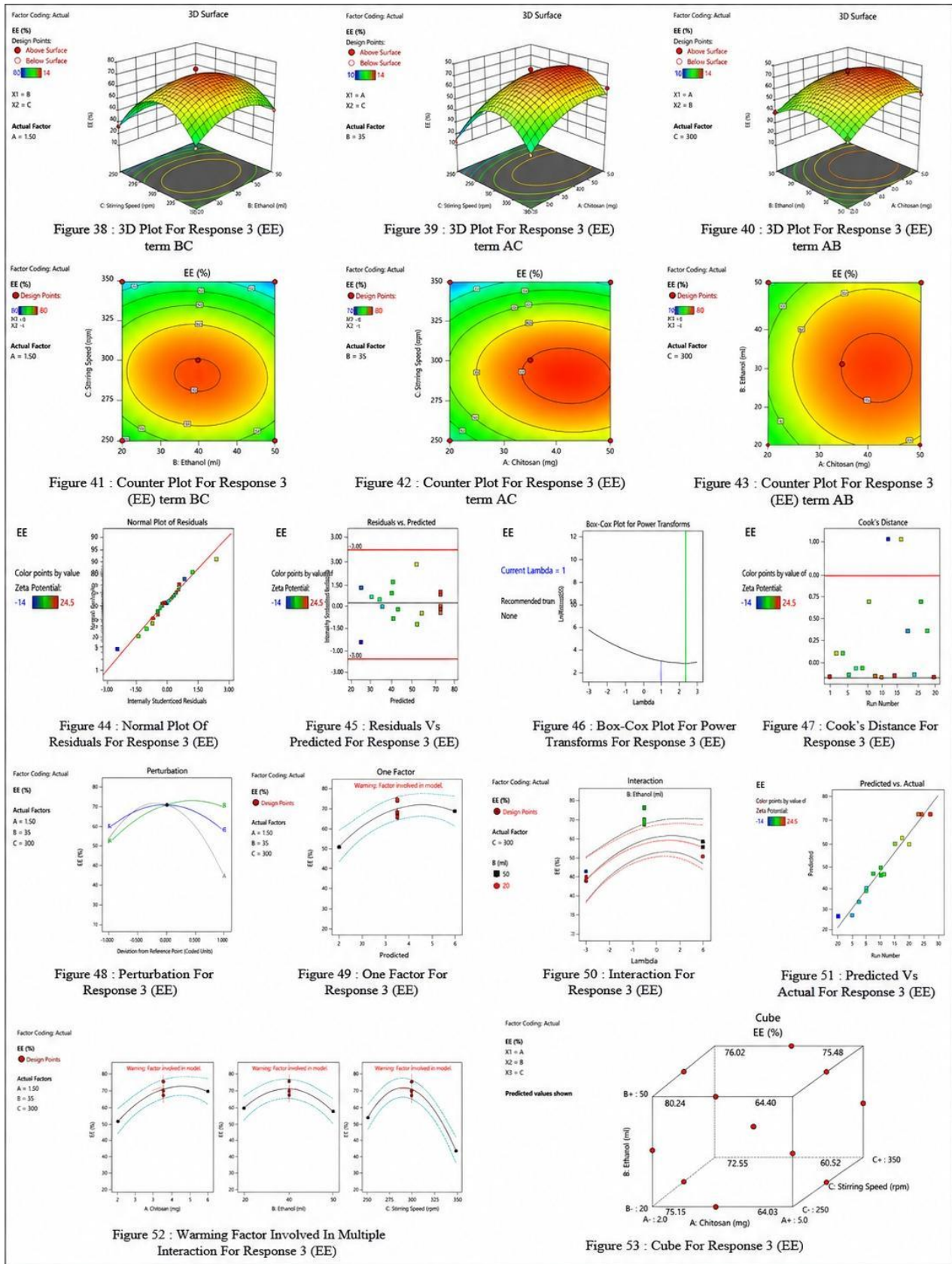
The **Predicted R²** of 0.6078 is not as close to the **Adjusted R²** of 0.9235 as one might normally expect; i.e. the difference is more than 0.2. This may indicate a large block effect or a possible problem with your model and/or data. Things to consider are model reduction, response transformation, outliers, etc. All empirical models should be tested by doing confirmation runs.

Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 12.779 indicates an adequate signal. This model can be used to navigate the design space.

Table 19 :Coefficients in Terms of Coded Factors for Response 3(EE)

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	90.06	1	0.4961	88.89	91.23	
A-Chitosan	1.81	1	0.3922	0.8851	2.74	1.0000
B-PEG 400	-0.1875	1	0.3922	-1.11	0.7399	1.0000
C-Stirring Speed	-2.00	1	0.3922	-2.93	-1.07	1.0000
AB	-0.1250	1	0.5547	-1.44	1.19	1.0000
AC	-0.5000	1	0.5547	-1.81	0.8116	1.0000
BC	-0.5000	1	0.5547	-1.81	0.8116	1.0000
A ²	-2.09	1	0.5406	-3.37	-0.8141	1.01
B ²	-2.59	1	0.5406	-3.87	-1.31	1.01
C ²	-5.47	1	0.5406	-6.75	-4.19	1.01

The coefficient estimate represents the expected change in response per unit change in factor value when all remaining factors are held constant. The intercept in an orthogonal design is the overall average response of all the runs. The coefficients are adjustments around that average based on the factor settings. When the factors are orthogonal the VIFs are 1; VIFs greater than 1 indicate multi-collinearity, the higher the VIF the more severe the correlation of factors. As a rough rule, VIFs less than 10 are tolerable.



Particle size

Pirfenidone nanoparticles with a spherical morphology were successfully prepared using low-molecular-weight chitosan via the solvent evaporation technique. Particle size was influenced by the addition of the PFD-containing organic phase into the chitosan solution in the presence of PEG 400 under magnetic stirring. The average particle size of chitosan loaded PED nanoparticles 91.7nm (Figure 54). Both the concentration and molecular weight of chitosan markedly affected particle size, with higher polymer content producing larger nanoparticles due to the formation of bigger emulsion droplets during emulsification.

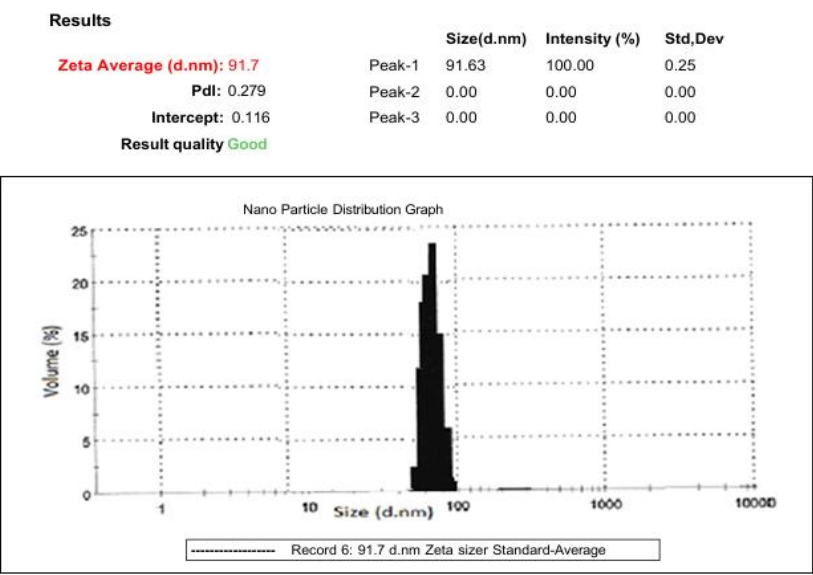


Figure 54 : Particle size of PFD

Zeta potential

The zeta potential increased proportionally with the amount of chitosan incorporated into the nanoparticle formulation. The measured values of zeta potential chitosan loaded pirfenidone gives 26.5mV indicating that the nanoparticle surface was predominantly composed of chitosan.(Figure 55) Positively charged nanoparticles can readily interact with negatively charged cell membranes, facilitating cellular interaction and release. The positive zeta potential confirms the formation of cationic chitosan nanoparticles. Moreover, the net positive surface charge promotes electrostatic attraction with negatively charged sialic acid residues on alveolar macrophages, enhancing cellular uptake of PFD-loaded nanoparticles.

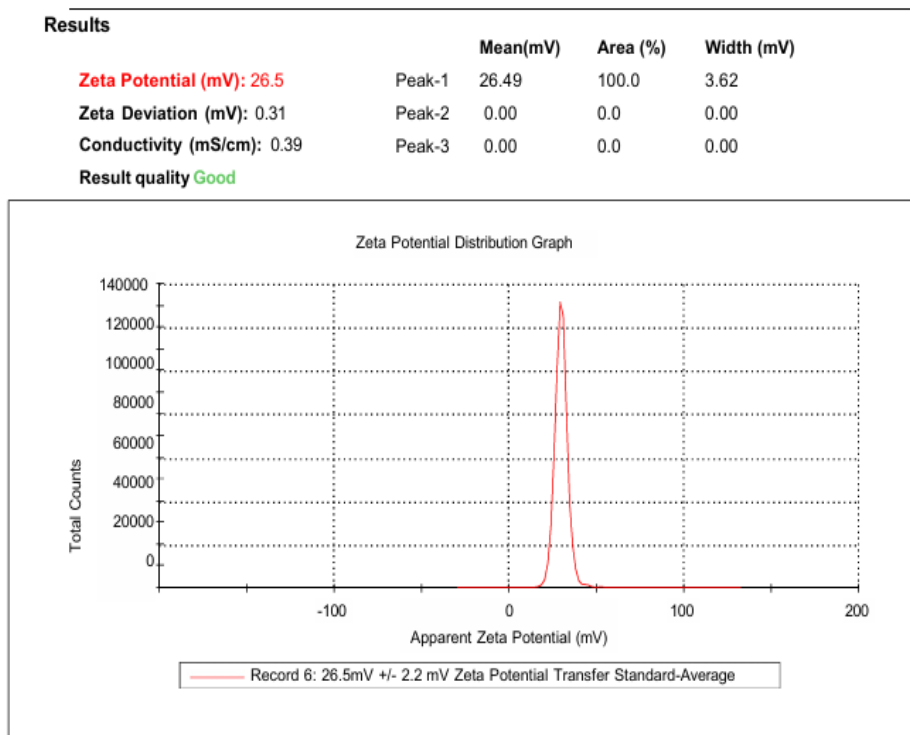


Figure 55: zeta potential of PFD

Polydispersity index (PDI)

Nanoparticles exhibiting a PDI below 0.4 are generally regarded as suitable for deep lung (alveolar) drug delivery according to reported studies. Polydispersity reflects the degree of variation in particle size distribution. The PDI values of pirfenidone nanoparticles prepared by the solvent evaporation method ranged is 0.279. An increase in PDI indicates greater heterogeneity in cross-linking, network structure, chain length, branching, and overall random organization.

Yield of nanoparticles

The percentage yield of chitosan nanoparticles for all formulations ranged between 55% and 54.32% . Although drug loss was minimal, the water solubility of pirfenidone led to partial drug diffusion into the aqueous phase, thereby reducing drug entrapment and limiting the nanoparticle yield to about 60%. Additionally, sonication time had a direct impact on yield, as prolonged sonication increased drug solubilization and consequently decreased the overall nanoparticle recovery.

% Entrapment efficiency

The drug entrapment efficiency of nanoparticles prepared with drug: polymer was found to be 90.12%. An overall increase in entrapment efficiency was observed with increasing polymer concentration. The improved entrapment is likely attributed to strong electrostatic interactions between the drug and the polymer matrix.

Drug release of CS-PFD-NPs

Pirfenidone nanoparticles were evaluated in vitro to study their drug release behavior. The release profile from chitosan nanoparticles was examined in phosphate buffer saline (pH 7.4) at 37 °C. As illustrated in the pure pirfenidone sample (F1, 10 mg) exhibited almost complete release (99.99%) at the initial sampling point. In contrast, PFD–chitosan nanoparticles showed an initial burst release of about 21%, followed by a sustained release reaching 93.45% over 12 hours. The burst phase may help achieve the desired lung plasma concentration rapidly, while the prolonged phase supports maintenance of therapeutic levels. The early release is attributed to drug molecules located near the particle surface that diffuse quickly upon contact with the dissolution medium. Compared with other methods, formulations prepared by solvent evaporation demonstrated extended release up to 12 hours, likely due to their denser electrostatic encapsulation and distinct particle structure.

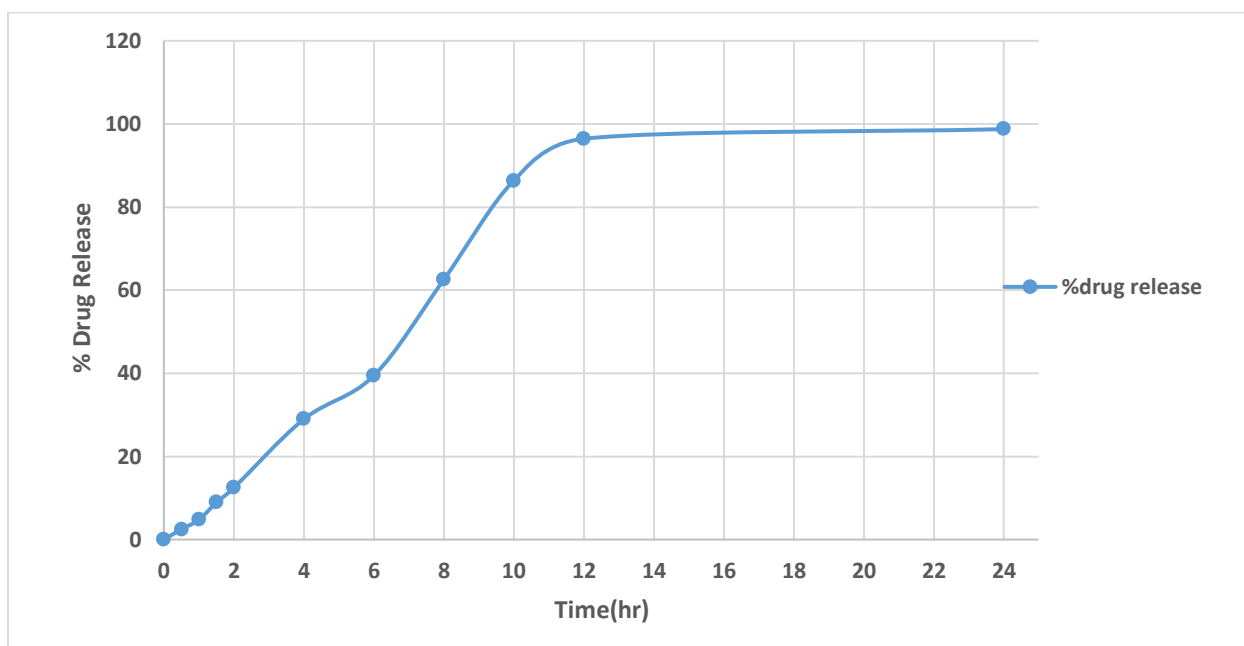


Figure 56: Drug release profile of PFD-loaded chitosan nanoparticles

Drug release kinetic study

The dissolution data were analyzed using different kinetic models, including zero-order, first-order, Higuchi, and Korsmeyer–Peppas and Hixson Crowell kinetic equations to characterize the release behavior of all six formulations. Based on the higher correlation coefficient (R^2) values, the drug release from the formulations was best described by zero-order kinetics and the Higuchi model.

Table 20: Correlation coefficient according to different kinetic equations

FORMULATION	% DRUG RELEASE	ZERO ORDER	FIRST ORDER	HIGUCH I PLOT	KORMEYE PEPPAAR	HIXSON CROWELL KINETIC	n VALUE
F ₃	93.45	0.9821	0.8644	0.9827	0.9378	0.7734	0.478

Optimized batch evaluation

Composition and evaluation of the optimized batch

The optimized formulation was prepared by dissolving 100 mg of PFD in the organic phase contained ethanol, and 100 mg of chitosan was dispersed in 20 mL of the aqueous phase with 1% PEG 400 as stabilizer. The organic phase was then slowly introduced into the aqueous phase under continuous stirring, and the remaining steps were carried out as described for nanoparticle preparation. Optimization was based on key parameters including mean particle size (91.7nm), zeta potential (26.5mV), strongly cationic surface charge, polydispersity index (0.279), entrapment efficiency (90.12%), and drug release at 12 h (93.45%). The finalized formulation was subsequently converted into an inhalable free-flowing powder to facilitate administration



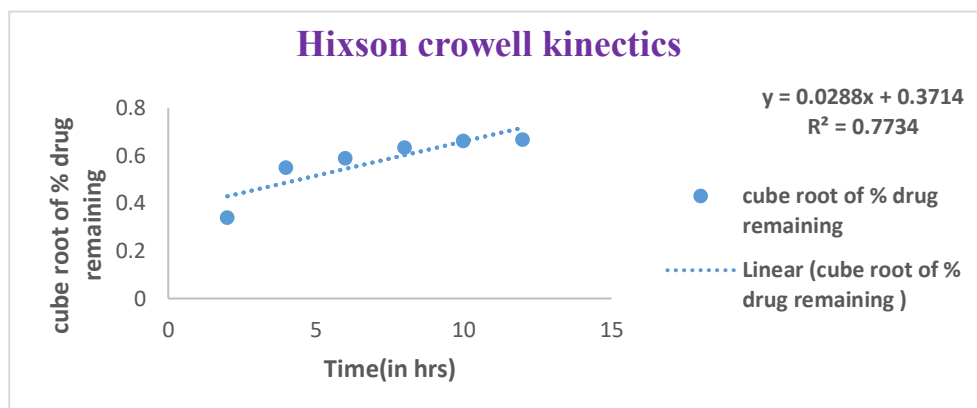
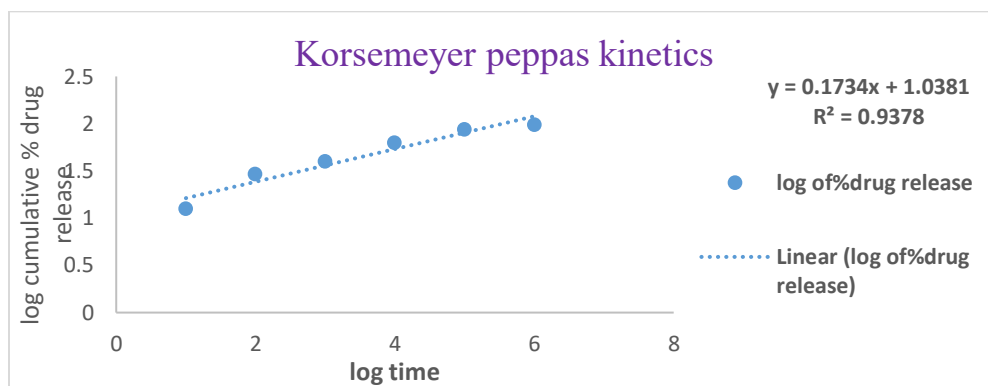
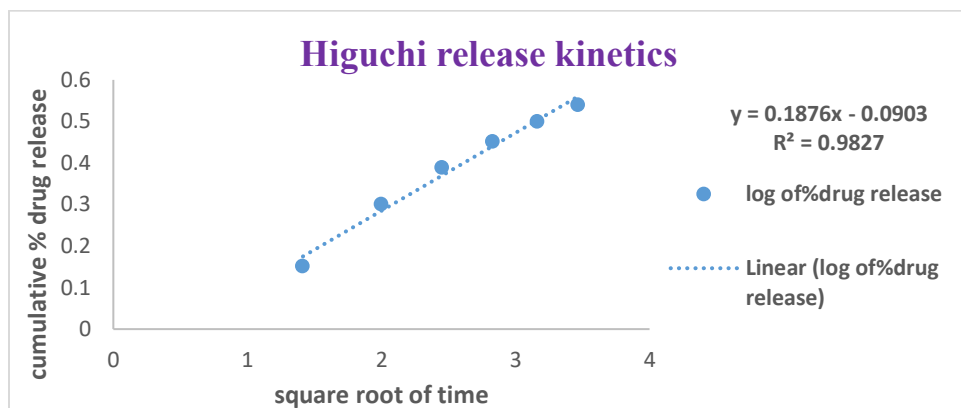
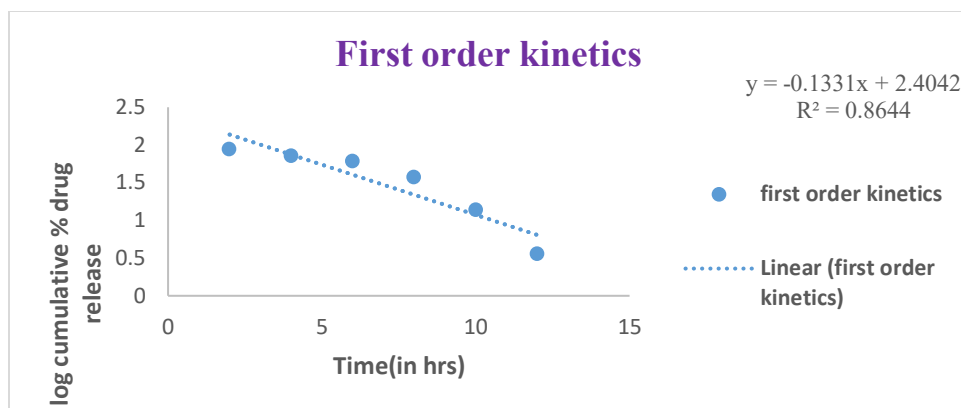


**Figure 57: drug release kinetics plot of optimized batch A) Zero order B)First order
C) Higuchi plot D)Korsmeyer peppas plot E)Hixson crowell plot**

In-vitro drug release study

The in-vitro drug release study demonstrated that pirfenidone was released gradually from the chitosan nanoparticles in phosphate buffer saline (pH 7.4) at 37 ± 0.5 °C. An initial burst release was observed during the first hour, which may be attributed to the drug present on the surface of the nanoparticles. This was followed by a sustained and controlled drug release pattern over the study period. Among the formulations, the optimized formulation showed a higher cumulative percentage drug release, reaching approximately 85–92% within 12 hours. The sustained release behavior indicates that chitosan nanoparticles effectively controlled the release of pirfenidone. The blank formulation containing pure pirfenidone powder exhibited a comparatively faster drug release than the nanoparticle formulations. These results suggest that the chitosan nanoparticle system is suitable for prolonged drug delivery of pirfenidone.

Stability studies

The stability evaluation of pirfenidone nanoparticles prepared using chitosan polymer revealed that the formulation remained stable for 45 days under 25 ± 2 °C temperature and 60 ± 5 % relative humidity as per International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use guidelines. The nanoparticles retained their original particle size, zeta potential, polydispersity index, entrapment efficiency, and drug release profile at both 8 h and 12 h. Throughout the storage period, no significant particle aggregation or alterations in physical properties were observed

Table 21 :Stability study of optimized batch (F3) of PFD NPs

Evaluation Parameter	Time period for sampling			
	Initial	After15 days	After 30 days	After45 days
Particle size (nm)	91.7	91.10	97.50	91.78
Zeta potential (mV)	26.5	26.7	26.17	26.34
%EE	90.12	90.25	90.37	90.20
PDI	0.279	0.296	0.254	0.274
% drug release at	62.54	62.54	62.32	62.96

8 hour				
%drug release at 12 hour	93.45	93.75	93.57	93.19

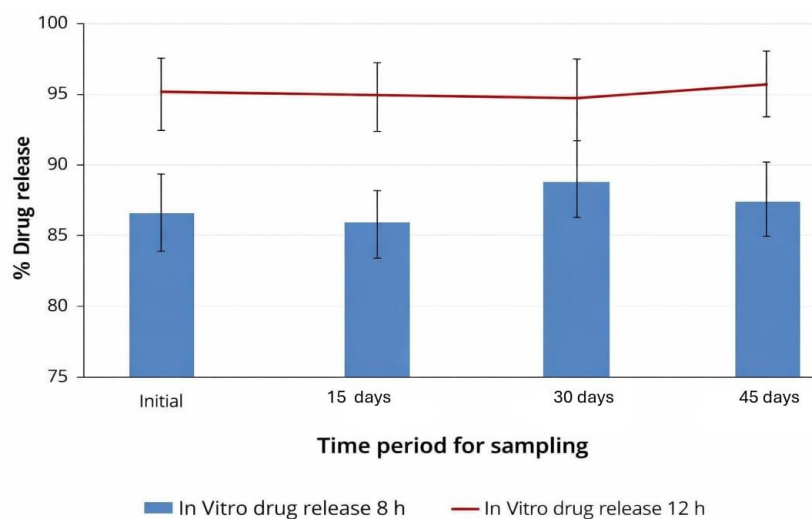


Figure 58 : Stability studies for %drug release study of PFD

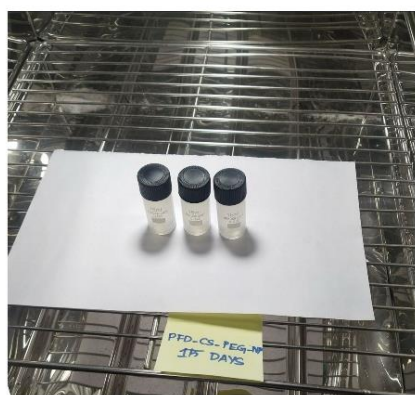


Figure 59 : Stability studies of PFD-CS-PEG 400-NPs for 15 days



Figure 60 : Stability studies of PFD-CS-PEG 400-NPs for 30 days

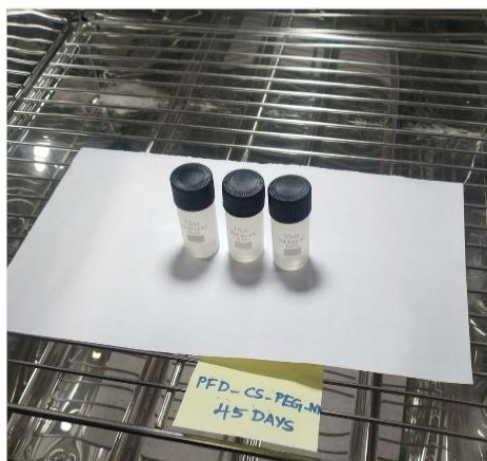


Figure 61 : Stability studies of PFD-CS-PEG 400-NPs for 45 days

DISCUSSION

The aim of the study was to develop pirfenidone-loaded nanoparticles to improve its therapeutic efficacy and reduce systemic side effects to the treatment of idiopathic pulmonary fibrosis. The optimized formulation showed particle size in the nanometer range with low PDI, indicating uniform distribution and good stability. The zeta potential values confirm the adequate surface charge, preventing the aggregation of nanoparticles. The formulation exhibited good entrapment efficiency, suggesting effective incorporation of the drug into the polymer matrix. In vitro drug release studies demonstrated a sustained release pattern, which is beneficial for prolonged therapeutic action and reduced dosing frequency. The solvent evaporation method proved a suitable technique for preparation stable nanoparticles. Optimization using Box-Behnken Design helped in identifying the ideal formulation parameters.

CONCLUSION

PFD nanoparticles were successfully prepared using the quasi-emulsion solvent evaporation technique. The formulation was evaluated for mean particle size, zeta potential, polydispersity index, surface morphology, entrapment efficiency, drug release, kinetic release behavior, stability, and in-vitro drug deposition. The optimized nanoparticles showed an average size of 91.7 nm and zeta potential of 26.5 mV, indicating a positively charged surface. Drug entrapment efficiency was 90.12%, with 93.45% drug release within 12 hours. Aerodynamic studies showed an MMAD of 1.28 μm , suggesting effective deep lung deposition and possible usefulness in treating idiopathic pulmonary fibrosis. Drug release followed Non-Fickian diffusion. Stability studies based on ICH guidelines confirmed that the optimized chitosan-based PFD nanoparticles remained stable for 45 days. Overall, this nanoparticle system may improve therapeutic efficiency by enhancing drug delivery to deep lung tissues at a lower dose.

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Conflict of interest

Material Support:

“The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. The active pharmaceutical ingredient (Pirfenidone) used in this research was kindly provided as a gift sample by [Allastir private Ltd]. The provider had no role in the study design, the collection, analysis, and interpretation of data, the writing of the report, or the decision to submit the article for publication.”

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