

RESEARCH PAPER

Quality by Design-Oriented Development and Optimization of Olopatadine HCl-Loaded Chitosan/Bovine Serum Albumin Nanogels for Ocular Delivery

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ABSTRACT

Ophthalmic conventional formulations suffer from a short precorneal residence time, which leads to low ocular bioavailability and frequent administration. This study aimed to develop and optimize olopatadine HCl-loaded bovine serum albumin/chitosan (BSA/CHS) nanogels as a sustained release ophthalmic delivery system using a Quality by Design (QbD) approach. Risk assessment and Design of Experimental were used to systematically identify critical formulation and process variables that impact nanogel characteristics. Nanogels were fabricated via pH-induced CHS/BSA self-assembly followed by thermal treatment to consolidate the matrix, then optimized to obtain the desired physicochemical properties. The optimized formulation was characterized using FTIR, PXRD, and SEM, while pH- and ionic responsiveness, *in vitro* release, and stability studies were also performed. The optimized nanogel formulation exhibited a particle size of 47.13 ± 1.6 nm, a polydispersity index of 0.268 ± 0.002 , a zeta potential of $+38.21 \pm 2.82$ mV, and an entrapment efficiency of $25.07 \pm 1.22\%$. The morphological analysis confirmed the formation of nanosized spherical particles. FTIR analysis confirmed the compatibility of the various formulation components, and PXRD findings were consistent with successful nanogel formation. The optimized nanogels displayed a sustained, pH-responsive drug-release profile and acceptable physical stability during storage. Results indicate that incorporating QbD principles offers a reliable and reproducible approach for developing nanogels as a potential platform for sustained ophthalmic drug delivery applications.

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INTRODUCTION

The advent of nanotechnology in drug product design increasingly demands a systematic, knowledge-driven approach rather than an empirical trial-and-error approach. Quality by Design (QbD) provides a structured, risk-based

framework that incorporates prior knowledge to identify sources of variability and ensure the consistent achievement of predefined product quality [1-3].

Nanogels are hydrogel-based polymeric nanoparticles that have attracted considerable

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interest for drug delivery [4]. QbD have increasingly been applied to optimize nanogel formulations [5-8].

In this work, nanogels containing Olopatadine HCl (OLO) were prepared via physical self-assembly driven by electrostatic interactions between oppositely charged polymers. The polymers for nanogel preparation are chitosan (CHS) and bovine serum albumin (BSA).

CHS, a linear biopolymer, is one of the most abundant polycationic, biocompatible, mucoadhesive, penetration-enhancing, and non-toxic polymers, widely used in pharmaceutical preparations [9, 10]. CHS (pK_a 6.3 – 6.5 [11]) is produced through an alkaline deacetylation reaction, where a higher degree of deacetylation (DDA) is desired for nanogel applications, as the presence of cationic amine groups offers better swelling characteristics [12]. CHS forms nanogel commonly by ionic gelation and electrostatic complexation [13-21].

BSA is frequently used as a complementary polymer with CHS due to its excellent drug-delivery characteristics, including water solubility, biocompatibility, and excellent potential for drug loading, crosslinking, and controlled release [21-27]. It is a polyampholyte with an isoelectric point (pI) between 4.5 and 5.1, possessing multiple hydrophobic and ionic binding domains to accommodate a wide variety of cargo [28-31].

Olopatadine HCl (OLO) is a second-generation antihistamine used for the treatment of allergic conjunctivitis [32]. Conventional ocular eyedrops exhibit poor bioavailability (<5%) due to rapid precorneal clearance [33-35]. Encapsulation of OLO within a mucoadhesive, sustained-release nanogel system may prolong residence time, improve bioavailability, and reduce dosing frequency [36-38]. Additionally, the cationic charge of CHS could enable interaction with the negatively charged mucin surface in the eye, which may help increase precorneal retention and extend drug residence on the ocular surface.

Although BSA/CHS nanogels have been widely investigated, few studies have systematically evaluated the combined influence of formulation variables and process parameters using a QbD framework to achieve reproducible ocular nanogel systems. Therefore, this study aims to fill the gap by applying a systematic QbD approach to develop and optimize OLO-loaded BSA/CHS nanogels for

ophthalmic delivery application. Through risk assessment and DoE, we seek to elucidate the critical effects of material attributes and process parameters on CQAs, ultimately defining a robust design space for reproducible formulation.

MATERIALS AND METHODS

Chemicals

Olopatadine hydrochloride was purchased from Beijing J&R Times Technology Co. (China). Bovine serum albumin (BSA) was purchased from Meryer Biochemical Technology Co. (China). Medium molecular weight chitosan (200-400 mPa-s, 95% DDA) and low molecular weight chitosan (<100 mPa-s, 90% DDA) were obtained from Rhawn Reagent (China) and Macklin Biochemical Co. (China), respectively. Glacial acetic acid was purchased from Central Drug House (India). Potassium chloride was purchased from Thomas Baker Pvt. Ltd. (India). Sodium chloride, sodium hydroxide, and Calcium chloride dihydrate were purchased from Alpha Chemika (India). Potassium phosphate monobasic, potassium phosphate dibasic, and sodium bicarbonate were purchased from Loba Chemie Pvt. Ltd. (India). Deionized water was used throughout the study, and its conductivity was verified using a conductivity meter (Omega, UK).

Method of Nanogel Preparation

The nanogel preparation method was derived from previously reported methods [19, 21]. Briefly, 0.5% (w/w) CHS solution was prepared by dispersing CHS in 0.5% (v/v) glacial acetic acid solution and allowing it to dissolve overnight. BSA 0.5% (w/w) was prepared in deionized water. To prepare drug-loaded nanogels, OLO is dissolved in the BSA solution. A predetermined amount of BSA solution was added dropwise to the CHS solution, with stirring, using a syringe pump (Cole-Parmer UK) at a specified rate to achieve the desired BSA:CHS ratio. The mixture was adjusted to the desired pH using 1 M NaOH and thermally equilibrated in a thermostatic water bath (set to 80 °C) with continuous agitation for a predetermined period. The obtained nanogel dispersion was centrifuged in a chilled centrifuge using an ultrafiltration tube (MWCO 10 kDa) at 2000 rpm for 60 min to separate the unloaded OLO. The amount of untrapped OLO was quantified using UV-visible spectrophotometry at 299.5 nm. The EE and DL

were defined as follows:

$$EE (\%) = [(Weight\ of\ OLO\ added - weight\ of\ OLO\ in\ supernatant) / (Weight\ of\ OLO\ added)] \times 100 \%$$

$$DL (\%) = [(Weight\ of\ OLO\ in\ nanogel) / (Weight\ of\ OLO\ in\ nanogel + Weight\ of\ polymers)] \times 100 \%$$

Following ultrafiltration, the retained nanogels were subsequently lyophilized for further characterization.

pH versus Zeta Potential Curve for BSA

The measurement of electrophoretic mobility was used to determine the Isoelectric point of BSA using the Zetasizer Nano-ZS instrument (Malvern Panalyticals - UK) at 25° C [39, 40]. 0.5% BSA solutions in water were prepared, and their pH was adjusted to the desired value using 1 M acetic acid and 1 M NaOH to simulate the ion type and strength used in nanogel preparation.

Quality by Design

Identifying QTPP and Selection of CQAs

The QTPP was defined in accordance with the specified ICH Q8 R(2) guidelines. The process began with defining the product's intended use, followed by selecting the appropriate dosage form and manufacturing method. Subsequently, critical considerations related to the drug substance, including its suitability for the selected formulation approach and its stability towards the manufacturing conditions, were evaluated. These assessments informed the identification of the CQAs necessary to ensure that the final product consistently meets the predefined quality requirements.

Risk Assessment

Qualitative risk assessment tools were applied in accordance with ICH Q9 guidelines to map and

prioritize risks. The assessment was conducted at the initiation of the study and iteratively updated as new information became available from preliminary studies and screening analysis. Inputs were derived from existing literature, expert judgment, and empirical experimentation data. In addition to product specifications, the assessment accounted for practical limitations and variability introduced by instruments, including operating settings, calibration status, and standard operating conditions. To mitigate subjectivity, a multidisciplinary consensus-based approach was adopted, combining risk identification using an Ishikawa diagram with risk analysis and evaluation through preliminary hazard analysis (PHA) and failure mode and effect analysis (FMEA) with risk priority number (RPN).

Risk Identification

Ishikawa fishbone diagrams were constructed using the 6m framework (material, machine, method, measurement, workforce, milieu) to capture all potential sources of variability. This aimed to exhaustively map all potential material attributes and process parameters that directly or indirectly affect the final product's CQAs for subsequent analysis.

Preliminary Hazard Analysis (PHA)

A qualitative PHA, adopted from ICH Q9 guidelines and published works [3, 41] was employed to rank the factors identified in the Ishikawa diagram (Table 1). Factors assigned as medium or high were then subjected to quantitative FMEA with RPN scoring, and low-risk factors were set for routine monitoring.

FMEA and RPN

To conduct FMEA, failure modes were categorized into formulation-related inputs (raw materials) and process-related inputs. The latter

Table 1. PHA Matrix.

Probability	Severity		
	Low	Medium	High
Low	Low	Low	Medium
Medium	Low	Medium	High
High	Medium	High	High

were further processed using unit operations (mixing, pH adjustment, denaturation, centrifugal separation). Each failure mode is assigned a 1-5 rating for severity (S), occurrence probability (O), and likelihood of detection (D), and the RPN is calculated as $RPN = O \times S \times D$. The rating scales are presented in Table 2, providing a standard basis for calculating RPN. Risk prioritization was then guided by the calculated RPN values and severity scales, with thresholds pre-defined before DoE to avoid bias (Table 3).

Design of Experiment

Selection of Factor Levels

The working ranges for each high-risk factor were determined through a critical analysis of literature reports and preliminary data on formulation and material attributes. The final levels were chosen to ensure practical feasibility, coverage of the lower and upper operational limits, and avoidance of conditions in which formulation instability or non-gelation might occur.

Definitive Screening Design

Definitive Screening Design (DSD) was selected for the screening phase to identify significant factors while accounting for potential curvature and two-factor interactions [42]. DSD was built using Design Expert Software with 7 continuous variables and one categorical, yielding a total of 18 runs (Table 4). Following initial model evaluation, the design was augmented using the coordinate

exchange algorithm under an I-optimality criterion to improve predictive performance and estimation efficiency [43]. Augmentation prioritized replication over exploration: of the 9 added runs, only 3 introduced new design points, while the remaining 6 strengthened the design through center-point triplication and selective duplication. All models were built using automatic model selection via forward regression and the AICc criterion, and were hierarchical. Diagnostic checks (residuals, leverage, and influence statistics) were performed for all the modeled responses.

Central Composite Design (CCD)

Following the screening design, statistically significant factors were further optimized using Response Surface Methodology. A CCD was employed to investigate linear, interaction, and curvature effects of the critical factors on CQAs and to identify optimal operating conditions within the studied ranges.

The CCD comprised two continuous factors (polymer ratio and pH) and one categorical factor (CHS MW). The design included replicated factorial and axial points, along with six center points distributed across two experimental blocks. A rotatable design was achieved using an alpha value of 1.41421 (Table 5). In total, 44 experimental runs were conducted, divided into 22 runs for each CHS MW. Experimental runs were randomized to minimize systematic bias, while center points were intentionally distributed in a

Table 2. FMEA Scoring Matrix.

Category	Rating Scale				
	1	2	3	4	5
Occurrence (O)	Rare	Unlikely	Possible	Likely	Very likely
Severity (S)	Negligible	Minor	Moderate	Serious	Critical
Detectability (D) ^a	Highly detectable	Detectable	Moderately detectable	Low detectability	Undetectable

Table 3. Decision Threshold for Risk Prioritization.

Priority level	RPN criteria	Action
High	≥ 60 or $S \geq 4$ ^a	Control or DoE investigation
Medium	30 – 60	Monitor (in-process checks) or follow up (screening DoE)
Low	< 30	Routine controls and noise candidates

^a Severity level 4 is chosen as the threshold for high priority because it represents failures with serious consequences.

nonrandom sequence to monitor process stability over time [43].

CCD data were analyzed using linear regression without Box-Cox transformation. Model reduction was performed when necessary to eliminate aliasing and/or overfitting as required. The design was performed in two blocks. Blocking was included as a nuisance variable to account for systematic variability.

Design Space Establishment and Multiple Response Optimization

The design space was established using overlay plots generated from the developed polynomial models. The criteria for the design space were defined as a particle size not exceeding 60 nm, a PDI not exceeding 0.3, a ZP between 30 and 50 mV, and an EE of at least 20 %.

The optimal formulation (opt-F) was identified within the established design space using a multiple-response optimization approach based on the four developed polynomial models. Optimization was performed using a desirability function to minimize particle size and PDI while maintaining ZP within the desired range of 30 to 50 mV. The EE model was excluded from the desirability optimization due to its low predictive power, and medium-MW CHS was used to achieve improved EE. Opt-F was subsequently prepared at the predicted levels of the independent variables and evaluated in quintuplicate to assess reproducibility and verify the validity of the model

prediction.

Evaluation and Characterization of Opt-F Determination of Particle Size, Polydispersity Index (PDI), and Zeta Potential (ZP)

The average particle diameter, PDI, and ZP of the nanogel were measured using a Zetasizer Nano-ZS instrument at 25 °C with a backscatter angle of 173°. The size and PDI were analyzed using a disposable polystyrene cuvette, while ZP was measured using disposable U-shaped capillary cells (DTS 1060, Malvern Panalyticals). Before measurement, the formulation was diluted with deionized water to achieve an optimum kilo counts per second range of 20–400. The particle diameter was calculated using the Stokes–Einstein equation. All measurements were performed in triplicate, with a minimum of 12 runs per size measurement and 30 per ZP measurement.

Morphological Study

Morphological analysis was performed using scanning electron microscope (Nova NanoSEM 450, Thermo Fisher Scientific (FEI), USA). Opt-F was diluted 1:30 using deionized water, and phosphotungstic acid to improve contrast. Images were taken at an accelerating voltage of 15 kV, from a working distance of 5 mm. Images were analyzed using ImageJ 1.53m software. The image was initially inverted to show the particles as black spots, then 30 isolated particles with a clear spherical shape and no apparent aggregation

Table 4. The 8 screening factors high (+1) and low (-1) levels for DSD.

Parameters	Unit	Type	Code	Levels	
				-1	+1
BSA:CHS ratio		Numerical	A	3:1	7:1
OLO concentration	mg/ dL %	Numerical	B	0.1	0.2
Addition rate	mL/min	Numerical	C	0.5	2
Stirring rate	rpm	Numerical	D	500	1500
Stirring duration	min	Numerical	E	30	90
pH		Numerical	F	4.4	4.5
Holding time	min	Numerical	G	20	60
CHS MW		Categorical	H	Low	Medium

Table 5. Parameters and levels for CCD.

Parameters	Type	Code	Levels				
			-α	-1	0	+1	+α
BSA:CHS ratio	Numerical	A	2.17:1	3:1	5:1	7:1	7.83:1
pH	Numerical	B	4.38	4.4	4.45	4.5	4.52
CHS MW	Categorical	C	-	Low	-	Medium	-

were measured as a representative sample.

pH-Responsiveness Profile

To evaluate the swelling behavior of opt-F, 0.5 mL were dispersed into buffer solutions of different pH values (4.5, 5, 5.5, 6, 6.8, 7.4), shaken for 10 min, and allowed to swell for 24h. The samples were then measured for particle size, PDI, and ZP. All experiments were performed in triplicates.

The effect of changing the ionic strength of opt-F on size, PDI, and ZP was also evaluated by dispersing them in NaCl solution of different ionic strength (from 0 to 200mM) and allowing them to equilibrate for 24 h.

In Vitro Drug Release Evaluation

Drug release studies were conducted using vertical Franz diffusion cells under standard conditions. The receptor media was continuously stirred at 700 rpm, using a 10 mm magnetic stirring bar, and the system was thermoregulated at 34 ± 2 °C [44, 45]. An aliquot of 1 mL of opt-F was placed in the donor compartment on a pre-soaked dialysis membrane (MWCO 8000–14000 Da). Release studies were performed under three different conditions: pH 5 using 0.01 M acetate buffer, pH 7.4 using 0.01 M phosphate buffer, and pH 7.4 using simulated lacrimal fluid (SLF) [46].

Samples were withdrawn from the receptor compartment and replaced with fresh medium. Sink conditions were maintained throughout the study [47]. The samples were analyzed using a UV spectrophotometer. All experiments were performed in triplicate.

The cumulative percentage released was calculated by the following sample replacement

correction and percentage released equations:

$$Q_n = C_n V + V_s \sum_{i=1}^{n-1} C_i$$

$$\% \text{ Release} = \frac{Q_n}{M_0} \times 100$$

where Q_n is the cumulative amount of drug released at the nth sampling point, C_n is the drug concentration in the receptor at that point, C_i is the drug concentration at the previous sampling points, V is the total volume of the receptor compartment, V_s is the volume of sample withdrawn at each sampling point, M_0 is the initial amount of drug in the donor compartment, n is the sampling point number.

Kinetic Modelling of In Vitro Drug Release Data

The cumulative release data obtained from drug release studies were evaluated using various mathematical models to estimate the possible OLO release mechanism. Model fitting was evaluated using the coefficient of determination (R^2). The models evaluated were zero-order, first-order, Higuchi, and Hixson-Crowell to analyze the release mechanism. Korsmeyer-Peppas and Weibull models were applied to evaluate the release mechanism. The mathematical equations employed are summarized in Table 6 [48, 49]. The Korsmeyer-Peppas model was evaluated up to 60%, while zero-order, first-order, Higuchi, Hixson-Crowell, and Weibull models were evaluated up to a plateau.

Table 6. Kinetic Modeling of In Vitro Drug Release Data.

Model	Equation ^a	Plot
Zero-order	$Q_t = K_0 t + Q_0$	Cumulative release vs time
First-order	$Q_t = Q_0 (1 - e^{-K_1 t})$	In cumulative remaining vs time
Higuchi	$Q_t = K_H t^{0.5}$	Cumulative release vs square root of time
Hixson-Crowell	$M_0^{1/3} - M_t^{1/3} = K_{HX} t$	Cube root of % remaining vs time
Korsmeyer-Peppas	$\frac{M_t}{M_\infty} = K_{KP} t^n$	log cumulative release vs log time
Weibull	$\frac{M_t}{M_\infty} = 1 - e^{-\alpha t^\beta}$	In [-ln (1-fraction released)] vs In time

^a Q_t represents the amount of drug released at time t , Q_0 is the initial amount of drug present, M_t is the amount of drug remaining at time t , M_∞ is the total amount of drug released at infinite time, n is the release exponent, α is the Weibull time scale parameter for drug release, β is the release curve shape parameter, K_0 , K_1 , K_H , K_{HX} , and K_{KP} are zero-order, first-order, Higuchi, Hixson-Crowell, and Korsmeyer-Peppas release constant, respectively.

Physical Stability Study

The short-term stability of opt-F was evaluated under two different storage conditions. Samples

were stored in sealed glass vials at ambient temperature ($25 \pm 5^\circ\text{C}$), and refrigeration ($4 \pm 2^\circ\text{C}$) for a period of 3 months. At predetermined

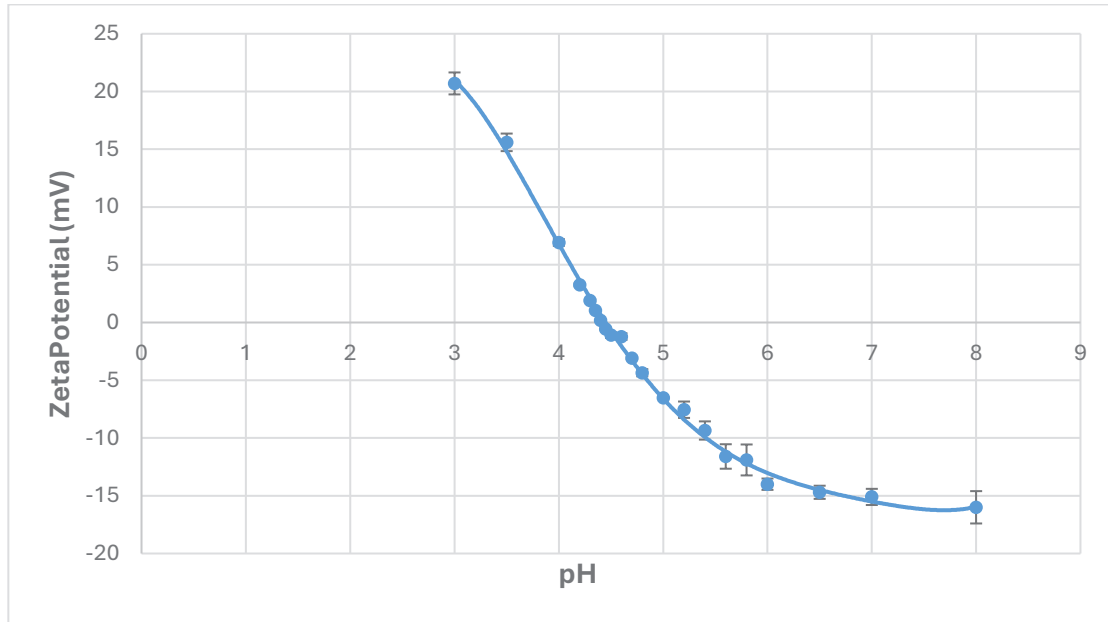


Fig. 1. Zetapotential measurement of BSA at different pH values (error bars represent mean $\pm$ SD (n=2)).

Table 7. QTPP for nanogel and its CQAs.

QTPP Element	Target	CQA	Justification
Dosage form	Nanogel	No	Ocular delivery improvement
Route of administration	Topical, ophthalmic	No	Easily administered, local delivery
Particle size	<100nm	Yes	Stability and Uniformity
PDI	<0.3	Yes	Enhance physical stability, prevent aggregation, and provide uniform drug release kinetics
ZP	[30-50 mV]	Yes	Physical stability
Shape	Spherical, deformable	Yes	Shape affects mucoadhesion and ocular retention
Clarity	Clear, colloidal.	Yes	Product stability, ease of detection of drug product instabilities
Aggregation	No visible sign	Yes	Product performance
EE	Maximum achievable	Yes	Encapsulation efficiency: maximized and optimized
Drug release	Controlled.	Yes	Controlled release with minimal initial burst
pH	6.6 – 7.8 optimal 4 – 8 tolerable	Yes	Optimal range improves patient compliance by reducing stinging, burning, and increased tear production. Tolerable range causes initial discomfort without causing chemical injury to ensure ocular compatibility with tear fluid.
Sterility	Sterile	Yes	Mandatory for ophthalmic product, prevent microbial contamination

intervals (0, 2 weeks, 1 month, and 3 months) samples were withdrawn and analyzed for CQAs (size, PDI, ZP). EE was evaluated at the 3-month time point to assess drug retention over the storage period. All tests were performed in triplicate.

Fourier Transform Infrared (FTIR) Study

An FTIR spectrophotometer was used to investigate the chemical structure of the drug

and polymer and their interactions within nanogels. Spectra were acquired using a Shimadzu IRAffinity-1 FTIR Spectrophotometer. Powdered samples and the nanogel formulation were mixed with KBr, ground to a fine powder, and pressed into thin, transparent disks. Prior to each sample measurement, a background scan was recorded using a KBr-only disk. Spectra were obtained over a wavenumber range of 4000-400 cm^{-1} .

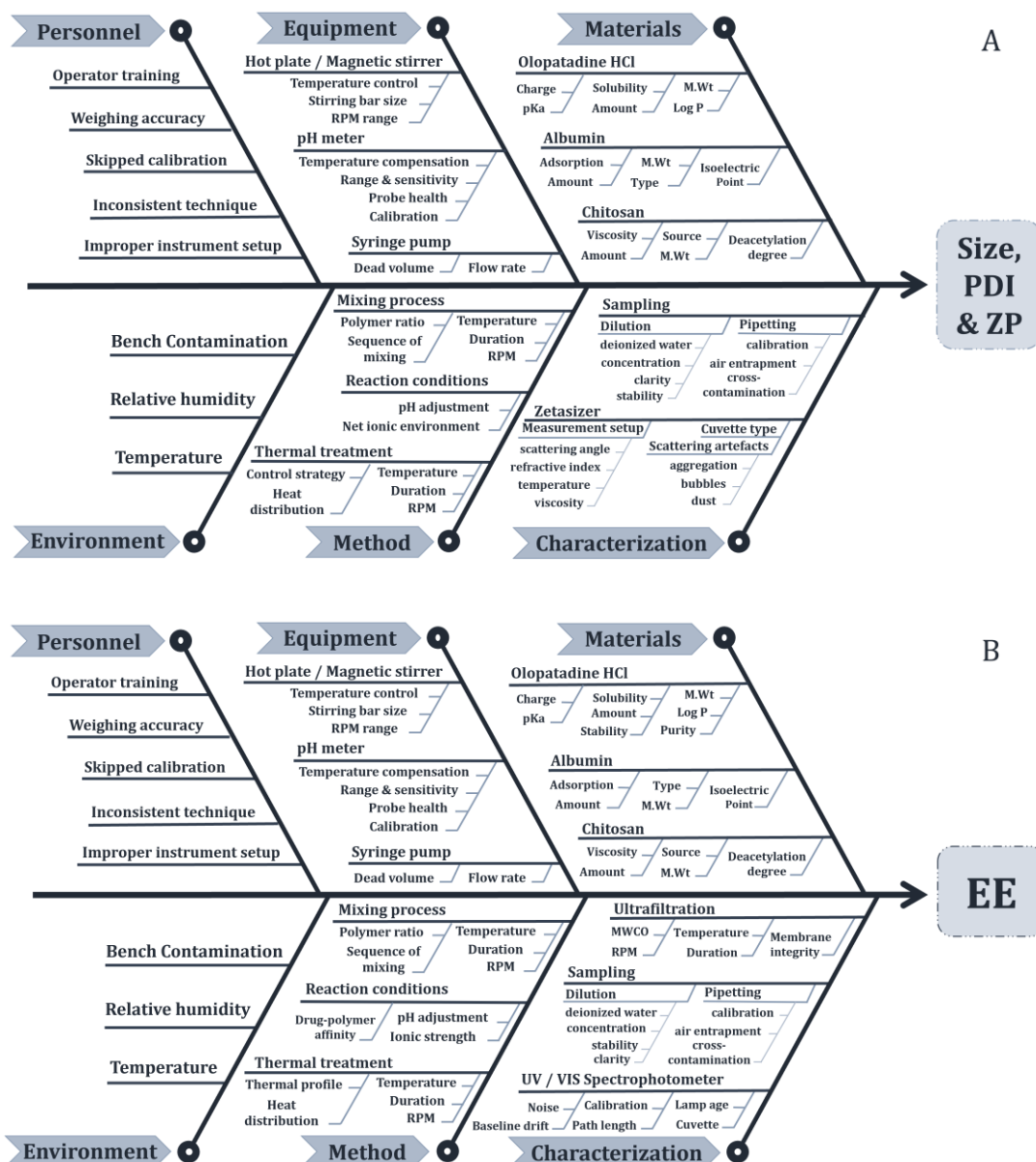


Fig. 2. Risk assessment via Ishikawa diagram defining the possible CMAs and CPPs in the preparation of OLO-loaded CHS/BSA nanogels for the CQAs, (A) particle size, PDI, and ZP, and (B) EE.

Powder X-ray Diffraction (PXRD)

Pure Olopatadine, polymers, a physical mixture, and a freeze-dried nanogel were subjected to PXRD analysis. The crystalline structure was analyzed using an X-ray diffractometer (X'Pert Pro, Malvern Panalytical) equipped with a Cu K α radiation source ($\lambda=1.5406$ Å) operating at 40 kV and 30 mA. Finely powdered samples were packed onto a standard sample holder to obtain a flat surface. Diffraction patterns were recorded over a 2θ range of 2° – 60° with a step size of 0.02° and a scan speed of $4.8^\circ/\text{min}$ at 25°C . Crystallinity and phase identification were determined by comparing the

diffraction patterns of the nanogel formulation with the pure drug.

Statistical Analysis

Data processing, basic descriptive statistics, and one-way ANOVA statistical analysis was performed using Microsoft Excel 365. The experiment was designed using Design-Expert 13.

RESULTS AND DISCUSSION**Determination of *pI* of BSA**

The zeta potential of BSA decreased from $+20.7 \pm 0.95$ mV at pH 3.0 to -16.0 ± 1.4 mV at pH 8.0,

Table 8. PHA for material attributes of OLO-loaded BSA/CHS nanogel.

CQAs	Material attributes														
	OLO					CHS					BSA			Water	
	Concentration	Solubility	pKa	Stability	Log P	Concentration	Source	MW	DDA	Viscosity	Concentration	Type	Isoelectric point	pH	Ionic strength
Size	M	M	L	L	M	H	L	H	H	M	H	H	H	M	M
PDI	L	L	L	L	L	M	L	M	M	M	M	H	M	L	L
ZP	L	L	M	L	L	M	L	H	H	L	M	H	H	M	H
EE	H	H	H	M	H	H	L	M	H	L	H	H	M	M	M

Table 9. PHA for process parameters of OLO-loaded BSA/CHS nanogel.

Table 1: PPI for process parameters of OLO loaded BSA:CHS nanogel.																				
CQAs	Process parameters																			
	Formulation					Mixing					Thermal treatment					Ultrafiltration				
	BSA: CHS	Polymer: OLO	pH	Ionic strength	Flow rate	RPM	Duration	Temperature	Bead size	Bead shape	Vessel size	Heat distribution	Temperature	Duration	RPM	Tube size	MWCO	Duration	RPM	Temperature
Size	L	M	H	M	M	M	M	M	M	M	M	H	H	H	H	L	L	L	L	L
PDI	H	L	H	L	M	H	H	M	M	M	M	H	M	H	H	L	L	L	L	L
ZP	H	L	H	H	M	M	L	M	L	L	L	M	M	M	L	L	L	L	L	L
EE	H	H	H	M	M	M	M	M	L	L	L	M	M	M	M	L	H	M	M	M

crossing zero between pH 4.4 ($+0.18 \pm 0.09$ mV) and pH 4.45 (-0.57 ± 0.14 mV) as shown in Fig. 1. Therefore, the isoelectric point was estimated

to be approximately pH 4.4-4.45 based on zeta potential reversal (charge neutrality point). The working pH range for nanogel preparation was set

Table 10. FMEA for material attributes of OLO-loaded BSA/CHS nanogel.

Unit	Failure mode	RPN	Priority	Corrective action
BSA	Type	16	High	Select a specific type and maintain throughout this work
	pI	48	High	Select a specific type and maintain it throughout this work
	Amount	40	Medium	Constant amount (0.5%w/w).
	Source	12	Low	Routine monitoring
CHS	MW	20	High	Tested via DoE screening analysis.
	Viscosity	4	Low	Routine monitoring
	DDA	20	High	DDA >90% maintained throughout this work.
	Amount	40	Medium	Constant amount (0.5%w/w).
	Salt	10	High	HCl salt selected
	pK _a	60	High	Batch check
OLO	Purity	20	High	Batch check
	Solubility	60	High	Use concentrations reasonably below saturation
	Stability	45	Medium	Batch check
	Amount	60	High	SOP for weighing, duplicate weighing, and content uniformity assay
Water	Log P	60	High	Batch check.
	Ionic strength	40	High	Check new batches periodically.

Table 11. FMEA for process parameters of OLO-loaded BSA/CHS nanogel.

Unit	Failure mode	RPN	Priority	Corrective action
Formulation	BSA: CHS	100	High	Test via DoE screening analysis
	Polymer: OLO	60	High	Test via DoE screening analysis
	pH	50	High	Test via DoE screening analysis
	Ionic strength	48	High	Use deionized water
Mixing	Flow rate	12	High	Test via DoE screening analysis
	RPM	8	High	Test via DoE screening analysis
	Duration	16	High	Test via DoE screening analysis
Thermal treatment	Heat distribution	45	High	Water bath with agitation
	Temperature	40	High	Thermostated water bath and probe check
	Duration	30	High	Test via DoE screening analysis
Ultrafiltration	RPM	6	Low	Routine monitoring
	MWCO	8	High	Controlled, 10 kDa MWCO

around this value.

Quality by Design

QTPP and Selection of CQAs

For nanogels intended to be administered as a topical, ophthalmic preparation, QTPP and CQAs were summarized in Table 7.

Risk Assessment

Risk identification

Risk identification using Ishikawa diagram is shown in Fig. 2.

Preliminary Hazard Analysis (PHA)

The outcomes of PHA are shown in Tables 8 and 9.

FMEA and RPN

Tables 10 and 11 outline FMEA and RPN for material attributes and process parameters for nanogel preparation.

Translation of Risk Assessment into Experimental Strategy

Following the comprehensive risk assessment, Fig. 3 consolidates the identified CMAs, CPPs, and CQAs into a QbD framework. This framework establishes a clear conceptual bridge between the qualitative risk evaluation and the subsequent

quantitative DoE, guiding factor selection and prioritization. The evident coupled influence of multiple interacting variables renders the one-factor-at-a-time approach inadequate to capture synergistic and antagonistic effects within the system and necessitates the use of a more sophisticated experimental approach.

Design of Experiment

Selection of Factor Levels

Working ranges of independent variables are listed in Table 12.

Screening Design

Data from the screening design (Table 13) were analyzed using linear regression, and no Box-Cox transformation was required for any response. No model was aliased or overfitted. All models met the adequacy criteria (Adeq. Precision >4, non-significant lack-of-fit, and Pred R^2 within 0.2 of Adj R^2) (Table 14). Some models had a low predicted R^2 , indicating that their predictive capability should be interpreted with caution despite acceptable model fit statistics.

A. Particle Size

The effects of the independent variable on PDI are illustrated in Fig. 4A. The most significant factor affecting the particle size was the pH with a high

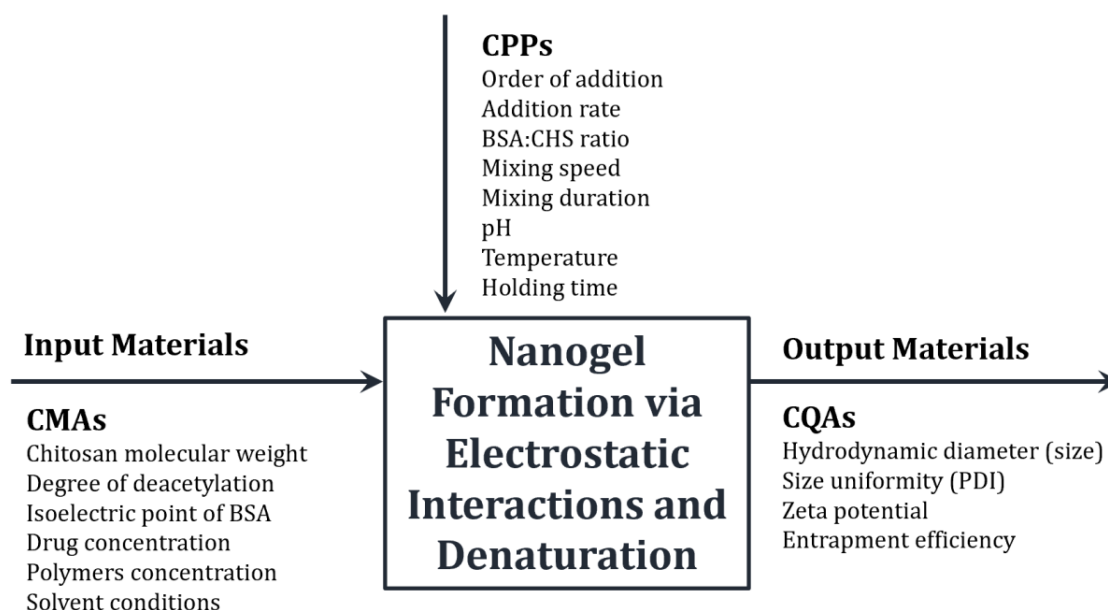


Fig. 3. Block Flow Diagram for nanogel formulation process and identification of the possible CMAs, CPPs, and CQAs.

positive coefficient estimate indicating an increase in particle size as the pH increased. The significant quadratic term suggests a nonlinear response; the curve of pH vs. particle size trend becomes steeper at the higher pH. A strong interaction between the pH and BSA: CHS ratio was also observed. The magnitude and complexity of the pH effect suggest a need for further optimization.

The second most important factor was holding time which had a strong positive influence. That was consistent with the hypothesis that longer heat exposure leads to increased BSA aggregation [57]. To minimize particle size, a 20 min holding time should be maintained in subsequent experiments.

Particularly, the particle size was strongly influenced by the BSA:CHS ratio, with the estimated coefficient being negative, suggesting that the higher the ratio the smaller the particle size. No quadratic term was found; and there were

two significant interaction terms, meaning that this factor affects system behavior in a complicated way.

Another important factor was CHS MW, with a positive coefficient estimate, which indicates that an increase in molecular weight increases the particle size. An interaction effect was noted between the stirring duration and the other processing conditions, implying that the effects of the processing conditions on particle formation are interdependent.

The addition rate was highly significant, with a negative coefficient estimate. The rapid addition of BSA caused more nucleation sites in the formation of the polyelectrolyte complex, which would lead to smaller particles because of the limited further growth. This is in line with literature reports that indicate that multiple nucleation events are favored in early stages of complexation [58]. The addition rate is recommended to be higher.

Table 12. High-risk independent variable and selected levels for screening.

Factor	Level (-1,+1)	Justification
CHS, BSA concentration	Fixed at 0.5% w/w	Lower concentration (0.1%) failed to form nanogels, while higher concentration (1%) caused turbidity and precipitation; fixing concentration prevented dominance and confounding in the screening design [19, 21, 50, 51].
CHS MW	Low, Medium.	Included due to conflicting literature reports regarding its influence on nanogel structure, stability, and physicochemical properties [50, 52].
BSA:CHS ratio	3:1 – 7:1	Ratio governs electrostatic interactions and protein aggregation; the selected range was aimed at minimizing heterogeneity and PDI [53, 54].
OLO concentration	0.1 – 0.2 g/dL	Higher concentrations caused instability by supersaturation and drug crystallization, while lower concentrations resulted in insufficient loading (<1%).
BSA addition rate	0.5 - 2 mL/min	Included due to its effect on interaction kinetics and nanogel properties reported in analogous self-assembly systems [6].
Stirring rate	500 - 1500 rpm	Influences energy input and dispersion; higher speeds are reported to increase particle size and PDI [55].
Stirring duration	30 – 90 min	Selected to capture effects of interaction kinetics and potential aggregation during prolonged mixing [55].
pH	4.4 – 4.5	Ensures CHS solubility and electrostatic interaction with BSA near its pI, while avoiding precipitation [19, 56].
Holding temperature	Fixed at 80 °C	Required for BSA denaturation and nanogel formation [19, 20, 22, 56].
Holding time	20 – 60 min	Selected to study the extent of protein denaturation and aggregation influencing nanogel size and PDI [19, 22].

OLO concentration also had a positive effect, albeit smaller, and had interactions with BSA: CHS ratio and addition rate, suggesting that this concentration had a complex effect on the particle formation process.

The stirring time had a significant positive effect, but small, with shorter stirring time producing a smaller particle size. The effect of stirring rate was

not significant.

B. Polydispersity Index

The effects of the independent variable on PDI are illustrated in Fig. 4B. The most significant factor is the CHS MW, with a positive coefficient estimate. The low MW CHS yields more uniform particles. The interaction term with OLO concentration

Table 13. Design matrix for the screening design with results.

No	Independent Variables ^a								Results (Mean $\pm$ SD)*			
	A	B	C	D	E	F	G	H	Size	PDI	ZP	EE
1	3	0.1	2	1500	60	4.4	20	M	72.5 $\pm$ 1.3	0.38 $\pm$ 0.01	33.4 $\pm$ 1.9	29.7 $\pm$ 2.2
2	3	0.15	0.5	500	90	4.4	60	M	66.3 $\pm$ 1.4	0.37 $\pm$ 0.01	41.2 $\pm$ 1.5	18.5 $\pm$ 1.3
3 ^b	3	0.2	1.25	500	30	4.5	20	M	92.9 $\pm$ 1.9	0.46 $\pm$ 0.09	36.3 $\pm$ 2.4	23.8 $\pm$ 1.2
4 ^c	5	0.15	1.25	1000	60	4.45	40	M	78.5 $\pm$ 0.7	0.37 $\pm$ 0.01	43.6 $\pm$ 1.6	24.4 $\pm$ 1.2
5	5	0.2	0.5	500	90	4.5	20	M	80.94 $\pm$ 3.27	0.47 $\pm$ 0.02	46.7 $\pm$ 2.9	22.5 $\pm$ 1.5
6	5	0.2	2	1500	90	4.5	60	M	104.5 $\pm$ 4.7	0.47 $\pm$ 0.07	38.5 $\pm$ 2.1	23.6 $\pm$ 1
7	7	0.1	0.5	1500	60	4.4	20	M	50.9 $\pm$ 1.4	0.37 $\pm$ 0.02	36.2 $\pm$ 1.9	26.3 $\pm$ 2
8	7	0.1	2	500	30	4.45	60	M	70.9 $\pm$ 1.9	0.32 $\pm$ 0.08	36.9 $\pm$ 3	29.6 $\pm$ 1.4
9 ^b	7	0.1	0.5	1000	90	4.5	20	M	73.4 $\pm$ 1.3	0.38 $\pm$ 0.02	36.2 $\pm$ 1.7	23.8 $\pm$ 1.8
10	7	0.2	0.5	1500	30	4.4	40	M	75 $\pm$ 1.8	0.46 $\pm$ 0.07	33 $\pm$ 1.6	21.4 $\pm$ 2.4
11	7	0.2	2	500	90	4.4	20	M	61.3 $\pm$ 0.9	0.35 $\pm$ 0.02	42.9 $\pm$ 1.1	23.4 $\pm$ 1.3
12	3	0.1	2	500	90	4.5	40	L	98.5 $\pm$ 0.8	0.39 $\pm$ 0.01	35.2 $\pm$ 0.7	25.1 $\pm$ 2
13	3	0.1	0.5	1500	30	4.5	60	L	107.9 $\pm$ 1.4	0.39 $\pm$ 0.01	30.7 $\pm$ 0.4	26.3 $\pm$ 1.2
14	3	0.2	0.5	1500	90	4.45	20	L	68.5 $\pm$ 3.1	0.34 $\pm$ 0.03	39.9 $\pm$ 2.5	20 $\pm$ 2.4
15	3	0.2	2	1000	30	4.4	60	L	75.6 $\pm$ 1.1	0.36 $\pm$ 0.03	41 $\pm$ 1.2	17.6 $\pm$ 1.6
16	5	0.1	0.5	500	30	4.4	20	L	50.2 $\pm$ 1.3	0.29 $\pm$ 0.02	30.1 $\pm$ 3.7	24 $\pm$ 0.5
17 ^c	5	0.15	1.25	1000	60	4.45	40	L	71.2 $\pm$ 1.9	0.36 $\pm$ 0.01	40.3 $\pm$ 6.6	25.2 $\pm$ 1.8
18	7	0.1	1.25	1500	90	4.4	60	L	60.4 $\pm$ 0.9	0.36 $\pm$ 0.04	40.4 $\pm$ 1.5	11.7 $\pm$ 1.9
19	7	0.15	2	1500	30	4.5	20	L	56.8 $\pm$ 0.6	0.28 $\pm$ 0.04	35.3 $\pm$ 0.2	20 $\pm$ 0.9
20	7	0.2	2	500	30	4.4	40	L	55.9 $\pm$ 1	0.29 $\pm$ 0.02	40.5 $\pm$ 1.5	18.2 $\pm$ 0.3
21	7	0.2	0.5	500	60	4.5	60	L	96.4 $\pm$ 0.9	0.37 $\pm$ 0.04	34.2 $\pm$ 2.7	20.2 $\pm$ 1.4

*All measurements were performed in triplicate and are reported as mean $\pm$ standard deviation

^a Factor codes: A, BSA:CHS ratio; B, OLO concentration (g/dL); C, addition rate (mL/min); D, stirring rate (rpm); E, stirring duration (min); F, pH; G, holding time (min); and H, CHS MW.

^b lack of fit points, prepared in duplicates.

^c center points, prepared in triplicate.

suggests a more complex response. This factor is a good candidate for optimization.

OLO concentration was also significant, with higher concentrations giving higher PDI value. Although pH did not reach statistical significance, its effect approached the significance threshold.

The addition rate and BSA:CHS ratio were not statistically significant as individual factors, but their interaction effect is significant. Therefore, its influence depends on formulation components. Stirring rate, stirring duration, and holding time did not significantly affect PDI.

C. Zeta potential

The effects of the independent variable on ZP are illustrated in Fig. 4C. The ZP model showed limited reliability, as evidenced by the relatively low R^2 value. However, all formulations were stable and within the acceptable CQA range. The model's shortcomings may reflect a combination of measurement variability and comparatively modest formulation-dependent changes in ZP, resulting in low signal-to-noise ratio. This behavior may be because the Zeta sizer measures ZP based on the particles' electrophoretic mobility, where the calculation assumes a monodisperse sample with approximately uniform shape, size, and surface charge [59]. This is not true for most samples, resulting in distorted measurements.

D. Entrapment Efficiency

The effects of the independent variable on EE are illustrated in Fig. 4D. CHS MW, which was significant in multiple response models with interaction and a significant positive effect on EE, was chosen for further optimization studies.

The EE was significantly negatively affected by OLO concentration, showing that as the drug concentration increased, the EE decreased. However, DL, a direct indicator of the amount of drug in the nanogel, showed interesting results. The OLO (0.1%) nanogels had lower DL than nanogels with higher concentrations of OLO (0.2%). This indicates that increasing drug concentration increased the absolute amount of drug incorporated into the nanogel matrix. The model results and practical considerations were used to fix OLO concentration at 0.2% for the optimization study.

pH exhibited significant linear and quadratic effects, a significant interaction term with holding time. Therefore, this was chosen as a parameter to be further optimized.

For EE, the effect of holding time was not significant alone, but resulted in significant interactions with pH. The net effect showed that longer holding times resulted in less entrapment and hence the lowest holding time was chosen for further experiments.

Table 14. Summary of regression model adequacy statistics for screening design.

Parameter	Outcome			
	Size	PDI	ZP	EE
Model	Significant	Significant	Significant	Significant
p-value*	< 0.0001	< 0.0001	0.0198	< 0.0001
Lack-of-fit	Not sign.	Not sign.	Not sign.	Not sign.
p-value*	0.4579	0.8178	0.1546	0.1661
R^2	0.9914	0.7602	0.5432	0.7776
Adjusted R^2	0.9841	0.6718	0.3749	0.6957
Predicted R^2	0.9726	0.5026	0.2707	0.5214
Adeq. Precision	41.9433	11.0566	7.0119	10.9423
Mean	75.4	0.3692	37.86	23.12
SD	1.95	0.0297	3.45	2.26
C.V. %	2.59	8.04	9.11	9.78

*Significance assessed at $\alpha=0.05$.

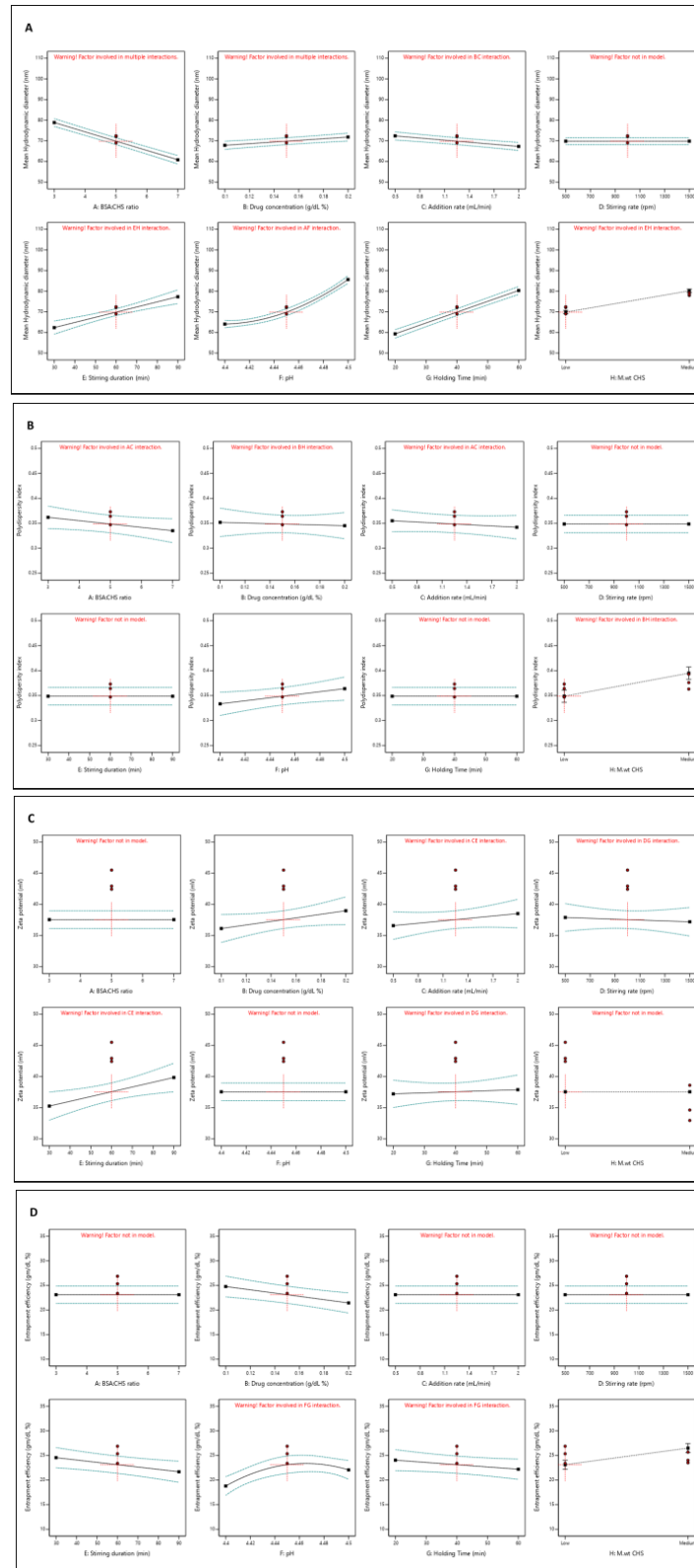


Fig. 4. Main Effect Plots for all Factors Affecting (A) Particle Size, (B) PDI, (C) ZP, and (D) EE.

The stirring duration had a negative effect on EE; therefore, the shortest stirring time was selected and was kept constant in the optimization design. However, polymer ratio, addition rate and stirring rate were not found to significantly affect EE in the experimental range studied.

Optimization

First Approach to Optimization with Screening Results

Table 15 outlines the levels of the factors used to create a follow-up DoE, selected based on screening results from the Design Expert

13 optimization tool. The formulation with the highest desirability, 0.93, for achieving minimum particle size and PDI while maximizing EE was selected. BSA: CHS ratio, pH, and CHS MW were selected for follow-up optimization.

Central Composite Design

Central composite design (CCD) was chosen for its ability to provide equal prediction variance across the design space, which is essential here given the likelihood that the optimum preparation condition lies at the periphery of the factor levels rather than at the center [60]. Table 16 presents

Table 15. Selection of factor levels for optimization based on screening results.

Parameters	Levels suggested by screening	Optimization Level(s)
BSA: CHS ratio	7:1	3:1 – 7:1
OLO concentration	0.1 mg/dL	0.2 mg/dL
Addition rate	2 mL/min	2 mL/min
Stirring rate	1500 rpm	1500 rpm
Stirring duration	30 min	30 min
pH	4.4	4.4 - 4.5
Holding time	20 min	20 min
CHS MW	Medium	Low - Medium

Table 16. Design Matrix for CCD with Results ^a.

No.	Point Type	Input Variables			Results Mean $\pm$ SD *			
		Ratio	pH	CHS MW	Particle size	PDI	ZP	EE
1	Axial	2.17	4.45	L	70.48 $\pm$ 1.24	0.346 $\pm$ 0.004	37.3 $\pm$ 0.14	22.17 $\pm$ 0.77
2	Factorial	3	4.4	L	55.98 $\pm$ 4.17	0.354 $\pm$ 0.004	40.42 $\pm$ 0.4	21.56 $\pm$ 1.28
3	Factorial	3	4.5	L	71.43 $\pm$ 4.68	0.351 $\pm$ 0.05	38.7 $\pm$ 3.06	18.54 $\pm$ 2.14
4	Axial	5	4.38	L	51.14 $\pm$ 1.26	0.281 $\pm$ 0.002	34.38 $\pm$ 1.81	20.66 $\pm$ 1.41
5	Center	5	4.45	L	57.3 $\pm$ 1.45	0.276 $\pm$ 0.027	36.99 $\pm$ 1.1	21.38 $\pm$ 3.16
6	Axial	5	4.52	L	65.47 $\pm$ 5.06	0.273 $\pm$ 0.016	32.43 $\pm$ 0.19	22.55 $\pm$ 0.23
7	Factorial	7	4.4	L	51.45 $\pm$ 0.49	0.328 $\pm$ 0.005	33.48 $\pm$ 1.15	21.66 $\pm$ 0.22
8	Factorial	7	4.5	L	71.44 $\pm$ 1.92	0.263 $\pm$ 0.006	32.22 $\pm$ 1.3	22.47 $\pm$ 0.52
9	Axial	7.83	4.45	L	51.94 $\pm$ 0.27	0.329 $\pm$ 0.012	30.87 $\pm$ 1.98	20.53 $\pm$ 4.78
10	Axial	2.17	4.45	M	73.11 $\pm$ 0.73	0.391 $\pm$ 0.01	40.08 $\pm$ 1.44	22.53 $\pm$ 0.6
11	Factorial	3	4.4	M	59.04 $\pm$ 0.32	0.362 $\pm$ 0.005	41.12 $\pm$ 0.87	25.06 $\pm$ 3.68
12	Factorial	3	4.5	M	78.13 $\pm$ 0.68	0.421 $\pm$ 0.002	40.57 $\pm$ 0.05	24.53 $\pm$ 1.39
13	Axial	5	4.38	M	44 $\pm$ 2.42	0.355 $\pm$ 0.000	40.87 $\pm$ 0.61	19.25 $\pm$ 0.45
14	Center	5	4.45	M	61.14 $\pm$ 2.1	0.331 $\pm$ 0.034	39.64 $\pm$ 0.68	24.21 $\pm$ 2.24
15	Axial	5	4.52	M	74.24 $\pm$ 1.1	0.387 $\pm$ 0.019	28.25 $\pm$ 0.87	27.54 $\pm$ 2.57
16	Factorial	7	4.4	M	46.8 $\pm$ 2.33	0.322 $\pm$ 0.011	37.47 $\pm$ 2.92	26.08 $\pm$ 0.84
17	Factorial	7	4.5	M	76.22 $\pm$ 1.59	0.385 $\pm$ 0.019	33.62 $\pm$ 2.14	21.02 $\pm$ 0.52
18	Axial	7.83	4.45	M	46.51 $\pm$ 1.19	0.263 $\pm$ 0.003	32.65 $\pm$ 0.02	26.65 $\pm$ 0.54

*All measurements were performed in triplicate.

the design matrix and the CCD results. A quartic polynomial regression model was fitted, allowing higher-order terms to capture complex, nonlinear relationships between formulation variables and the responses. All resulting models are shown in Table 17. Unless otherwise specified below, all models met the adequacy criteria (Adequate Precision >4, non-significant lack of fit, and Pred R² within 0.2 of Adj R²). Fig. 5 shows the response surface plots for particle size, PDI, ZP, and EE.

Modeled Responses Equations

Particle Size = $59.22 - 7.96 A + 7.96 B + 1.53 C + 1.86 AB - 1.31 AC + 2.23 BC + 0.75 A^2 - 0.15 B^2 + 2.54 A^2B - 0.84 A^2C + 5.64 AB^2 + 3.8 A^2B^2$

PDI = $0.306 - 0.0201 A + 0.0071 B + 0.0277 C - 0.0055 AB - 0.0221 AC + 0.004 BC + 0.008 A^2 + 0.0043 B^2 + 0.0103 ABC + 0.0017 A^2B - 0.0179 A^2C - 0.0029 AB^2 + 0.0104 B^2C + 0.0439 A^2B^2 + 0.0215 A^2BC + 0.0275 AB^2C$

ZP = $38.31 - 2.45 A - 2.6 B + C - 0.3561 AB + 0.0874 AC - 1.9 BC - 1.61 A^2 - 2.28 B^2 - 0.4685 ABC + 1.68 A^2B + 0.043 A^2C - 0.5523 AB^2 + 2.92 A^2B^2 + 1.73 A^2BC$

EE = $22.85 + 0.32 A - 3.89 B + 1.41 C - 0.09 AB + 0.10 AC + 0.35 BC - 0.19 B^2 - 1.04 ABC + 2.91 B^3$

Particle Size

Several previous studies have established that BSA-CHS interactions are spontaneous and driven primarily by hydrophobic interactions

[61]. The driving force for CHS-BSA interaction is pH-dependent. At pH values where BSA is at or below its pI, the driving forces of interaction are hydrophobic association and H-bonding. By raising the pH slightly, BSA becomes negatively charged, and the driving force is a combination of hydrophobic and electrostatic interactions. The higher the pH, the more influential the electrostatic interactions become, leading to the formation of larger nanogels. Up to a point where the particles cross into micro-sized, at which turbidity and eventually precipitation occur. For CHS, as pH increases, a fraction of its NH₃⁺ groups deprotonate, weakening electrostatic repulsion and enhancing hydrophobic and hydrogen-bond driven associations both intramolecularly and supramolecular with other CHS molecules and BSA. This promotes the formation of larger nanogels, particularly for medium-MW CHS. In contrast, low-MW CHS remains more soluble and less prone to pH-induced aggregation, showing weaker intramolecular collapse and smaller supramolecular structure [62].

For low-MW CHS, the pH shows a steep slope at higher BSA ratios. In the pH range of 4.4-4.44, where the pI of BSA was previously established, the particles are smaller, and the increase in size with pH rise is less drastic when the BSA:CHS ratio is high. Here, the electrostatic interactions between CHS and BSA are minimal, and the driving force of nanogel formation is hydrophobic interactions and

Table 17. Summary of Regression Model Adequacy Statistics for CCD.

Parameter	Outcome			
	Size	PDI	ZP	EE
Model	Significant	Significant	Significant	Significant
<i>p-value*</i>	< 0.0001	< 0.0001	< 0.0001	0.0025
Lack-of-fit	Not sign.	Not sign.	Not sign.	Significant.
<i>p-value*</i>	0.2013	0.2568	0.6061	0.0239
R ²	0.9638	0.9168	0.9159	0.5056
Adjusted R ²	0.9493	0.8656	0.8739	0.3707
Predicted R ²	0.9203	0.7552	0.7681	0.1436
Adeq Precision	25.2775	13.5239	15.8227	7.6053
Mean	61.03	0.3309	36.56	22.7
SD	2.32	0.0175	1.34	2.31
C.V. %	3.8	5.29	3.66	10.18

*Significance assessed at α=0.05.

H-bonding, which is why there is little change in nanogel size at this pH range. However, once the pH is at or above 4.45, a sharper increase in size is observed, indicating a greater contribution of electrostatic interactions between BSA and CHS due to a more pronounced negative charge on BSA in this range. The lower BSA amount is less affected by the change in pH, as the relationship appears nearly linear. The fewer BSA molecules present in the solution, the less pronounced the pH changes have, as the net negative charge on BSA is proportionately less impactful. Larger particles are probably produced here, due to a greater impact of CHS's intermolecular bonding.

For medium-MW CHS, the same trends are observed in pH interactions with the ratio, but with a higher magnitude. Both smaller and larger particles are produced here than with low-MW CHS.

The longer chain of the medium-MW CHS undergoes a greater net loss of charge per chain due to NH_3^+ deprotonation, thereby enabling pronounced conformational rearrangement as pH rises [63]. The impact of this effect is higher in the case of higher MW CHS due to its longer chains, allowing interchain interactions and conformational collapse [64].

Medium-MW CHS (95% DDA) has more amine groups than low-MW CHS (90% DDA), giving it a

higher charge density at low pH. As pH increases, both lose charge due to deprotonation, but because medium-MW CHS starts with more ionizable sites, a given pH shift produces a larger absolute change in total positive charge, despite the underlying per-site protonation curve being similar [65].

Polydispersity Index

CHS MW was the most significant factor that positively influenced PDI, that is, as MW increased the PDI increased. This may be a result of more entanglements of the chains and less mixing efficiency at higher molecular weights, resulting in less uniform particle formation. This trend has been observed in earlier studies, where low-MW CHS produced more homogeneous dispersions of nanoparticles [66, 67].

A significant interaction between CHS MW and the BSA:CHS ratio was found, indicating that the effect of polymer ratio on PDI was dependent upon MW. The lower MW CHS systems had more uniform particle size distribution as a function of formulation ratio, while higher MW CHS systems were more sensitive to changes in the formulation ratio.

Zeta potential

The ZP model exhibited clear, expected

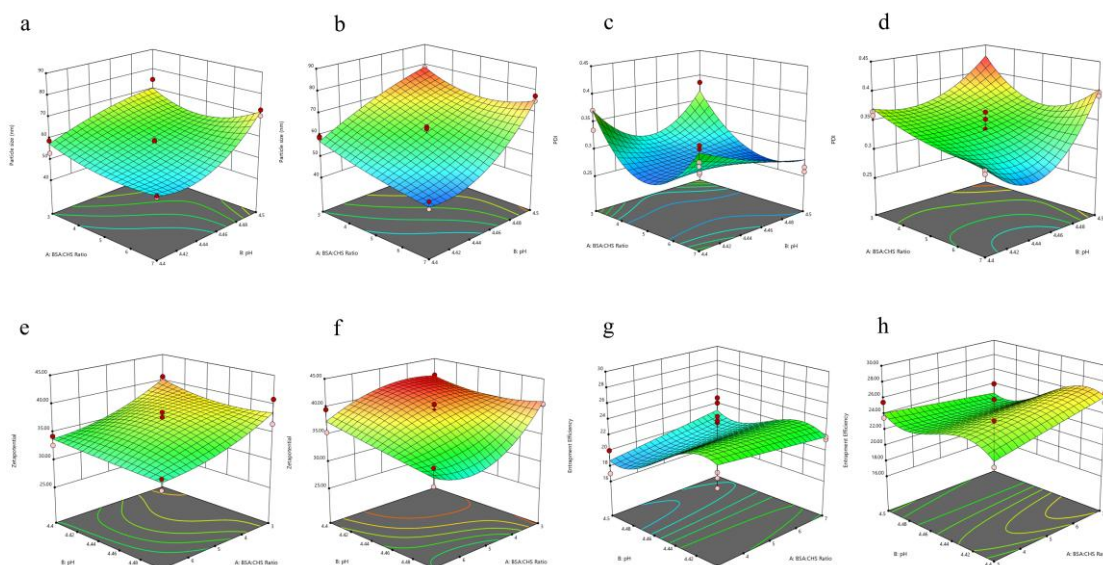


Fig. 5. Surface plots showing the effects of BSA:CHS ratio and pH on (a, b) particle size, (c, d) PDI, (e, f) zeta potential, and (g, h) entrapment efficiency for low- and high-MW CHS nanogel formulations, respectively.

behavior, where pH was the most significant factor, negatively affecting ZP. Increasing pH decreases the nanogel's positive surface charge density, likely due to the pH-dependent ionization of both CHS and BSA. The degree of protonation of the amine groups on CHS decreases with increasing pH, reducing its positive charge density. Simultaneously, BSA becomes increasingly negatively charged as pH increases. The combined effect produced an overall decrease in the ZP.

The BSA:CHS ratio negatively affected ZP, with the surface charge density decreasing as the proportion of BSA increased. This effect is likely due to a relative decrease in CHS content within the formulation with increasing BSA proportion, thereby lowering the net contribution of positively charged amine groups.

The increase in CHS MW resulted in a significant increase in ZP. As previously established in the particle size model, higher-MW CHS contains a greater number of charged functional groups per polymer chain, producing an overall increase in positive surface charge.

Furthermore, a prominent interaction in the model is between CHS MW and pH. The medium-MW CHS exhibits a stronger pH-dependent response compared to low-MW CHS. This behavior is consistent with its higher DDA, which increases the sensitivity of CHS protonation to changes in

pH, contributing to a more pronounced variation in ZP across the studied pH range.

D. Entrapment Efficiency

The reduced cubic model for EE demonstrates weak explanatory power, indicating that only half of the observed variability can be attributed to the investigated formulation factors. The low predicted R^2 value and significant lack of fit suggest the model is misleading in both its interpretation of factor effects and its forward predictions.

CHS MW was the only factor for which a consistent and reliable effect on EE could be deduced from the model. Medium MW CHS resulted in higher EE compared to low MW CHS. This behavior can be attributed to differences in polymer chain length and network architecture. The shorter chains of low-MW CHS provide reduced steric hindrance, facilitating drug diffusion out of the crosslinked polymeric network and resulting in greater drug loss. In contrast, medium MW CHS forms a denser network with higher viscosity, slowing drug diffusion. Additionally, the higher charge density of medium MW CHS enhances electrostatic interactions with BSA, increasing the crosslinking density within the network and thereby improving drug retention in the nanogel matrix.

Low to moderate EE is not uncommon in

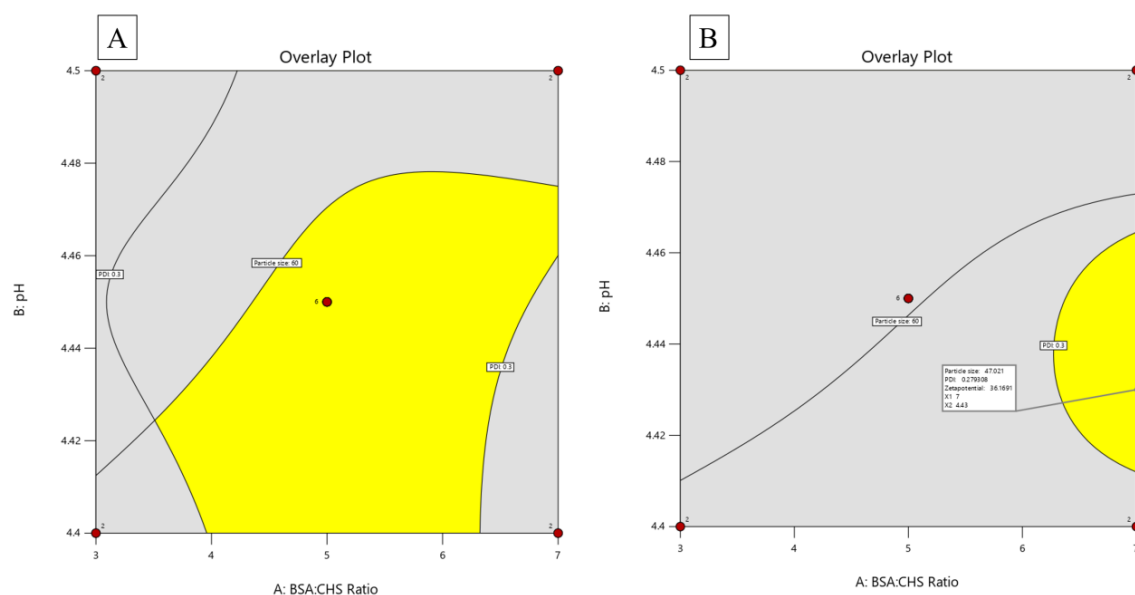


Fig. 6. Overlay plot showing the design space for A) Low MW CHS, and B) Medium MW CHS and selected point of maximum desirability.

nanogels when loading is passive and governed mainly by drug diffusion into the nanogel matrix. This mechanism-driven limitation arises from: (1) limited hydrophobic domains for drug partitioning, (2) competitive electrostatic interactions between the drug and polymers, as OLO is predominantly positively charged in this pH range, (3) high drug solubility in the aqueous phase leading to diffusion out of the polymeric network, and (4) drug loss during purification [68-70]. Lazaridou et al. prepared CHS nanogels containing deferoxamine mesylate, a hydrophilic drug, and obtained EE ranging from 18.96% to 44.45% [71]. Sahu et al. prepared similar CHS nanogels and loaded them with bleomycin (another hydrophilic drug), with a moderate EE of 55% [72]. For Doxorubicin, Zare et al. obtained 40% EE with CHS nanogels [73], and Wang et al. prepared BSA/CHS nanogels, measuring EE of 46.3% [21].

Radeva et al. prepared similar BSA/CHS nanogels loaded with doxorubicin and obtained

an EE of 74.7%. This discrepancy with the current work may be attributed to the lower theoretical DL (9.1%) used in their study versus the (28.6%) used here [19]. Increasing drug concentration was previously observed to reduce EE in PLGA nanoparticles, where drug entrapment is also diffusion-driven [74], as well as in modified CHS nanogels [75] suggesting a balance is required when improving DL without unnecessary loss of the drug.

Multiple Response Optimization and Model Validation

The design space is shown in Fig. 6, the yellow region represents the factor combinations that simultaneously satisfy all specified response requirements for low- and medium-MW CHS. The design space obtained for low MW-CHS was broader, indicating greater formulation robustness and tolerance to factor variation. In contrast, the design space for medium-MWCHS was narrower,

Table 18. Opt-F and experimental validation results.

Category	Parameter	Optimized level	Prediction interval (95%)	Experimental Value *	RSD (%)
Independent variable	BSA:CHS	7:1			
	pH	4.43			
	CHS MW	Medium			
Response	Size (nm)		43.66 – 50.38	47.13±1.6	3.4
	PDI		0.253 – 0.306	0.268±0.002	0.7
	ZP (mV)		36.69-38.44	+38.21±2.82	7.4
	EE %		23.28 – 29.42	25.07±1.215%	4.8

* Results reported as mean ± SD (n=5).

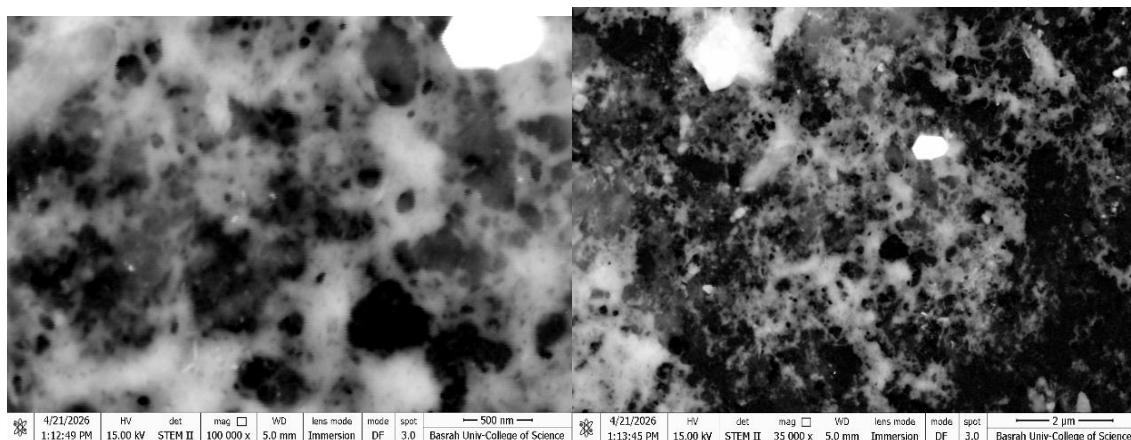


Fig. 7. SEM image of the optimal nanogel formulation showing dispersed spherical particles (black spots) and dense aggregations (large, irregular spots).

suggesting that tighter control of formulation variables is required to consistently achieve the desired product attributes.

The flagged point in Fig. 6B indicates the selected opt-F, corresponding to the highest desirability value (0.858) within the acceptable region. Experimental responses from five independent opt-F preparations were the mean fell within the 95% prediction intervals (Table 18), confirming model adequacy.

The optimal BSA:CHS ratio was the highest investigated level (7:1), as it consistently yielded the smallest particle sizes while maintaining sufficient polymer interactions for nanogel formation. The optimal pH was 4.43, which is close to the experimentally determined pI of BSA, where a balance of non-electrostatic and weak electrostatic interactions promotes stable network formation. Medium-MW CHS MW was the optimal polymer grade, because it yielded higher EE across all experiments.

Characterization of Nanogels Morphological Study

The SEM images are shown in Fig. 7 Particle size analysis revealed a range of 31-69 nm, with a mean particle diameter of 48.8 ± 9.2 nm ($n=42$).

The SEM micrographs demonstrated a heterogeneous particle distribution, with some particles appearing aggregated into irregular structures, while others retained a well-defined

spherical morphology. The observed aggregation is likely due to drying during sample preparation, as CHS-based nanoparticles are known to exhibit adhesive interactions [76, 77]. Additionally, partial loss of sphericity was observed in some particles, attributable to dehydration-induced deformation, a behavior commonly reported for nanogels due to their inherent structural flexibility [78].

pH and Ionic Strength Response of BSA/CHS Nanogel

Fig. 8 shows the pH-dependent changes in particle size, PDI, and ZP. As pH increases above the pI of BSA, more unionized carboxylic groups become deprotonated, resulting in a greater density of negatively charged sites. The resulting electrostatic repulsion relaxes and expands the polymer network, increasing water uptake and swelling. BSA is most compact near its pI and adopts a more expanded conformation as pH increases [79, 80].

In the CHS-containing nanogel, swelling is influenced by the protonation and deprotonation of amino groups. As pH increases from 4.5 to 6, the positive charge density of CHS decreases, reducing electrostatic compaction and increasing swelling. However, sufficient electrostatic interactions between negatively charged BSA and protonated CHS groups remain to preserve nanogel integrity [55]. At pH 7.4, reduced CHS protonation and solubility resulted in decreased electrostatic

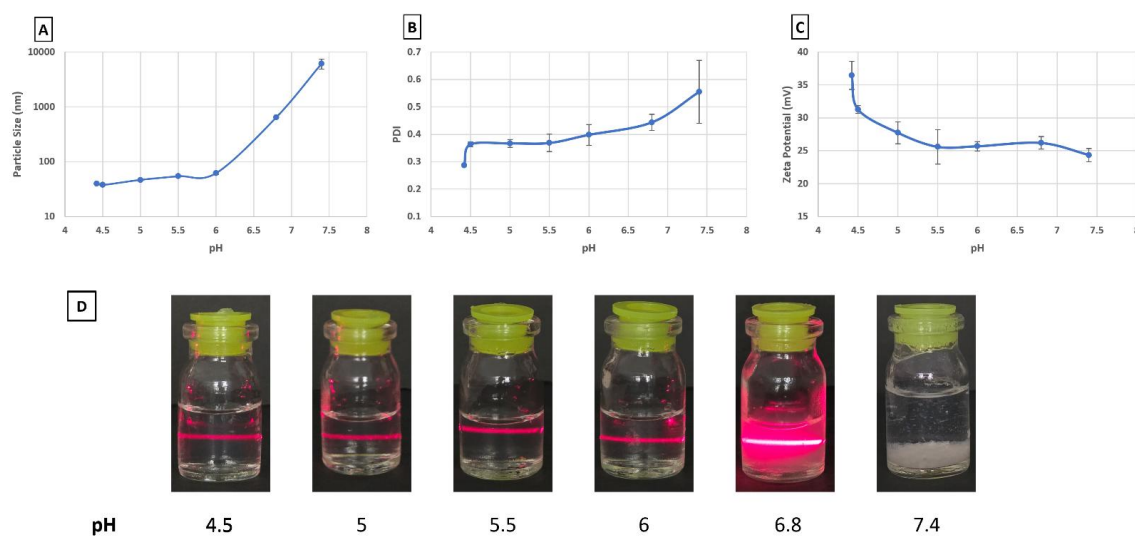


Fig. 8. pH-responsive changes in nanogel size (A), polydispersity (B), and surface charge (C). (D) Photo showing Tyndall effect at different pH values.

stabilization and increased particle aggregation and subsequent nanogel precipitation.

Table 19 demonstrates the effect of salt addition from 0 to 200mM, using NaCl as the model electrolyte at pH 4.5. Nanogels showed a moderate increase in size with increasing salt concentration, which indicates that salt addition might have weakened electrostatic interactions within the network by the effect of counterions, resulting in polymer chain relaxation and increased water uptake [81]. This observation is supported by the reduction in ZP with increasing salt concentration, indicating electrostatic shielding and compression of the electrical double layer. PDI did not change significantly, suggesting that salt

addition did not induce particle aggregation or broaden the particle size distribution. The effect of salt addition on NIPAM. is similar in that increasing salt concentration led to nanogel swelling and eventual aggregation at high salt concentrations, as well as positive charge screening [82].

In Vitro Drug Release and Kinetic Analysis

The *in vitro* release profile was evaluated using Franz diffusion cells at 34 ± 2 °C under sink conditions. The cumulative percentage of drug released within 24 h is shown in Fig. 9 and the kinetic model fitting is shown in Table 20.

The control OLO solution exhibited rapid diffusion into the receptor compartment,

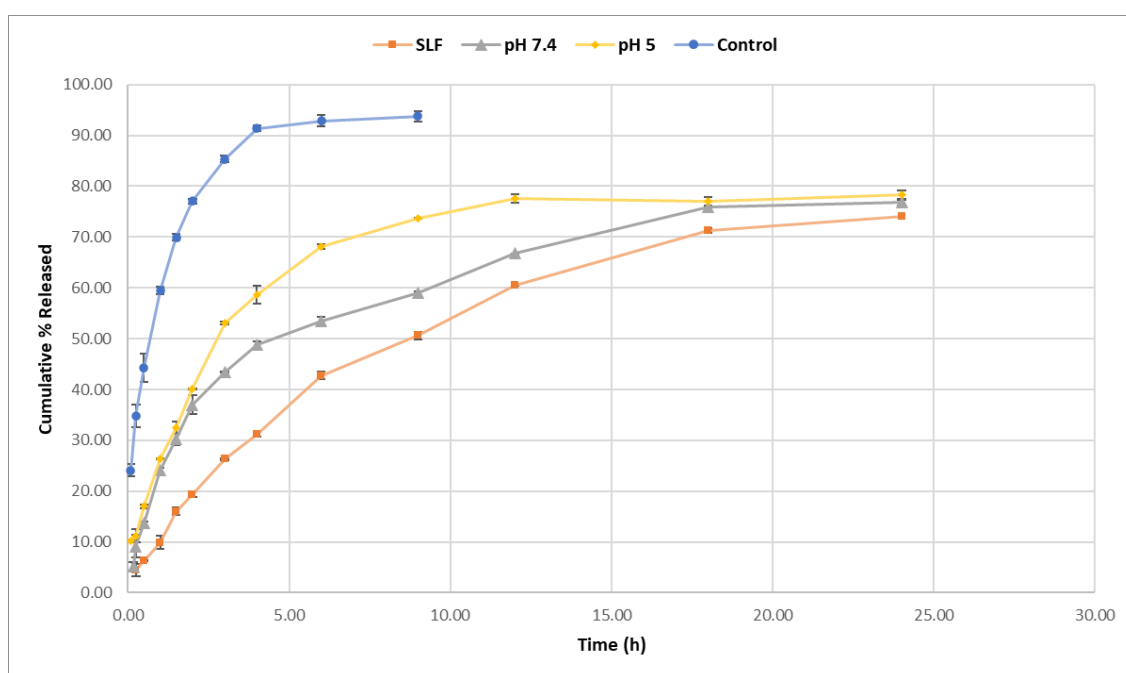


Fig. 9. In vitro cumulative release profile of OLO from BSA/CHS nanogels in pH 5 buffer pH 7.4 buffer, and SLF compared with aqueous drug control solution.

Table 19. Effect of ionic strength in opt-F size, PDI, and ZP.

Release Kinetics	Control	pH 5	pH 7.4	SLF
Zero order	0.878	0.824	0.801	0.89
First order	0.995	0.929	0.929	0.965
Higuchi	0.999	0.957	0.947	0.983
Hixson - Crowell	0.97	0.897	0.863	0.944
Korsmeyer-Peppas	0.996	0.956	0.963	0.992
	(n=0.354)	(n=0.499)	(n=0.539)	(n=0.706)
Weibull	0.983	0.97	0.975	0.995
	(β = 0.542)	(β = 0.605)	(β = 0.642)	(β = 0.79)

reaching near-complete recovery within 4 h, indicating minimal diffusional resistance during the initial phase. However, at later points, the decreasing concentration gradient likely increased the contribution of membrane permeation resistance [83]. Therefore, only the first 4 h of the control profile were included in the release kinetics analysis. Interpretation of dialysis-based release data should be cautious, because the measured profile reflects both drug liberation from the nanogel and diffusion across the dialysis membrane. The control solution was best described by the Higuchi model, suggesting minor diffusional resistance imposed by the dialysis membrane [84, 85].

The discrepancy between the relatively low measured EE and the absence of a pronounced burst release suggests a multiple drug association state within the nanogel system. The fraction of OLO classified as “free” during centrifugal ultrafiltration may be weakly associated with the nanogel surface or the hydrated polymer domains, resulting in an underestimation of EE. These interactions may still retard diffusion, thereby contributing to the observed sustained-release behavior. Similarly, the incomplete release observed after 24 h may indicate a tightly

associated drug fraction within the nanogel core, which may require polymer erosion or structural degradation for complete release [47].

The release profiles exhibit sustained release behavior in all investigated media, with Higuchi generally providing the best fit, indicating predominantly diffusion-driven release [86, 87]. Korsmeyer-Peppas analysis indicated anomalous transport, reflecting contributions from both diffusion and polymer relaxation, with diffusion remaining the dominant mechanism, a pattern commonly reported for porous CHS-based nanocarriers [19, 75]. Weibull analysis similarly indicated diffusion-controlled release, with an increasing contribution of polymer relaxation, particularly in SLF. The poor fit for the Hixson-Crowell model suggests that changes in particle surface area or erosion were not the primary governing mechanisms for release.

At pH 5, diffusion is the primary mechanism governing the release, although the n value suggests a minor contribution from polymer swelling may also be present. This limited contribution of polymer relaxation, combined with the protonation and high solubility ($> 20\text{ mg/ml}$ [88]) of OLO at these pH values, creates a larger diffusion gradient, resulting in a faster release

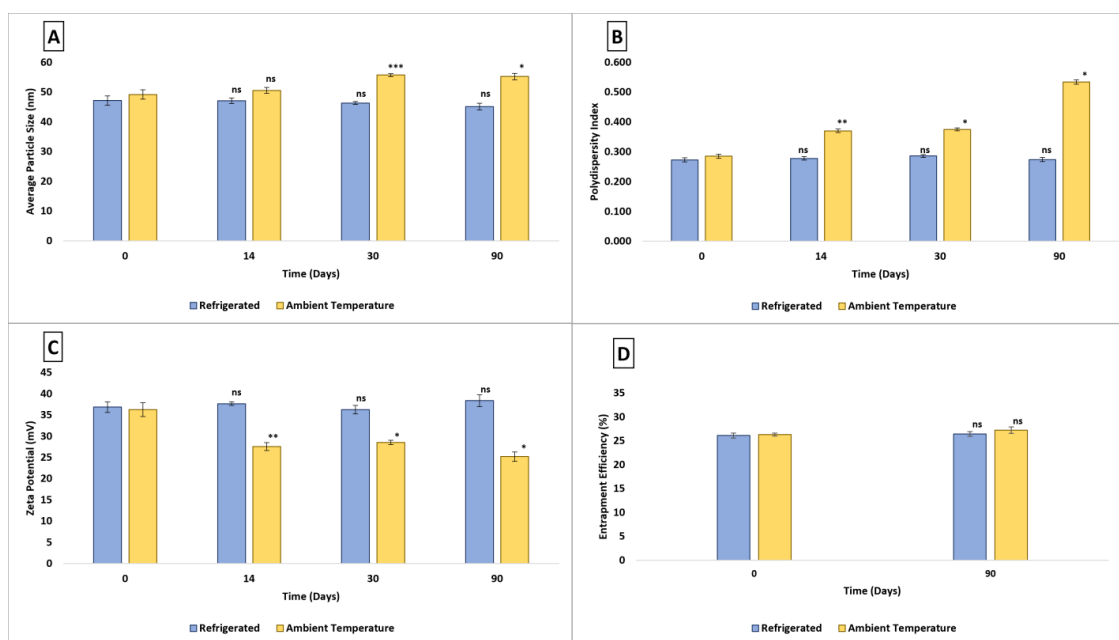


Fig. 10. Effect of storage conditions on A) particle size, B) PDI, C) zeta potential, and D) entrapment efficiency of nanogel over 90 days. (ns=not significant, * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$)

profile.

At pH 7.4, the transport mechanism remains anomalous, and the n value indicates a slightly greater contribution from polymer relaxation, which may result from reduced protonation of CHS at higher pH values, thereby decreasing electrostatic interactions between polymer chains and producing a looser matrix structure. However, OLO is less soluble at alkaline pH values, resulting in reduction of the driving force of diffusion and increased contribution from polymer relaxation to

the release kinetics.

For SLF, a major mechanistic shift is observed, in which polymer relaxation significantly contributes to the release kinetics, while the diffusion contribution is lower. The higher ionic strength likely promotes electrostatic screening of NH_3^+ groups in CHS, altering chain interactions and increasing polymer chain hydration and mobility [89]. However, the overall rate of release is slower, most likely due to a common-ion effect, whereby chloride ions from NaCl reduce the apparent

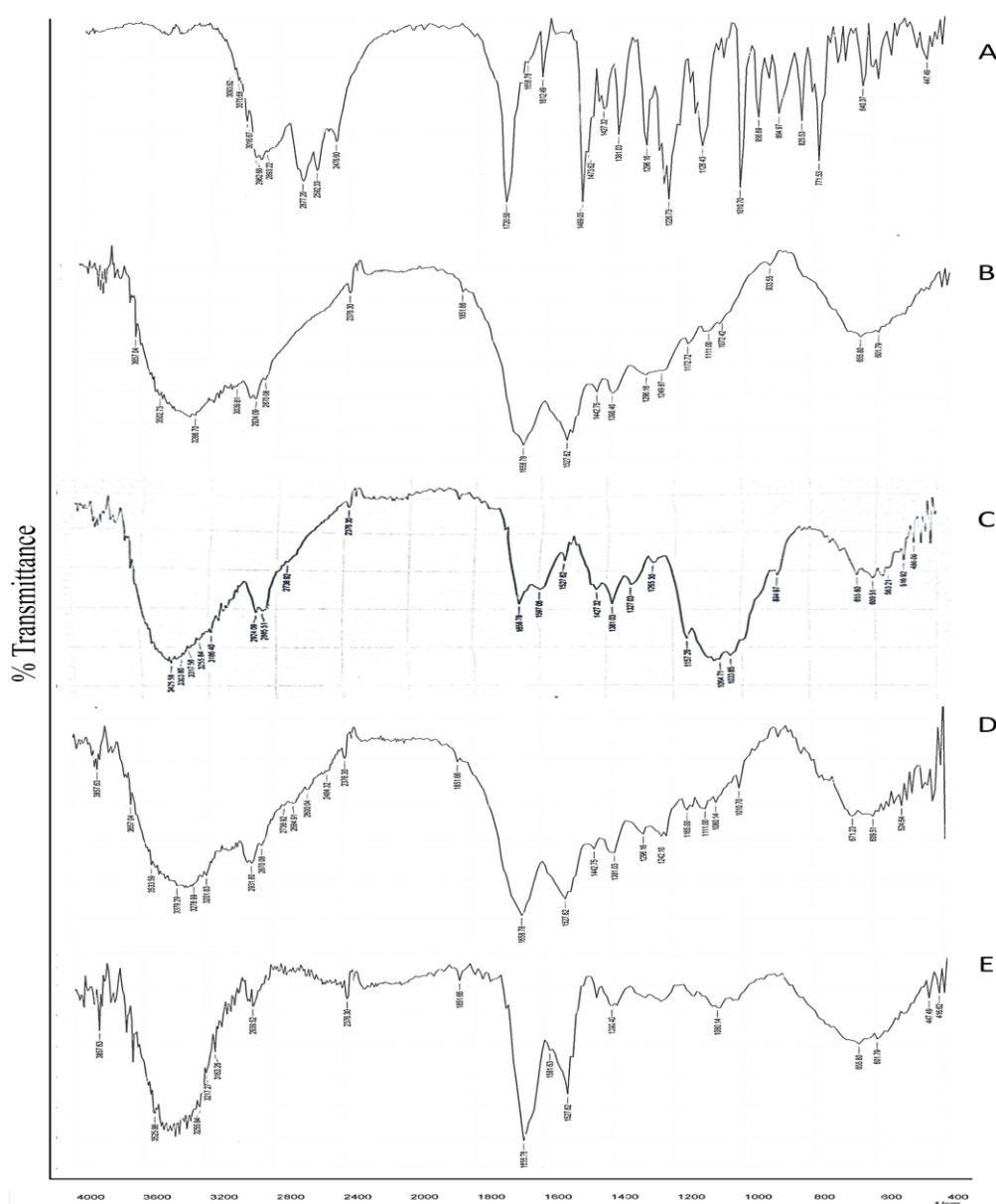


Fig. 11. FTIR Spectrum for A) OLO, B) BSA, C) CHS, D) Physical Mixture, E) OLO-Loaded Nanogels.

solubility of OLO, reducing the diffusional driving force.

Physical Stability Study

Samples stored at both refrigerated and ambient temperature showed no observable changes in odor, color, or clarity, and no visible precipitation occurred during the 3-month study period. Fig. 10 and shows the effect of storage conditions on nanogel properties at each time point.

The short-term stability study demonstrated a clear effect of temperature on the physical stability of opt-F. Samples stored at 4 °C exhibited good physical stability, as evidenced by the minimal variation in particle size, PDI, and ZP throughout storage. In contrast, samples stored at ambient temperature (25 ± 5 °C) exhibited significant changes in all these parameters. Average particle size increased by approximately 12% after 3 months, while PDI increased by 58% and ZP

decreased by 21.9%. This decreased stability at ambient temperature may be attributed to increased Brownian motion, which promotes interparticle collisions while reducing electrostatic stabilization.

Fourier Transformed Infrared Spectroscopy (FTIR)

FTIR spectra (Fig. 11) was employed to evaluate the chemical compatibility and intermolecular interactions among OLO, BSA, and CHS [90]. The spectra for OLO, CHS, and BSA are standard and comparable to the literature [9, 91, 92].

Physical mixture spectrum shows a combination of the three spectra of OLO, BSA, and CHS, with spectral features dominated by BSA, due to its high ratio (BSA: CHS ratio was 7:1) and low concentration of OLO. Characteristic OLO bands overlapped and appeared only in the fingerprint region. The absence of new absorption bands or significant shifts indicates chemical compatibility

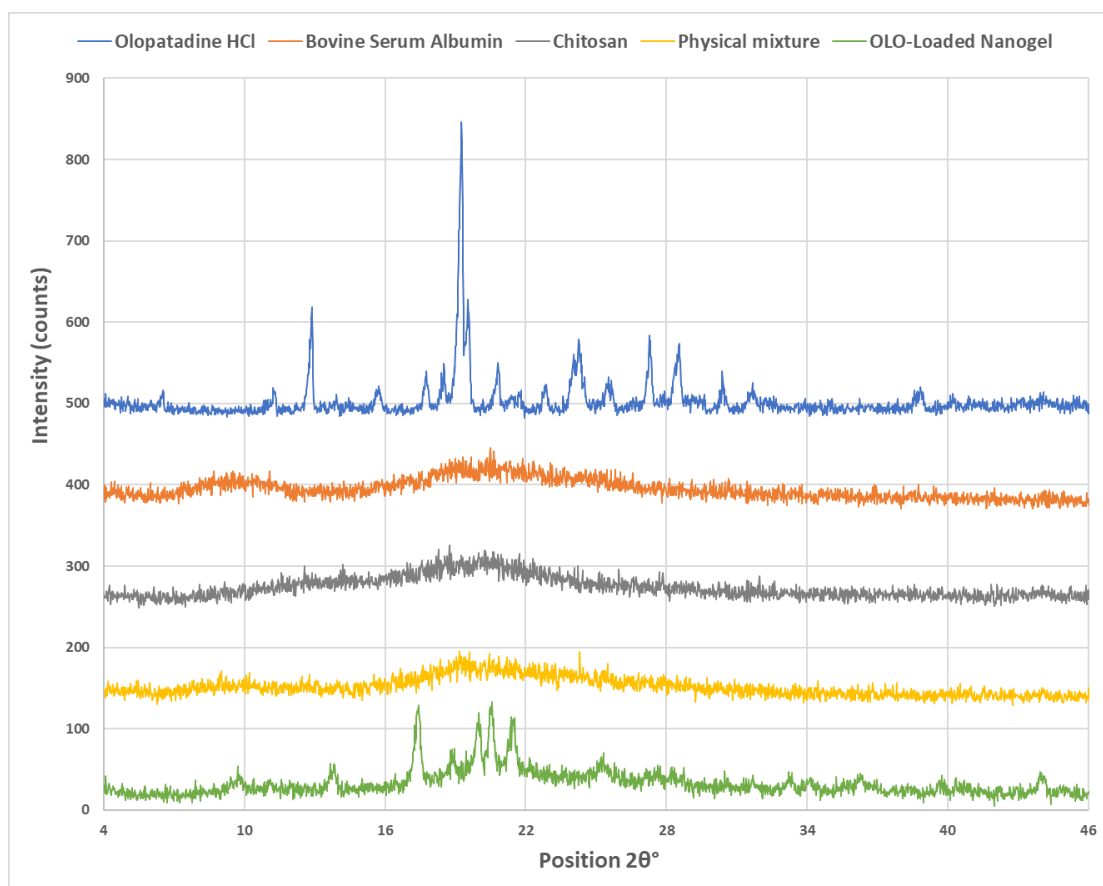


Fig. 12. Powder X-ray diffraction patterns of the pure components and freeze-dried nanogel.

among the materials.

Opt-F spectrum showed a narrower OH stretching band than the physical mixture. This behavior can be attributed to the presence of a more uniform, structured H-bonding environment in the wet formulation, resulting from intermolecular interactions. In contrast, the dry physical mixture exhibits more heterogeneous H-bonding, resulting in greater band broadening [90, 93].

Powder X-Ray Diffraction

PXRD patterns are shown in Fig. 12. OLO exhibits sharp Bragg reflections, characteristic of a highly crystalline material. The spectrum is similar to the one reported in the literature for form A of OLO [91]. BSA and CHS exhibit broad, diffuse halos, indicative of their amorphous nature [94-96].

The physical mixture exhibits a diffractogram similar to that of the polymers, with no characteristic drug diffraction peaks. This behavior is likely due to the low drug content, peak masking effect of the polymeric matrix, and partial loss of crystallinity during grinding, which may have resulted in and partial loss of crystallinity during grinding [97-100].

The freeze-dried opt-F exhibits distinct crystalline reflections that did not correspond to any of the starting materials used in the preparation. The peaks were consistent with the α -polymorphic form of mannitol that was used as a lyoprotectant during freeze-drying [101, 102]. These peaks dominated the diffraction pattern, potentially obscuring any subtle structural ordering within the nanogel matrix.

CONCLUSION

In the present study, OLO-loaded BSA/CHS nanogels for ocular drug delivery have been successfully formulated and optimized by a systematic QbD approach. Critical material attributes and process parameters were identified and optimized with risk assessment tools and Design of Experiments, resulting in an optimized formulation with nanoscale dimensions, narrow size distribution, appropriate zeta potential and entrapment efficiency, and sustained drug release behaviour. The nanogel system also exhibited pH- and ionic strength-responsive behavior, physical stability under tested storage conditions, and successful drug incorporation. The results demonstrate the utility of QbD principles for the

reproducible development of nanogels. Further in vivo and clinical studies are warranted to evaluate its therapeutic efficacy and clinical translation.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

REFERENCES

1. ter Horst JP, Turimella SL, Metsers F, Zwiers A. Implementation of Quality by Design (QbD) Principles in Regulatory Dossiers of Medicinal Products in the European Union (EU) Between 2014 and 2019. *Therapeutic Innovation and Regulatory Science*. 2021;55(3):583-590.
2. Yu LX. Pharmaceutical Quality by Design: Product and Process Development, Understanding, and Control. *Pharm Res*. 2008;25(4):781-791.
3. Lee S-H, Kim J-K, Jee J-P, Jang D-J, Park Y-J, Kim J-E. Quality by Design (QbD) application for the pharmaceutical development process. *Journal of Pharmaceutical Investigation*. 2022;52(6):649-682.
4. Gupta J, Sharma G. Nanogel: A versatile drug delivery system for the treatment of various diseases and their future perspective. *Drug Delivery and Translational Research*. 2024;15(2):455-482.
5. Kesharwani D, Das Paul S, Paliwal R, Satapathy T. Exploring potential of diacerin nanogel for topical application in arthritis: Formulation development, QbD based optimization and pre-clinical evaluation. *Colloids Surf B Biointerfaces*. 2023;223:113160.
6. Mohtashamian S, Boddohi S, Hosseinkhani S. Preparation and optimization of self-assembled chondroitin sulfate-nisin nanogel based on quality by design concept. *Int J Biol Macromol*. 2018;107:2730-2739.
7. Saraogi GK, Tholiya S, Mishra Y, Mishra V, Albutti A, Nayak P, et al. Formulation Development and Evaluation of Pravastatin-Loaded Nanogel for Hyperlipidemia Management. *Gels*. 2022;8(2):81.
8. Li J, Kong X, Chen H, Lu M, Liu X, Wang L. Optimization of Laponite Nanogel with Curcumin Incorporation: A Quality by Design Approach. *Gels*. 2025;11(9):677.
9. Marei NH, El-Samie EA, Salah T, Saad GR, Elwahy AHM. Isolation and characterization of chitosan from different local insects in Egypt. *Int J Biol Macromol*. 2016;82:871-877.
10. Abdou ES, Nagy KSA, Elsabee MZ. Extraction and characterization of chitin and chitosan from local sources. *Bioresour Technol*. 2008;99(5):1359-1367.
11. Ardean C, Davidescu CM, Nemeş NS, Negrea A, Ciopec M, Duteanu N, et al. Factors Influencing the Antibacterial Activity of Chitosan and Chitosan Modified by Functionalization. *Int J Mol Sci*. 2021;22(14):7449.
12. Novikov VY, Derkach SR, Konovalova IN, Dolgopyatova NV, Kuchina YA. Mechanism of Heterogeneous

- Alkaline Deacetylation of Chitin: A Review. *Polymers*. 2023;15(7):1729.
13. Azadi A, Hamidi M, Rouini M-R. Methotrexate-loaded chitosan nanogels as 'Trojan Horses' for drug delivery to brain: Preparation and in vitro/in vivo characterization. *Int J Biol Macromol*. 2013;62:523-530.
14. Bruno SG, Martínez SM, Costa Gobbato C, Quinteros DA, Alaimo A, Pérez OE. Chitosan-TPP Nanogels for Ocular Delivery of Folic Acid: Release Profile, Corneal Permeation, and Mucoadhesion Assessment. *Pharmaceutics*. 2025;17(4):424.
15. Algharib SA, Dawood A, Zhou K, Chen D, Li C, Meng K, et al. Designing, structural determination and biological effects of rifaximin loaded chitosan- carboxymethyl chitosan nanogel. *Carbohydr Polym*. 2020;248:116782.
16. Ducepe N, Tabrizian M. Factors influencing the transfection efficiency of ultra low molecular weight chitosan/hyaluronic acid nanoparticles. *Biomaterials*. 2009;30(13):2625-2631.
17. Veloso SRS, Marta ES, Rodrigues PV, Moura C, Amorim CO, Amaral VS, et al. Chitosan/Alginate Nanogels Containing Multicore Magnetic Nanoparticles for Delivery of Doxorubicin. *Pharmaceutics*. 2023;15(9):2194.
18. Zhou K, Wang X, Chen D, Yuan Y, Wang S, Li C, et al. Enhanced Treatment Effects of Tilmicosin Against *Staphylococcus aureus* Cow Mastitis by Self-Assembly Sodium Alginate-Chitosan Nanogel. *Pharmaceutics*. 2019;11(10):524.
19. Radeva L, Zaharieva MM, Spassova I, Kovacheva D, Pencheva-El Tibi I, Najdenski H, et al. Biopolymeric Nanogel as a Drug Delivery System for Doxorubicin—Improved Drug Stability and Enhanced Antineoplastic Activity in Skin Cancer Cells. *Pharmaceutics*. 2024;17(2):186.
20. Yu S, Hu J, Pan X, Yao P, Jiang M. Stable and pH-Sensitive Nanogels Prepared by Self-Assembly of Chitosan and Ovalbumin. *Langmuir*. 2006;22(6):2754-2759.
21. Wang Y, Xu S, Xiong W, Pei Y, Li B, Chen Y. Nanogels fabricated from bovine serum albumin and chitosan via self-assembly for delivery of anticancer drug. *Colloids Surf B Biointerfaces*. 2016;146:107-113.
22. Ay Şenyiğit Z, Coşkunmeriç N, Çağlar EŞ, Öztürk İ, Atılhan Gündoğdu E, Siafaka Pl, et al. Chitosan-bovine serum albumin-Carbopol 940 nanogels for mupirocin dermal delivery: ex-vivo permeation and evaluation of cellular binding capacity via radiolabeling. *Pharmaceutical Development and Technology*. 2021;26(8):852-866.
23. Boeris V, Farruggia B, Picó G. Chitosan-bovine serum albumin complex formation: A model to design an enzyme isolation method by polyelectrolyte precipitation. *J Chromatogr B*. 2010;878(19):1543-1548.
24. Kurukji D, Norton I, Spyropoulos F. Fabrication of sub-micron protein-chitosan electrostatic complexes for encapsulation and pH-Modulated delivery of model hydrophilic active compounds. *Food Hydrocolloids*. 2016;53:249-260.
25. Gao Y, Luo D, Li X, Xue B, Xie J, Sun T. Preparation and characterization of bovine serum albumin/chitosan composite nanoparticles for delivery of Antarctic krill peptide. *Journal of the Science of Food and Agriculture*. 2024;105(1):162-170.
26. Mardikasari SA, Katona G, Sipos B, Ambrus R, Csóka I. Preparation and Optimization of Bovine Serum Albumin Nanoparticles as a Promising Gelling System for Enhanced Nasal Drug Administration. *Gels*. 2023;9(11):896.
27. Malaiya A, Kenwat R, Mamgain A, Nayak P, Parker A, Paliwal SR, et al. Intranasal resveratrol delivery to the brain with chitosan-decorated bovine serum albumin nanoparticles: Advancing Alzheimer's management in old female rats through QbD-based optimization, in vitro evaluation, and in vivo exploration. *Int J Biol Macromol*. 2025;311:143300.
28. Li Y, Lee J, Lal J, An L, Huang Q. Effects of pH on the Interactions and Conformation of Bovine Serum Albumin: Comparison between Chemical Force Microscopy and Small-Angle Neutron Scattering. *The Journal of Physical Chemistry B*. 2008;112(12):3797-3806.
29. Raghuvanshi VS, Yu B, Browne C, Garnier G. Reversible pH Responsive Bovine Serum Albumin Hydrogel Sponge Nanolayer. *Frontiers in Bioengineering and Biotechnology*. 2020;8.
30. Adamczyk O, Szota M, Rakowski K, Prochownik M, Doveiko D, Chen Y, et al. Bovine Serum Albumin as a Platform for Designing Biologically Active Nanocarriers—Experimental and Computational Studies. *Int J Mol Sci*. 2023;25(1):37.
31. Kubiak-Ossowska K, Tokarczyk K, Jachimska B, Mulheran PA. Bovine Serum Albumin Adsorption at a Silica Surface Explored by Simulation and Experiment. *The Journal of Physical Chemistry B*. 2017;121(16):3975-3986.
32. Kam KW, Chen LJ, Wat N, Young AL. Topical Olopatadine in the Treatment of Allergic Conjunctivitis: A Systematic Review and Meta-analysis. *Ocul Immunol Inflamm*. 2016;25(5):668-682.
33. Ahmed S, Amin MM, Sayed S. Ocular Drug Delivery: a Comprehensive Review. *AAPS PharmSciTech*. 2023;24(2).
34. Abore KW, Fole EB, Abebe MT, Tekle NF, Tilahun RB, Chinkey FD, et al. Adherence and factors influencing adherence to glaucoma medications among adult glaucoma patients in Ethiopia: A systematic review and meta-analysis. *PLoS One*. 2024;19(3):e0293047.
35. Newman-Casey PA, Rhee DJ, Robin AL, Mansberger SL. Patient Challenges with Glaucoma Eye Drops: A Need to Identify Nonadherence and Facilitate Appropriate Support and Disease Management. *Ophthalmology Glaucoma*. 2025;8(4):327-330.
36. Zafaryab M, Vig K. Biomedical Application of Nanogels: From Cancer to Wound Healing. *Molecules*. 2025;30(10):2144.
37. Wu Y, Tao Q, Xie J, Lu L, Xie X, Zhang Y, et al. Advances in Nanogels for Topical Drug Delivery in Ocular Diseases. *Gels*. 2023;9(4):292.
38. Bairagi RD, Reon RR, Hasan MM, Sarker S, Debnath D, Rahman MT, et al. Ocular drug delivery systems based on nanotechnology: a comprehensive review for the treatment of eye diseases. *Discover Nano*. 2025;20(1).
39. Salis A, Boström M, Medda L, Cugia F, Barse B, Parsons DF, et al. Measurements and Theoretical Interpretation of Points of Zero Charge/Potential of BSA Protein. *Langmuir*. 2011;27(18):11597-11604.
40. Rodriguez-Loya J, Lerma M, Gardea-Torresdey JL. Dynamic Light Scattering and Its Application to Control Nanoparticle Aggregation in Colloidal Systems: A Review. *Micromachines*. 2023;15(1):24.
41. Charoo NA, Ali AA. Quality risk management in pharmaceutical development. *Drug Development and Industrial Pharmacy*. 2012;39(7):947-960.
42. Jones B, Nachtsheim CJ. A Class of Three-Level Designs for Definitive Screening in the Presence of Second-Order Effects. *Journal of Quality Technology*. 2011;43(1):1-15.
43. Jones R. Design and Analysis of Experiments (fifth edition), Douglas Montgomery, John Wiley and Sons, 2001, 684 pages, £33.95. *Qual Reliab Eng Int*. 2002;18(2):163-163.

44. Derel HG. Method Development and Validation of in Vitro Release Test for Fluorometholone Ophthalmic Suspension using Franz Diffusion Cell System. *Global Journal of Pharmacy and Pharmaceutical Sciences*. 2021;9(2).
45. Naik P, Shah S, Heaney J, Hanson R, Nagarsenker M. Influence of Test Parameters on Release Rate of Hydrocortisone from Cream: Study Using Vertical Diffusion Cell. *Dissolution Technologies*. 2016;23(3):14-20.
46. Borkar S, Baumli P, Vance T, Sharma E, Shi X, Wu JY, et al. Elucidating the roles of electrolytes and hydrogen bonding in the dewetting dynamics of the tear film. *Proceedings of the National Academy of Sciences*. 2024;121(31).
47. Zambito Y, Pedreschi E, Di Colo G. Is dialysis a reliable method for studying drug release from nanoparticulate systems?—A case study. *Int J Pharm*. 2012;434(1-2):28-34.
48. Siepmann J. Modeling of drug release from delivery systems based on hydroxypropyl methylcellulose (HPMC). *Adv Drug Del Rev*. 2001;48(2-3):139-157.
49. Mathematical modeling of drug release from swellable polymeric nanoparticles. *Journal of Applied Pharmaceutical Science*. 2017.
50. Coşkunmeriç N, Üstündağ Okur N, Okur ME, Ayla Ş, Voltaş A, Karavana SY. Promising nanogels loaded with usnic acid for oral ulcer treatment: development, characterization, and in vivo evaluation in rabbits. *Pharmaceutical Development and Technology*. 2021;26(4):431-443.
51. Barone G, Giancola C, Verdoliva A. DSC studies on the denaturation and aggregation of serum albumins. *Thermochim Acta*. 1992;199:197-205.
52. Kleine-Brueggeney H, Zorzi GK, Fecker T, El Gueddari NE, Moerschbacher BM, Goycoolea FM. A rational approach towards the design of chitosan-based nanoparticles obtained by ionotropic gelation. *Colloids Surf B Biointerfaces*. 2015;135:99-108.
53. Vaezifar S, Razavi S, Golozar MA, Karbasi S, Morshed M, Kamali M. Effects of Some Parameters on Particle Size Distribution of Chitosan Nanoparticles Prepared by Ionic Gelation Method. *J Cluster Sci*. 2013;24(3):891-903.
54. Li G, Huang J, Chen T, Wang X, Zhang H, Chen Q. Insight into the interaction between chitosan and bovine serum albumin. *Carbohydr Polym*. 2017;176:75-82.
55. Preparation of an Environmentally Friendly Formulation of the Insecticide Nicotine Hydrochloride through Encapsulation in Chitosan/Tripolyphosphate Nanoparticles. *American Chemical Society (ACS)*.
56. Hong Y-H, McClements DJ. Formation of Hydrogel Particles by Thermal Treatment of β -Lactoglobulin–Chitosan Complexes. *Journal of Agricultural and Food Chemistry*. 2007;55(14):5653-5660.
57. Borzova VA, Markossian KA, Chebotareva NA, Kleymenov SY, Poliansky NB, Muranov KO, et al. Kinetics of Thermal Denaturation and Aggregation of Bovine Serum Albumin. *PLoS One*. 2016;11(4):e0153495.
58. Meka VS, Sing MKG, Pichika MR, Nali SR, Kolapalli VRM, Kesharwani P. A comprehensive review on polyelectrolyte complexes. *Drug Discovery Today*. 2017;22(11):1697-1706.
59. Bhattacharjee S. DLS and zeta potential – What they are and what they are not? *Journal of Controlled Release*. 2016;235:337-351.
60. Lundstedt T, Seifert E, Abramo L, Thelin B, Nyström Å, Pettersen J, et al. Experimental design and optimization. *Chemometrics Intellig Lab Syst*. 1998;42(1-2):3-40.
61. Bekale L, Agudelo D, Tajmir-Riahi HA. Effect of polymer molecular weight on chitosan–protein interaction. *Colloids Surf B Biointerfaces*. 2015;125:309-317.
62. Chang S-H, Lin H-TV, Wu G-J, Tsai GJ. pH Effects on solubility, zeta potential, and correlation between antibacterial activity and molecular weight of chitosan. *Carbohydr Polym*. 2015;134:74-81.
63. Pérez-Pacheco Y, Tylkowski B, García-Valls R. Chitosan Micro/Nanocapsules in Action: Linking Design, Production, and Therapeutic Application. *Molecules*. 2025;30(2):252.
64. Rinaudo M. Chitin and chitosan: Properties and applications. *Prog Polym Sci*. 2006;31(7):603-632.
65. Wang QZ, Chen XG, Liu N, Wang SX, Liu CS, Meng XH, et al. Protonation constants of chitosan with different molecular weight and degree of deacetylation. *Carbohydr Polym*. 2006;65(2):194-201.
66. Ho HMK, Day RM, Craig DQM. An Investigation into the Effects of Processing Factors on the Properties and Scaling-Up Potential of Propranolol-Loaded Chitosan Nanogels. *Pharmaceutics*. 2024;16(5):662.
67. Carrasco-Sandoval J, Aranda M, Henríquez-Aedo K, Fernández M, López-Rubio A, Fabra MJ. Impact of molecular weight and deacetylation degree of chitosan on the bioaccessibility of quercetin encapsulated in alginate/chitosan-coated zein nanoparticles. *Int J Biol Macromol*. 2023;242:124876.
68. Herdiana Y, Febrina E, Nurhasanah S, Gozali D, Elamin KM, Wathoni N. Drug Loading in Chitosan-Based Nanoparticles. *Pharmaceutics*. 2024;16(8):1043.
69. Radeva L, Yoncheva K. Nanogels—Innovative Drug Carriers for Overcoming Biological Membranes. *Gels*. 2025;11(2):124.
70. Novartis Campus, East Hanover, New Jersey, United States. *Transforming Landscapes: De Gruyter*; 2020. p. 148-155.
71. Lazaridou M, Christodoulou E, Nerantzaki M, Kostoglou M, Lambropoulou D, Katsarou A, et al. Formulation and In-Vitro Characterization of Chitosan-Nanoparticles Loaded with the Iron Chelator Deferoxamine Mesylate (DFO). *Pharmaceutics*. 2020;12(3):238.
72. Sahu P, Kashaw SK, Kushwah V, Sau S, Jain S, Iyer AK. pH responsive biodegradable nanogels for sustained release of bleomycin. *Bioorganic and Medicinal Chemistry*. 2017;25(17):4595-4613.
73. Zare M, Mohammadi Samani S, Sobhani Z. Enhanced Intestinal Permeation of Doxorubicin Using Chitosan Nanoparticles. *Advanced Pharmaceutical Bulletin*. 2018;8(3):411-417.
74. Struzek A-M, Scherließ R. Quality by Design as a Tool in the Optimisation of Nanoparticle Preparation—A Case Study of PLGA Nanoparticles. *Pharmaceutics*. 2023;15(2):617.
75. Tao Q, Zhong J, Wang R, Huang Y. Ionic and Enzymatic Multiple-Crosslinked Nanogels for Drug Delivery. *Polymers*. 2021;13(20):3565.
76. Rusu AG, Chiriac AP, Nita LE, Rosca I, Rusu D, Neamtu I. Self-Assembled Nanocarriers Based on Modified Chitosan for Biomedical Applications: Preparation and Characterization. *Polymers*. 2020;12(11):2593.
77. Salehi R, Rasouli S, Hamishehkar H. Smart thermo/pH responsive magnetic nanogels for the simultaneous delivery of doxorubicin and methotrexate. *Int J Pharm*. 2015;487(1-2):274-284.
78. Muñana-González S, Veloso-Fernández A, Ruiz-Rubio L, Pérez-Álvarez L, Vilas-Vilela JL. Covalent Cross-Linking as a Strategy to Prepare Water-Dispersible Chitosan Nanogels.

- Polymers. 2023;15(2):434.
79. Sennaroglu Bostan M. Protein-Modulated Stimuli-Responsive Hydrogels Based on Methacrylated Bovine Serum Albumin and pNIPAm: pH- and Temperature-Dependent Drug Release Behavior. *Gels*. 2026;12(3):263.
 80. Chi Z, Hong B, Ren X, Cheng K, Lu Y, Liu X. Investigation on the conformational changes of bovine serum albumin in a wide pH range from 2 to 12. *Spectrosc Lett*. 2018;51(6):279-286.
 81. Kobayashi H, Winkler RG. Universal conformational properties of polymers in ionic nanogels. *Sci Rep*. 2016;6(1).
 82. Ayazbayeva AY, Shakhvorostov AV, Gussenov IS, Seilkhanov TM, Aseyev VO, Kudaibergenov SE. Temperature and Salt Responsive Amphoteric Nanogels Based on N-Isopropylacrylamide, 2-Acrylamido-2-methyl-1-propanesulfonic Acid Sodium Salt and (3-Acrylamidopropyl) Trimethylammonium Chloride. *Nanomaterials*. 2022;12(14):2343.
 83. Schwarzl R, Du F, Haag R, Netz RR. General method for the quantification of drug loading and release kinetics of nanocarriers. *Eur J Pharm Biopharm*. 2017;116:131-137.
 84. Modi S, Anderson BD. Determination of Drug Release Kinetics from Nanoparticles: Overcoming Pitfalls of the Dynamic Dialysis Method. *Mol Pharm*. 2013;10(8):3076-3089.
 85. Siepmann J, Siepmann F. Sink conditions do not guarantee the absence of saturation effects. *Int J Pharm*. 2020;577:119009.
 86. Ritger PL, Peppas NA. A simple equation for description of solute release I. Fickian and non-fickian release from non-swelling devices in the form of slabs, spheres, cylinders or discs. *Journal of Controlled Release*. 1987;5(1):23-36.
 87. Siepmann J. Mathematical modeling of bioerodible, polymeric drug delivery systems. *Adv Drug Del Rev*. 2001;48(2-3):229-247.
 88. Łaszcz M, Trzcińska K, Witkowska A, Ciesielska A, Badowska-Roślonek K, Kuziak K. Structural and Physicochemical Studies of Olopatadine Hydrochloride Conformational Polymorphs. *J Pharm Sci*. 2016;105(8):2419-2426.
 89. López-León T, Carvalho ELS, Seijo B, Ortega-Vinuesa JL, Bastos-González D. Physicochemical characterization of chitosan nanoparticles: electrokinetic and stability behavior. *Journal of Colloid and Interface Science*. 2005;283(2):344-351.
 90. Dendisová M, Jenišťová A, Parchaňská-Kokaislová A, Matějka P, Prokopec V, Švecová M. The use of infrared spectroscopic techniques to characterize nanomaterials and nanostructures: A review. *Anal Chim Acta*. 2018;1031:1-14.
 91. Velez P, Busso J. Blatant and Subtle Forms of Discrimination and Affective States. *PsycEXTRA Dataset: American Psychological Association (APA)*; 2006.
 92. Retnakumari A, Setua S, Menon D, Ravindran P, Muhammed H, Pradeep T, et al. Molecular-receptor-specific, non-toxic, near-infrared-emitting Au cluster-protein nanoconjugates for targeted cancer imaging. *Nanotechnology*. 2009;21(5):055103.
 93. Drabczyk A, Kudłacik-Kramarczyk S, Głąb M, Kędzierska M, Jaromin A, Mierzwiński D, et al. Physicochemical Investigations of Chitosan-Based Hydrogels Containing Aloe Vera Designed for Biomedical Use. *Materials*. 2020;13(14):3073.
 94. Hancock BC, Zografi G. Characteristics and Significance of the Amorphous State in Pharmaceutical Systems. *J Pharm Sci*. 1997;86(1):1-12.
 95. Gaur M, Maurya S, Akhtar MS, Yadav AB. Synthesis and Evaluation of BSA-Loaded PLGA-Chitosan Composite Nanoparticles for the Protein-Based Drug Delivery System. *ACS Omega*. 2023;8(21):18751-18759.
 96. Yadav P, Yadav AB. Preparation and characterization of BSA as a model protein loaded chitosan nanoparticles for the development of protein-/peptide-based drug delivery system. *Future Journal of Pharmaceutical Sciences*. 2021;7(1).
 97. Makoto O, Nobuyoshi K. Effect of grinding on the crystallinity and chemical stability in the solid state of cephalothin sodium. *Int J Pharm*. 1990;62(1):65-73.
 98. Newman AW, Byrn SR. Solid-state analysis of the active pharmaceutical ingredient in drug products. *Drug Discovery Today*. 2003;8(19):898-905.
 99. Newman JA, Schmitt PD, Toth SJ, Deng F, Zhang S, Simpson GJ. Parts per Million Powder X-ray Diffraction. *Anal Chem*. 2015;87(21):10950-10955.
 100. Yu L. Amorphous pharmaceutical solids: preparation, characterization and stabilization. *Adv Drug Del Rev*. 2001;48(1):27-42.
 101. Yang Y, Liu J, Hu A, Nie T, Cheng Z, Liu W. A Critical Review on Engineering of d-Mannitol Crystals: Properties, Applications, and Polymorphic Control. *Crystals*. 2022;12(8):1080.
 102. Cares-Pacheco MG, Vaca-Medina G, Calvet R, Espitalier F, Letourneau JJ, Rouilly A, et al. Physicochemical characterization of d-mannitol polymorphs: The challenging surface energy determination by inverse gas chromatography in the infinite dilution region. *Int J Pharm*. 2014;475(1-2):69-81.