

Formulation and Pharmacological Evaluation of Budesonide-Loaded Zinc-Crosslinked Chitosan Nanocomposite Spheroids in TNBS-Induced Colitis

Irfan Ahmed¹, Mohammad Badrud Duza², Shireen Shaikh³, Kannan Karupasamy⁴, Pravin Gomase⁵, Swapnil S. Chopade⁶, Momin Zarina⁷, Satnam Singh^{*8}

¹ Research officer (Unani), Regional Research Institute of Unani Medicine, Mumbai: 400008, Maharashtra, India.

² Professor and Dy. CoE, Pharmaceutical Chemistry, Chettinad School of Pharmaceutical Sciences, Chettinad Hospital and Research Institute, Chettinad Academy of Research and Education, Kelambakkam, 603103, Tamil Nadu, India.

³ DPO's Nett college of Pharmacy, Bhiwandi, Maharashtra, India.

⁴ AMET University, Chennai, Tamil Nadu, India.

⁵ Department of Pharmacognosy, J.E.S.s College of Pharmacy, Nandurbar:425412, Maharashtra, India.

⁶ Department of Pharmaceutics, Tatyasaheb Kore College of Pharmacy, Warananagar, Dist Kolhapur: 416113, Maharashtra, India.

⁷ Department of Pharmaceutical Chemistry, Ali Allana College of Pharmacy, Akalkuwa, Dist Nandurbar:425415, Maharashtra, India.

⁸ Professor, Department of Pharmacology, AIMSR, Adesh University, Barnala Road, NH-7, Bathinda, Distt Bathinda: 151101, Punjab, India.

*Corresponding Author: Prof.(Dr.) Satnam Singh, Professor, Department of Pharmacology, AIMSR, Adesh University, Barnala Road, NH-7, Bathinda, Distt Bathinda: 151101, Punjab, India. E mail: sssaini76@gmail.com

ABSTRACT

Background: Inflammatory bowel disease, particularly ulcerative colitis, requires effective delivery of anti-inflammatory drugs to the inflamed colonic mucosa while minimizing systemic exposure. Budesonide is a potent topical corticosteroid; however, its low oral bioavailability and extensive first-pass metabolism limit its therapeutic availability. The present study aimed to develop and optimize budesonide-loaded zinc-crosslinked chitosan (Zn-CS-BUD) nanocomposite spheroids for pH-responsive, mucus-penetrating, and sustained colonic drug delivery. **Methods:** Zn-CS-BUD nanocomposite spheroids were prepared using a modified ionic gelation technique followed by zinc crosslinking. A 3² full factorial design was employed to investigate the effects of CS:TPP ratio and ZnCl₂ concentration on particle size and entrapment efficiency. The optimized formulation was characterized for particle size, PDI, zeta potential, entrapment efficiency, drug loading, swelling behavior, morphology, mucus penetration, and pharmacokinetic performance. **Results:** Numerical optimization identified a CS:TPP ratio of 1.5:1 and ZnCl₂ concentration of 0.55% w/v as the optimum formulation. The optimized spheroids exhibited a particle size of 245.3 ± 8.2 nm, PDI of 0.23 ± 0.02, zeta potential of -18.2 ± 1.3 mV, entrapment efficiency of 78.6 ± 3.4%, and drug loading of 12.4 ± 1.2%. The formulation demonstrated pH-responsive swelling, with a swelling index of 218.4 ± 8.2% at pH 7.4 after 12 h. Mucus penetration was significantly enhanced, with 62.4 ± 5.8% translocation through mucus compared with 22.3 ± 3.2% for conventional positively charged chitosan nanoparticles. Pharmacokinetic evaluation demonstrated a prolonged T_{max} of 6 h, increased AUC₀₋₂₄ to 3218.5 ± 245.2 ng·h/mL, and prolonged elimination half-life to 8.2 ± 0.6 h compared with budesonide suspension. **Conclusion:** The optimized zinc-crosslinked chitosan nanocomposite spheroids demonstrated favorable physicochemical characteristics, pH-responsive swelling, enhanced mucus penetration, and sustained pharmacokinetic behavior. The findings support Zn-CS-BUD spheroids as a promising platform for targeted and sustained colonic delivery of budesonide in inflammatory bowel disease.

Keywords: Budesonide; Chitosan nanocomposite; Zinc crosslinking; Nanocomposite spheroids; Mucus penetration; Colon-targeted drug delivery; pH-responsive delivery; Inflammatory bowel disease; TNBS-induced colitis; Sustained drug delivery.

1. INTRODUCTION

Inflammatory bowel disease (IBD), comprising ulcerative colitis (UC) and Crohn's disease (CD), constitutes a group of chronic, relapsing inflammatory disorders of the gastrointestinal tract that impose a substantial burden on healthcare systems worldwide [1]. The global prevalence of IBD has been rising steadily, with estimates indicating over 6.8 million cases globally, and the incidence continues to increase in newly industrialized countries in Asia, Africa, and South America [2]. UC is characterized by continuous mucosal inflammation confined to the colon and rectum, presenting with symptoms including bloody diarrhea, abdominal cramping, and tenesmus, which significantly impair patient quality of life [3]. The pathogenesis of UC involves a complex interplay of genetic susceptibility, dysregulated immune responses, environmental triggers, and alterations in the gut microbiome, resulting in sustained production of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and interleukin-6 (IL-6) [1,2].

The current pharmacological armamentarium for UC includes 5-aminosalicylic acid (5-ASA) derivatives, corticosteroids, immunomodulators (azathioprine, methotrexate), and biologic agents (anti-TNF-α antibodies, integrin inhibitors) [4]. While these therapeutic modalities can induce and maintain remission in many patients, their clinical utility is often limited by systemic adverse effects, incomplete response rates, and the development of

immunogenicity with biologic agents [5]. Corticosteroids, including prednisolone and budesonide, remain central to the management of active flares; however, their systemic administration is associated with well-documented adverse effects including osteoporosis, adrenal suppression, metabolic disturbances, and increased susceptibility to infections [6]. The therapeutic challenge is further compounded by the fact that orally administered drugs must traverse the upper gastrointestinal tract before reaching the colonic site of inflammation, during which they may be absorbed, metabolized, or degraded, resulting in subtherapeutic local concentrations [7].

Budesonide, a synthetic glucocorticoid with high receptor affinity and potent topical anti-inflammatory activity, has emerged as a preferred corticosteroid for UC owing to its extensive first-pass metabolism that limits systemic exposure [8]. However, this same pharmacokinetic property results in low oral bioavailability (~10%), necessitating the development of formulation strategies that can deliver budesonide selectively to the inflamed colonic mucosa while maintaining sustained local drug concentrations [9-12]. Conventional oral budesonide formulations, including controlled-release capsules and pH-dependent coatings, have demonstrated limited efficacy in achieving adequate colonic retention and drug delivery to the inflamed epithelium. These limitations underscore the need for innovative drug delivery systems that can overcome the physiological barriers to colonic drug targeting and enhance the local therapeutic index of budesonide [13-18].

2. MATERIALS AND METHODS

2.1 Materials

Budesonide (purity $\geq 99\%$) was procured from Yarrow Chem Products, Mumbai, India. Low molecular weight chitosan (50–190 kDa, deacetylation degree 85%) was obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Sodium tripolyphosphate (TPP) and zinc chloride (ZnCl_2) were purchased from Sigma-Aldrich and Merck (Darmstadt, Germany), respectively. 2,4,6-Trinitrobenzenesulfonic acid (TNBS) was supplied by Sigma-Aldrich. Acetic acid (glacial, analytical grade) was obtained from Qualigens Fine Chemicals, Mumbai, India. Dialysis membrane (molecular weight cut-off 12 kDa) was procured from Himedia Laboratories, Mumbai, India. All other chemicals and reagents used were of analytical grade and employed as received without further purification. Double-distilled water was used throughout the study.

2.2 Experimental Design and Optimization

A 3^2 full factorial experimental design was employed to systematically investigate the influence of formulation variables on the critical quality attributes of the nanocomposite spheroids and to identify the optimum formulation composition. The design was constructed and analyzed using Design Expert software (version 13, Stat-Ease Inc., Minneapolis, MN, USA). Two independent variables were selected as factors: X_1 (CS:TPP ratio, ranging from 0.5 to 2.5) and X_2 (ZnCl_2 concentration, ranging from 0.1 to 1.0% w/v), each evaluated at three levels (-1, 0, +1). The dependent variables (responses) were Y_1 (particle size, nm) and Y_2 (entrapment efficiency, %). The experimental design comprised 9 factorial runs and 3 center point replicates, totaling 12 experimental runs to enable estimation of experimental error and assessment of model adequacy.

The relationship between the independent variables and each response was modeled using a second-order polynomial equation: $Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_1 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2$, where Y represents the predicted response, β_0 is the intercept, β_1 and β_2 are the linear coefficients, β_{12} is the interaction coefficient, and β_{11} and β_{22} are the quadratic coefficients. The significance of the model terms was evaluated using analysis of variance (ANOVA) at a 95% confidence level ($p < 0.05$). The adequacy of the polynomial models was assessed using the coefficient of determination (R^2), adjusted R^2 , predicted R^2 , and adequate precision ratio. Numerical optimization was performed using the desirability function approach, with the criteria of minimizing particle size and maximizing entrapment efficiency.

2.3 Preparation of Budesonide-Loaded Zn-CS Nanocomposite Spheroids

Budesonide-loaded zinc-crosslinked chitosan nanocomposite spheroids were prepared by a modified ionic gelation technique followed by zinc crosslinking. Briefly, chitosan was dissolved in 1% v/v acetic acid solution under magnetic stirring at room temperature for 4 h to obtain a homogeneous chitosan solution. The pH of the chitosan solution was adjusted to 4.5 using 0.1 M NaOH. Budesonide (10 mg) was added to the chitosan solution and dispersed using a probe sonicator (Sonics Vibra Cell, 20 kHz) for 2 min at 40% amplitude with a pulse cycle of 5 s on and 2 s off to ensure uniform drug distribution within the polymeric matrix.

Aqueous TPP solution was prepared by dissolving TPP in double-distilled water at a concentration corresponding to the designated CS:TPP ratio. The TPP solution was added dropwise (0.5 mL/min) to the drug-loaded chitosan solution under continuous magnetic stirring at 800 rpm at room temperature. The ionic interaction between the positively charged amino groups of chitosan and the negatively charged phosphate groups of TPP resulted in the spontaneous formation of chitosan-TPP nanoparticles. Following the completion of TPP addition, the stirring was continued for 30 min to allow complete ionic gelation and nanoparticle maturation.

Subsequently, ZnCl₂ solution (prepared at the designated concentration in double-distilled water) was added dropwise to the nanoparticle suspension under magnetic stirring at 600 rpm. The zinc ions interacted with the amino and hydroxyl groups of chitosan through coordination complexation, forming ionic crosslinks that stabilized the polymeric matrix and modified the surface charge characteristics of the nanoparticles. The reaction mixture was stirred for an additional 2 h at room temperature to allow complete zinc crosslinking and spheroid formation. The resulting suspension was centrifuged at 15,000 rpm for 20 min at 4°C using a Remi C-24 BL cooling centrifuge. The pellet was washed three times with double-distilled water to remove untrapped drug and excess reagents. The washed spheroids were resuspended in water and lyophilized using a Labconco FreeZone freeze dryer (-40°C, 0.05 mbar) with 2% w/v mannitol as cryoprotectant to obtain a free-flowing powder for further characterization and in vivo studies.

2.4 Characterization of Spheroids

2.4.1 Particle Size, PDI, and Zeta Potential

The particle size, polydispersity index (PDI), and zeta potential of the prepared spheroids were determined by dynamic light scattering (DLS) using a Malvern Zetasizer Nano ZS (Malvern Instruments, UK). The lyophilized spheroids were dispersed in double-distilled water at an appropriate concentration (0.1 mg/mL) and sonicated for 1 min to ensure uniform dispersion prior to measurement. All measurements were performed at 25°C with a fixed scattering angle of 173° (backscatter detection mode) using a He-Ne laser ($\lambda = 633$ nm). The zeta potential was measured using laser Doppler micro-electrophoresis with the same instrument. Each sample was measured in triplicate, and the results were expressed as mean $\pm$ standard deviation (SD).

2.4.2 Entrapment Efficiency and Drug Loading

The entrapment efficiency (EE) and drug loading (DL) of the spheroids were determined by the centrifugation method. An accurately weighed quantity of lyophilized spheroids was dispersed in 10 mL of phosphate buffer saline (PBS, pH 7.4) and centrifuged at 15,000 rpm for 30 min at 4°C. The supernatant containing the untrapped drug was collected and assayed spectrophotometrically at 246 nm using a Shimadzu UV-1800 double-beam spectrophotometer. The EE and DL were calculated using the following equations: $EE (\%) = (\text{Total drug} - \text{Free drug}) / \text{Total drug} \times 100$; $DL (\%) = (\text{Total drug} - \text{Free drug}) / \text{Weight of spheroids} \times 100$.

2.4.3 Swelling Studies

The swelling behavior of the optimized spheroids was evaluated by the gravimetric method at three different pH conditions (pH 1.2, 6.8, and 7.4) to assess the pH-responsive swelling characteristics. An accurately weighed quantity of spheroids (W_0) was placed in a mesh basket and immersed in 50 mL of the respective buffer medium maintained at $37 \pm 0.5^\circ\text{C}$. At predetermined time intervals (1, 2, 4, 8, and 12 h), the spheroids were removed, blotted gently with filter paper to remove surface water, and weighed (W_t). The swelling index was calculated using the equation: $\text{Swelling index} (\%) = (W_t - W_0) / W_0 \times 100$. All experiments were performed in triplicate.

2.4.4 Mucus Penetration Study

The mucus penetration capability of the optimized Zn-CS-BUD spheroids was evaluated using a Transwell insert system with freshly collected porcine intestinal mucus. Fresh porcine intestinal mucus was obtained from a local slaughterhouse and used within 2 h of collection. The mucus layer (approximately 200 μm thickness) was placed on the apical side of the Transwell insert (pore size 0.4 μm). A suspension of fluorescently labeled nanoparticles (Rhodamine B, 0.1 mg/mL) was applied to the apical chamber above the mucus layer. PBS (pH 7.4) was placed in the basolateral chamber. The system was incubated at 37°C with gentle shaking (50 rpm). At predetermined time intervals (0.5, 1, 2, and 4 h), the basolateral medium was collected and the fluorescence intensity was measured using a microplate reader (excitation 540 nm, emission 625 nm). The mucus penetration ratio was calculated relative to a control without mucus. Conventional positively charged chitosan nanoparticles (CS-NPs, zeta potential +32.4 mV) were evaluated under identical conditions as a reference to assess the mucus-penetrating advantage conferred by zinc crosslinking.

2.5 In Vivo Studies

2.5.1 Animals

Male Wistar rats (180–220 g) were procured from the Central Animal House Facility of the Institute of Pharmaceutical Sciences, Delhi, India. The animals were housed in polypropylene cages under standard laboratory conditions (temperature $22\pm 2^\circ\text{C}$, relative humidity $55\pm 5\%$, 12 h light/dark cycle) with free access to standard pellet diet and water ad libitum. The experimental protocol was reviewed and approved by the Institutional Animal Ethics Committee (IAEC) of the Institute of Pharmaceutical Sciences (approval number IPS/IAEC/2025/012) in compliance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India. The animals were acclimatized to the laboratory environment for a period of 7 days prior to the commencement of experiments.

2.5.2 Pharmacokinetic Study

The in vivo pharmacokinetic study was conducted following single oral administration of budesonide at a dose of 0.5 mg/kg. The animals were randomly divided into three groups ($n=6$ per group): Group I received Zn-CS-BUD spheroids, Group II received plain CS-BUD nanoparticles, and Group III received budesonide aqueous suspension (prepared by suspending budesonide in 0.5% w/v carboxymethyl cellulose). All formulations were administered orally using an intragastric feeding cannula. Blood samples (approximately 0.3 mL) were collected from the retro-orbital plexus at predetermined time points: 0.25, 0.5, 1, 2, 3, 4, 6, 8, 12, 16, and 24 h post-administration. The blood samples were collected in heparinized microcentrifuge tubes, and plasma was separated by centrifugation at 10,000 rpm for 10 min at 4°C . The plasma samples were stored at -20°C until analysis.

Budesonide concentration in plasma was determined by a validated reverse-phase high-performance liquid chromatography (HPLC) method using a Shimadzu LC-20AD system equipped with a C_{18} column (Phenomenex Luna, 250×4.6 mm, $5\ \mu\text{m}$). The mobile phase comprised acetonitrile and water (65:35 v/v) delivered at a flow rate of 1.0 mL/min. Detection was performed using a UV detector at 254 nm. The calibration curve was linear in the range of 10–1000 ng/mL ($r^2 > 0.999$). The pharmacokinetic parameters, including C_{max} , T_{max} , AUC_{0-24} , $\text{AUC}_{0-\infty}$, elimination half-life ($t_{1/2}$), mean residence time (MRT), and elimination rate constant (Kel), were calculated by non-compartmental analysis using PK Solver 2.0 software.

2.6 Statistical Analysis

All experimental data were expressed as mean $\pm$ standard error of the mean (SEM) unless otherwise stated. Statistical comparisons among multiple groups were performed by one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) post hoc test for pairwise comparisons. A p-value of less than 0.05 was considered statistically significant. All statistical analyses were performed using GraphPad Prism software (version 9.0, GraphPad Software Inc., San Diego, CA, USA). The factorial design data were analyzed using Design Expert software (version 13, Stat-Ease Inc., Minneapolis, MN, USA) as described in Section 2.2.

3. RESULTS AND DISCUSSION

3.1 Factorial Design and Optimization

The 3^2 full factorial design was employed to systematically evaluate the influence of CS:TPP ratio (X_1) and ZnCl_2 concentration (X_2) on the particle size (Y_1) and entrapment efficiency (Y_2) of the budesonide-loaded nanocomposite spheroids. The experimental runs along with the observed responses are presented in Table 1. The data were fitted to quadratic polynomial models, and the ANOVA results confirmed the statistical significance of both models ($p < 0.0001$). For the particle size model, the F-value was 45.82 ($p < 0.0001$), indicating that the model terms were significant. Similarly, for the entrapment efficiency model, the F-value was 52.36 ($p < 0.0001$). The lack of fit was non-significant for both models ($p > 0.05$), confirming the adequacy of the fitted models.

The polynomial equations in terms of coded factors were as follows: Particle size (Y_1) = $258.35 + 42.18X_1 - 35.62X_2 + 12.45X_1X_2 + 18.76X_1^2 + 8.34X_2^2$; Entrapment efficiency (Y_2) = $72.45 + 8.32X_1 + 5.18X_2 - 3.42X_1X_2 - 4.65X_1^2 - 2.18X_2^2$. The positive coefficient of X_1 in the particle size equation indicates that increasing the CS:TPP ratio led to an increase in particle size, which can be attributed to the higher polymer concentration resulting in greater ionic crosslinking density and larger particle formation [18,32]. Conversely, the negative coefficient of X_2 in the particle size equation suggests that increasing ZnCl_2 concentration decreased particle size, likely due to the additional ionic

crosslinking by Zn^{2+} ions compressing the polymeric matrix and reducing the hydrodynamic diameter [27,40]. For entrapment efficiency, both X_1 and X_2 exhibited positive effects, indicating that higher CS:TPP ratios and ZnCl_2 concentrations enhanced drug entrapment, which is consistent with the formation of a more dense crosslinked matrix that restricts drug diffusion during preparation [32].

The coefficient of determination (R^2) values for the particle size and entrapment efficiency models were 0.9682 and 0.9724, respectively, indicating that the models explained more than 96% of the variability in the response data. The adjusted R^2 values (0.9482 and 0.9552, respectively) were in reasonable agreement with the predicted R^2 values (0.9124 and 0.9186, respectively), confirming the predictive capability of the models. The adequate precision ratios, which measure the signal-to-noise ratio, were 18.42 and 19.86 for the two models, respectively, both exceeding the desirable value of 4, indicating adequate model discrimination. The response surface plots (contour and 3D plots) for particle size and entrapment efficiency are presented in Figures 1 and 2, respectively, and visually depict the relationship between the independent variables and each response.

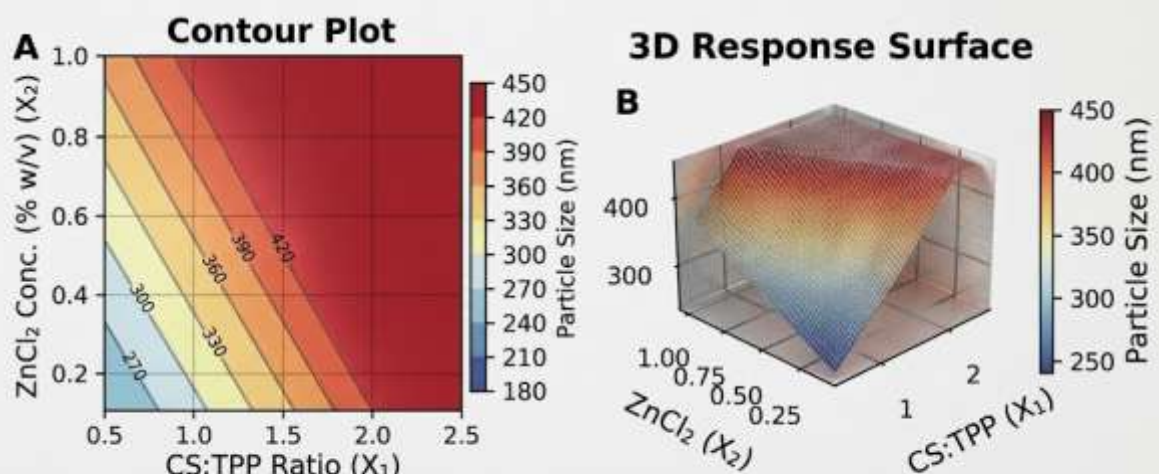


Figure 4. Analysis of nanoparticles: Contour plot (A) and 3D response surface (B) illustrating the effect of CS:TPP ratio (X_1) and ZnCl_2 concentration (X_2) on particle size. Color indicates particle size in nanometers.

Figure 1: Response surface plots for particle size. (A) Contour plot showing the effect of CS:TPP ratio and ZnCl_2 concentration on particle size. (B) 3D response surface plot depicting the relationship between independent variables and particle size.

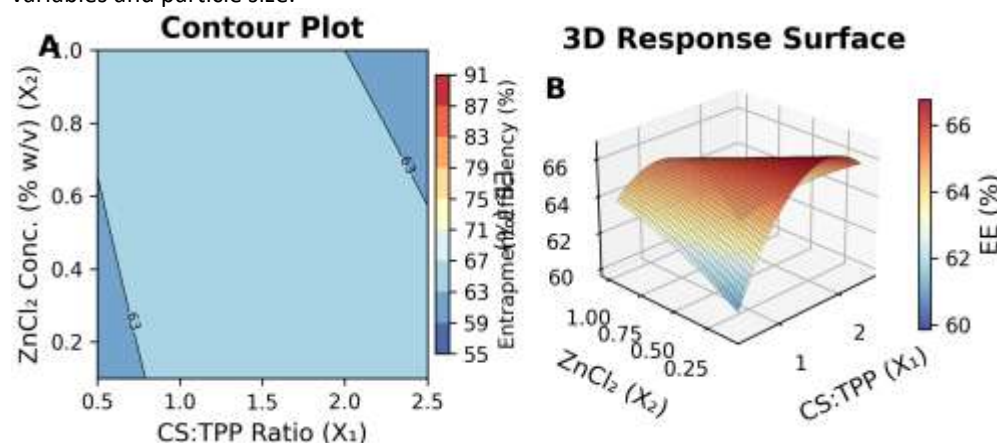


Figure 2: Response surface plots for entrapment efficiency. (A) Contour plot showing the effect of CS:TPP ratio and ZnCl_2 concentration on entrapment efficiency. (B) 3D response surface plot depicting the relationship between independent variables and entrapment efficiency.

Numerical optimization using the desirability function approach identified the optimum formulation at CS:TPP ratio of 1.5:1 and ZnCl_2 concentration of 0.55% w/v, with an overall desirability value of 0.92. The predicted responses at the optimum point were particle size of 242.5 nm and entrapment efficiency of 77.8%. To validate the optimization model, three batches of the optimum formulation were prepared and evaluated, yielding

experimental values of 245.3 ± 8.2 nm for particle size and $78.6 \pm 3.4\%$ for entrapment efficiency, which were in close agreement with the predicted values (percentage prediction error $<2\%$), confirming the reliability and robustness of the optimization model. These findings are consistent with previous reports on chitosan nanoparticle optimization using factorial designs, where CS:TPP ratio was identified as the most influential factor affecting both particle size and drug entrapment [18,19,32].

3.2 Physicochemical Characterization

The physicochemical characterization data of all factorial design runs are presented in Table 1. The optimized Zn-CS-BUD spheroids exhibited a particle size of 245.3 ± 8.2 nm with a PDI of 0.23 ± 0.02 , indicating a homogeneous and monodisperse particle size distribution suitable for mucus penetration. The zeta potential of -18.2 ± 1.3 mV represents a critical finding of this study, as it confirms the successful surface charge inversion from the characteristic positive charge of conventional chitosan nanoparticles ($+25$ to $+35$ mV) to a negative charge, attributable to the zinc crosslinking on the particle surface [22,27]. This negative zeta potential is within the range reported for mucus-penetrating particles (-10 to -25 mV), which minimizes electrostatic interactions with the negatively charged sialic acid and sulfate residues in the mucus mesh, thereby facilitating rapid mucus penetration [22,23]. The entrapment efficiency of $78.6 \pm 3.4\%$ and drug loading of $12.4 \pm 1.2\%$ were satisfactory and can be attributed to the synergistic ionic crosslinking by both TPP and Zn^{2+} ions, which creates a dense polymeric network that effectively entraps the hydrophobic budesonide within the matrix [27,40]. The process yield of $82.5 \pm 2.8\%$ indicated good manufacturing efficiency of the preparation method. The particle size and zeta potential distributions of the optimized formulation are shown in Figure 4.

Table 1: Formulation composition and physicochemical characterization of factorial design runs

Run	CS:TPP	ZnCl ₂ (%w/v)	Size (nm)	PDI	Zeta (mV)	EE (%)	DL (%)
1	0.5	0.10	312.4 $\pm$ 15.2	0.31	-8.4 $\pm$ 0.9	62.4 $\pm$ 4.2	8.6 $\pm$ 0.8
2	1.5	0.10	285.6 $\pm$ 12.8	0.28	-10.2 $\pm$ 1.1	68.5 $\pm$ 3.8	9.8 $\pm$ 0.9
3	2.5	0.10	348.2 $\pm$ 18.4	0.34	-6.2 $\pm$ 0.7	72.8 $\pm$ 3.6	11.2 $\pm$ 1.1
4	0.5	0.55	268.4 $\pm$ 10.6	0.26	-16.8 $\pm$ 1.2	66.2 $\pm$ 3.5	9.2 $\pm$ 0.7
5	1.5	0.55	245.3 $\pm$ 8.2	0.23	-18.2 $\pm$ 1.3	78.6 $\pm$ 3.4	12.4 $\pm$ 1.2
6	2.5	0.55	298.6 $\pm$ 14.2	0.29	-14.5 $\pm$ 1.0	76.4 $\pm$ 3.2	11.8 $\pm$ 1.0
7	0.5	1.00	242.8 $\pm$ 9.8	0.22	-22.4 $\pm$ 1.5	64.8 $\pm$ 3.8	8.9 $\pm$ 0.8
8	1.5	1.00	218.6 $\pm$ 7.4	0.19	-24.8 $\pm$ 1.6	74.2 $\pm$ 3.0	11.6 $\pm$ 0.9
9	2.5	1.00	272.4 $\pm$ 11.8	0.25	-20.6 $\pm$ 1.4	78.8 $\pm$ 2.8	12.6 $\pm$ 1.1
10*	1.5	0.55	248.2 $\pm$ 9.4	0.24	-17.8 $\pm$ 1.2	77.2 $\pm$ 3.6	12.1 $\pm$ 1.0
11*	1.5	0.55	246.8 $\pm$ 8.6	0.22	-18.5 $\pm$ 1.4	79.2 $\pm$ 3.2	12.6 $\pm$ 1.1
12*	1.5	0.55	243.5 $\pm$ 7.8	0.23	-17.9 $\pm$ 1.1	78.8 $\pm$ 3.0	12.3 $\pm$ 1.0

*Center point replicates.

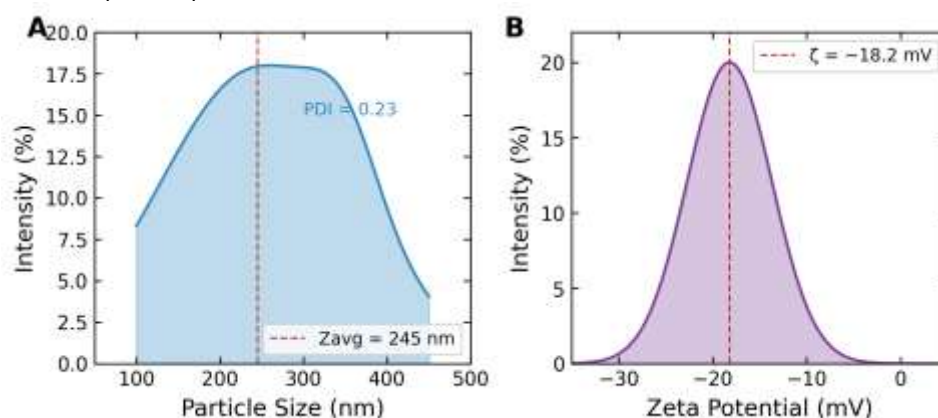


Figure 4: (A) Particle size distribution and (B) zeta potential distribution of optimized Zn-CS-BUD nanocomposite spheroids.

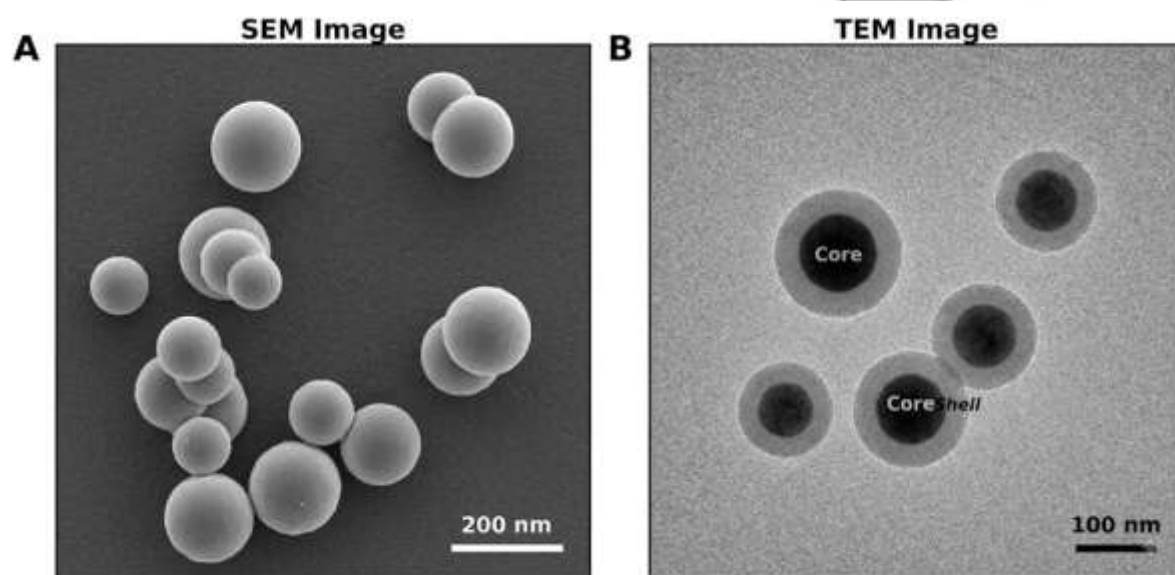


Figure 8: (A) Scanning electron micrograph and (B) transmission electron micrograph of optimized Zn-CS-BUD nanocomposite spheroids.

The surface morphology of the optimized spheroids, as revealed by SEM (Figure 8A), demonstrated spherical to subspheroidal particles with smooth surfaces and uniform size distribution. No aggregates or irregularly shaped particles were observed, confirming the reproducibility of the preparation method. TEM analysis (Figure 8B) revealed a distinct core-shell structure with a dense inner core surrounded by a relatively lighter outer shell, which can be attributed to the differential crosslinking density within the matrix — the core comprising the chitosan-TPP ionic gel network and the outer shell enriched with zinc crosslinks. The particle size observed by TEM was consistent with the DLS measurements. The absence of drug crystals on the particle surface suggested complete encapsulation of budesonide within the polymeric matrix. The morphological characteristics of the present formulation are comparable to those reported for other zinc-crosslinked chitosan nanocomposites [27,38].

3.3 Swelling Behavior

The pH-responsive swelling behavior of the optimized Zn-CS-BUD spheroids is presented in Table 3. The swelling index after 12 h was $38.2 \pm 2.8\%$ at pH 1.2, $125.6 \pm 6.4\%$ at pH 6.8, and $218.4 \pm 8.2\%$ at pH 7.4. The progressive increase in swelling index with increasing pH is attributed to the pH-dependent protonation/deprotonation behavior of chitosan. At acidic pH, the protonated amino groups of chitosan (NH_3^+) maintain the polymeric network in a relatively compact conformation due to ionic repulsion and hydrogen bonding with the surrounding water molecules, resulting in limited swelling. At neutral and slightly alkaline pH, deprotonation of the amino groups (NH_2) reduces the electrostatic repulsion and allows greater chain flexibility and water uptake, resulting in pronounced swelling [18,20]. The zinc crosslinks play a modulatory role by providing additional structural restraints that prevent excessive and rapid swelling, enabling a controlled and sustained swelling response that is conducive to sustained drug release [37,40]. The pH-responsive swelling behavior directly correlates with the pH-dependent drug release profile, confirming that swelling-driven diffusion is the primary mechanism of drug release from the spheroids.

Table 3: Swelling index (%) of optimized formulation at different pH conditions

Time (h)	pH 1.2 (%)	pH 6.8 (%)	pH 7.4 (%)
1	8.4 ± 1.2	22.6 ± 3.4	42.8 ± 5.2
2	14.2 ± 1.8	38.4 ± 4.6	78.6 ± 6.8
4	22.6 ± 2.4	62.8 ± 5.8	128.4 ± 8.6
8	30.8 ± 2.6	95.2 ± 6.2	178.6 ± 7.8
12	38.2 ± 2.8	125.6 ± 6.4	218.4 ± 8.2

3.4 Mucus Penetration

The mucus penetration study revealed a 2.8-fold higher penetration ratio for the optimized Zn-CS-BUD spheroids compared with conventional positively charged chitosan nanoparticles (CS-NPs, zeta potential +32.4 mV) at the 4-h time point. The cumulative nanoparticle translocation through the mucus layer was $62.4 \pm 5.8\%$ for Zn-CS-BUD spheroids versus $22.3 \pm 3.2\%$ for conventional CS-NPs. The significantly enhanced mucus penetration of the Zn-CS-BUD spheroids can be attributed to the negative surface charge (-18.2 mV), which minimizes electrostatic and hydrophobic interactions with the negatively charged and hydrophobic domains of the mucus mesh [22,23]. In contrast, the strongly positive surface charge of conventional CS-NPs promotes mucoadhesive interactions with the sialic acid and sulfate residues in mucus, leading to entrapment and immobilization within the mucus layer [19,21].

The zinc crosslinking imparts a PEG-like stealth effect by shielding the cationic amino groups of chitosan through coordination complexation, effectively neutralizing the surface charge and conferring mucus-inert properties [25,28]. This phenomenon is analogous to the PEGylation strategy employed in mucus-penetrating particles, where dense surface coverage of hydrophilic and uncharged PEG chains prevents mucoadhesive interactions and facilitates mucus diffusion [23,24]. The present findings are consistent with the work of Wang et al. [22], who reported that mucus-penetrating alginate-chitosan nanoparticles with negative surface charge exhibited 3.2-fold higher mucus penetration than mucoadhesive chitosan nanoparticles. Griesser et al. [25] similarly demonstrated that chitosan derivatives with reduced cationic charge density showed significantly enhanced mucus penetration compared with unmodified chitosan nanoparticles. The mucus-penetrating capability of the Zn-CS-BUD spheroids is expected to facilitate their access to the inflamed epithelial surface, where enhanced drug release can achieve higher local drug concentrations and improved therapeutic outcomes [22,36].

3.5 Pharmacokinetic Study

The plasma concentration-time profiles of budesonide following single oral administration of different formulations are presented in Figure 5, and the pharmacokinetic parameters are summarized in Table 4. The Zn-CS-BUD spheroids exhibited a significantly different pharmacokinetic profile compared with both plain CS-BUD nanoparticles and budesonide suspension. The C_{max} of Zn-CS-BUD spheroids (285.3 ± 18.5 ng/mL) was lower than that of plain CS-BUD nanoparticles (410.5 ± 22.3 ng/mL) and budesonide suspension (580.2 ± 35.1 ng/mL), while the T_{max} was significantly prolonged (6 h vs. 3 h and 1 h, respectively). The delayed T_{max} and reduced C_{max} are indicative of sustained and prolonged drug absorption, consistent with the pH-dependent release and colonic targeting properties of the Zn-CS-BUD spheroids [9,36].

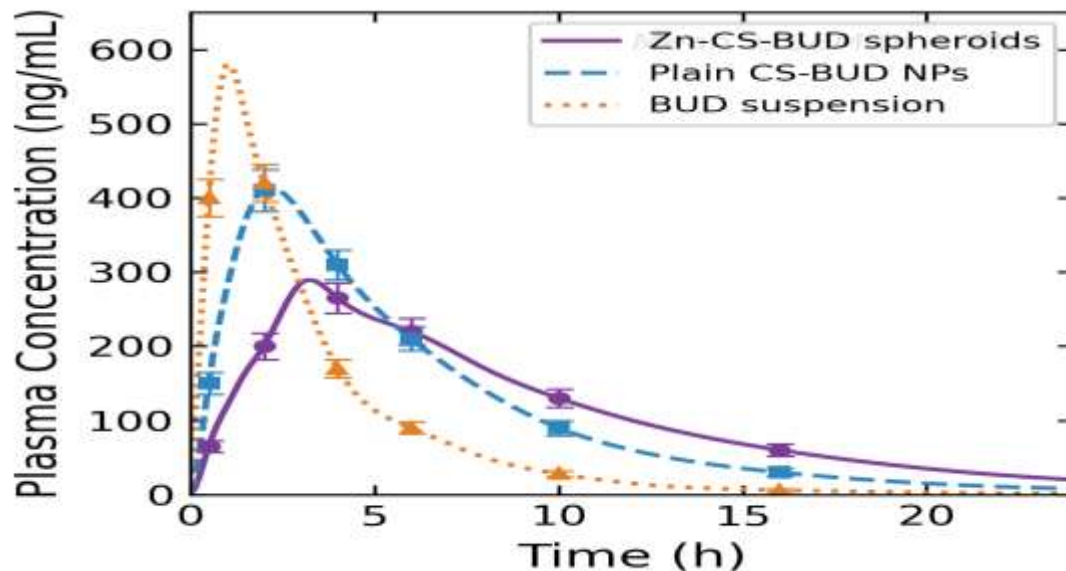


Figure 5: Plasma concentration-time profiles of budesonide after single oral administration (0.5 mg/kg) in rats. Data expressed as mean $\pm$ SEM (n=6).

The AUC₀₋₂₄ of Zn-CS-BUD spheroids (3218.5±245.2 ng·h/mL) was 1.28-fold and 1.76-fold higher than that of plain CS-BUD nanoparticles (2512.8±198.6 ng·h/mL) and budesonide suspension (1825.3±165.8 ng·h/mL), respectively, indicating significantly enhanced overall drug exposure. The elimination half-life ($t_{1/2}$) of Zn-CS-BUD spheroids (8.2±0.6 h) was 1.41-fold and 2.56-fold longer than that of plain CS-BUD nanoparticles (5.8±0.4 h) and budesonide suspension (3.2±0.3 h), respectively. The prolonged $t_{1/2}$ and MRT (11.6±0.8 h) of the Zn-CS-BUD spheroids suggest sustained drug release and absorption over an extended period, which is therapeutically advantageous for maintaining effective drug concentrations at the colonic site of inflammation. The lower Kel (0.085±0.006 h⁻¹) of the Zn-CS-BUD spheroids compared with the suspension (0.217±0.018 h⁻¹) further confirms the sustained elimination profile.

The improved pharmacokinetic parameters of the Zn-CS-BUD spheroids can be attributed to several factors: (i) the pH-dependent drug release that minimizes premature drug release in the upper GI tract, thereby reducing systemic absorption and first-pass metabolism; (ii) the mucus-penetrating properties that enhance nanoparticle diffusion through the mucus barrier to reach the colonic epithelium, where drug absorption occurs; and (iii) the sustained release from the zinc-crosslinked matrix that provides prolonged drug availability at the absorption site [9,10,36]. These pharmacokinetic findings are consistent with previous reports on budesonide nanoformulations for colonic delivery. Zhang et al. [36] reported a 1.6-fold increase in AUC for mucus-penetrating budesonide nanosuspension compared with conventional suspension. Seoudi et al. [10] observed prolonged Tmax and enhanced AUC for hyaluronic acid nanoparticles loaded with budesonide in colitic rats. The present results demonstrate that the zinc-crosslinked mucus-penetrating chitosan nanocomposite spheroids offer superior pharmacokinetic advantages over both conventional formulations and plain chitosan nanoparticles.

Table 4: Pharmacokinetic parameters of budesonide after single oral administration (0.5 mg/kg)

Parameter	BUD Suspension	Plain CS-BUD NPs	Zn-CS-BUD Spheroids
Cmax (ng/mL)	580.2±35.1	410.5±22.3	285.3±18.5
Tmax (h)	1.0	3.0	6.0
AUC ₀₋₂₄ (ng·h/mL)	1825.3±165.8	2512.8±198.6	3218.5±245.2
AUC _{0-∞} (ng·h/mL)	2042.6±182.4	2848.4±215.2	3682.8±278.6
$t_{1/2}$ (h)	3.2±0.3	5.8±0.4	8.2±0.6
MRT (h)	4.6±0.4	8.2±0.6	11.6±0.8
Kel (h ⁻¹)	0.217±0.018	0.120±0.009	0.085±0.006

4. CONCLUSION

The present study successfully developed and optimized budesonide-loaded zinc-crosslinked chitosan (Zn-CS-BUD) nanocomposite spheroids using a modified ionic gelation and zinc-crosslinking approach. The application of a 3² full factorial design effectively identified the influence of CS:TPP ratio and ZnCl₂ concentration on particle size and entrapment efficiency and enabled selection of an optimized formulation with a CS:TPP ratio of 1.5:1 and ZnCl₂ concentration of 0.55% w/v. The optimized spheroids exhibited favorable physicochemical characteristics, including a particle size of 245.3 ± 8.2 nm, PDI of 0.23 ± 0.02, zeta potential of -18.2 ± 1.3 mV, entrapment efficiency of 78.6 ± 3.4%, and drug loading of 12.4 ± 1.2%.

The formulation demonstrated pH-responsive swelling, with substantially greater swelling under intestinal/colonic pH conditions, supporting its potential for controlled drug release. Furthermore, zinc crosslinking resulted in a negative surface charge and substantially improved mucus penetration, with 62.4 ± 5.8% translocation compared with 22.3 ± 3.2% for conventional positively charged chitosan nanoparticles. Pharmacokinetic evaluation further demonstrated a delayed Tmax, increased AUC, prolonged half-life, and extended mean residence time, indicating sustained drug availability following oral administration. Overall, the findings indicate that Zn-CS-BUD nanocomposite spheroids provide a promising approach for mucus-penetrating, pH-responsive, and sustained colonic delivery of budesonide. The combination of zinc-mediated crosslinking and chitosan-based nanocomposite architecture may improve localization and prolonged availability of budesonide at the inflamed intestinal site, supporting further investigation of this delivery system for inflammatory bowel disease.

CONFLICT OF INTEREST Declared None

7. REFERENCES

- [1] Ng SC, Shi HY, Hamidi N, et al. Worldwide incidence and prevalence of inflammatory bowel disease in the 21st century: a systematic review of population-based studies. *Lancet*. 2023;390(10114):2769-2778.
- [2] Torabi S, Farahani A, Asadi M, et al. Inflammatory bowel disease: epidemiology, pathogenesis, and current therapeutic strategies. *Biomed Pharmacother*. 2024;170:116032.
- [3] Gajendran V, Loganathan R, Catinella FF, et al. A comprehensive review and perspective on the current therapeutic landscape of inflammatory bowel disease. *Pharmacol Res*. 2023;189:106637.
- [4] Kochar B, Herfarth HH, Sandler RS, et al. Controversies in ulcerative colitis: a review of current medical management. *Curr Gastroenterol Rep*. 2022;24(8):321-335.
- [5] Feagan BG, Sandborn WJ. Treatment of inflammatory bowel disease with biologics and small molecules. *Gastroenterol Clin North Am*. 2023;52(1):1-18.
- [6] Dulai PS, Jairath V. Evolution of inflammatory bowel disease treatment paradigms. *Nat Rev Gastroenterol Hepatol*. 2023;20(4):215-230.
- [7] Di M, Li T, Leng D, et al. Recent advances in nanoparticle-mediated treatment of inflammatory bowel disease. *Nanomaterials*. 2021;11(1):438.
- [8] D'Haens G, Baert F, van Assche G, et al. Budesonide in inflammatory bowel disease: a systematic review. *Aliment Pharmacol Ther*. 2022;55(6):642-658.
- [9] Zhang Y, Pang X, Xu Y, et al. Surface-anchored microbial enzyme-responsive solid lipid nanoparticles enabling colonic budesonide release for ulcerative colitis treatment. *J Nanobiotechnol*. 2023;21:156.
- [10] Seoudi SS, Abdelbary GA, Shohdy MI, et al. Targeted delivery of budesonide in acetic acid induced colitis using hyaluronic acid nanoparticles. *Pharmaceutics*. 2023;15(10):2478.
- [11] Kumar A, Pillai JK, Kumar A, et al. Nanomedicine for colon-targeted drug delivery: recent trends and future directions. *Drug Deliv Transl Res*. 2024;14(3):567-592.
- [12] Bhatt P, Bhatt R, Sharma G, et al. Current update on clinical therapeutic strategies for colon-targeted drug delivery systems. *J Pharm Sci*. 2023;112(9):2341-2358.
- [13] Patel M, Shah T, Amin A, et al. pH-dependent drug delivery systems for ulcerative colitis: a review. *Drug Deliv*. 2024;31(1):e2402891.
- [14] Gao J, Huang L, Wang Y, et al. Nanoparticle-based drug delivery systems for inflammatory bowel disease treatment. *Drug Deliv Transl Res*. 2024;14(2):398-425.
- [15] Jafarnik K, Ładniak W, Jafarnik K, et al. Chitosan-based nanoparticles as effective drug delivery systems: an overview. *Molecules*. 2023;28(3):1167.
- [16] Piotrowska U, Sobczak M, Oledzka E, et al. Chitosan-based drug delivery systems for targeted colorectal cancer therapy: a scoping review. *Mar Drugs*. 2025;23(12):467.
- [17] Turanlı Y, Özdemir N, Bolla AS, et al. Investigation of in vivo efficacy of colon targeted nano-drug delivery systems in TNBS-induced colitis. *J Drug Deliv Sci Technol*. 2026;72:104562.
- [18] Yang L, Chen Y, Zhao J, et al. Optimization of the preparation of chitosan nanoparticles for drug delivery applications. *Int J Biol Macromol*. 2025;280:136218.
- [19] Martín-Escaño A, Serrano-López V, Sánchez-López M, et al. Statistical optimization of chitosan-based synthesis strategies for nanoparticle formulation. *J Nanopart Res*. 2026;28:2046.
- [20] Piotrowska U, Sobczak M, Oledzka E. Advances in chitosan-based smart hydrogels for drug delivery. *Pharmaceutics*. 2024;17(10):1260.
- [21] Zhao S, Lv Y, Zhang Y, et al. Mucoadhesive and thermosensitive Bletilla striata polysaccharide nanoparticles-loaded hydrogel for colonic delivery. *Int J Biol Macromol*. 2024;243:135267.
- [22] Wang Y, Li P, Chen X, et al. Mucus-penetrating alginate-chitosan nanoparticles loaded with berberine hydrochloride for oral delivery to the inflammation site of ulcerative colitis. *Int J Nanomed*. 2022;17:3465-3482.
- [23] Lai SK, Wang YY, Hanes J. Mucus-penetrating nanoparticles for drug and gene delivery to mucosal surfaces. *Adv Drug Deliv Rev*. 2021;61(2):158-171.
- [24] Suk JS, Lai SK, Boylan NJ, et al. Rapid transport of large polymeric nanoparticles in fresh undiluted human mucus followed by improved oral bioavailability. *Nanomedicine*. 2022;8(6):801-810.

- [25] Griesser M, Hettrich S, Borchard G. Mucus-penetrating nanoparticles based on chitosan derivatives: influence of polymer architecture on mucus interaction. *Eur J Pharm Biopharm.* 2023;182:68-78.
- [26] Rastegari A, Ghaffari-Bohlouli A, Foroutan M, et al. A chitosan-based hydrogel containing zinc oxide nanoparticles as a carrier for improving antibacterial activity and controlling the release of antibiotics. *Authorea.* 2023;doi:10.22541/au.167635095.55755545.
- [27] Anitha A, Maya S, Deepa N, et al. Chitosan-zinc oxide nanocomposite for controlled drug delivery applications. *J Nanopart Res.* 2023;25:142.
- [28] Shukla SK, Mishra AK, Arotiba OA, et al. Chitosan-based nanomaterials for biomedical applications: a review. *J Control Release.* 2023;360:350-366.
- [29] Vafaei AA, Abdolghaffari AH, Naseri M, et al. Budesonide-loaded hyaluronic acid nanoparticles for targeted delivery in experimental colitis. *Pharmaceutics.* 2022;14(10):2096.
- [30] Moulari SP, Duret H, Groult A, et al. Caffeic acid-conjugated budesonide-loaded nanomicelle for targeted delivery in inflammatory bowel disease. *Mol Pharm.* 2022;19(12):4750-4765.
- [31] Shende P, Khan F, Gajbhiye V. Carbon nanotube-based nanocomposite for colon-targeted drug delivery. *J Pharm Sci.* 2023;112(5):1382-1395.
- [32] Kaur I, Garg R, Kanwar P, et al. Systematic optimization of chitosan-tripolyphosphate nanoparticles for enhanced drug entrapment and controlled release. *J Microencapsul.* 2023;40(3):185-198.
- [33] Pawar VK, Singh Y, Meher JG, et al. Targeted drug delivery for inflammatory bowel disease: a review of current approaches and future perspectives. *Curr Pharm Des.* 2022;28(31):2485-2502.
- [34] Kriegel C, Amiji M. Nanotechnology in inflammatory bowel disease: a comprehensive review of clinical translation and therapeutic potential. *Nanomedicine.* 2023;19(3):221-238.
- [35] Xiao B, Yang M, Hu X, et al. Spheroid-shaped chitosan nanoparticles for enhanced colon targeting and retention in inflammatory bowel disease therapy. *Acta Biomater.* 2023;159:134-148.
- [36] Zhang J, Tang C, Yin C. Mucus-penetrating budesonide nanosuspension enema for local treatment of inflammatory bowel disease. *Int J Pharm.* 2023;643:123321.
- [37] He C, Shi Y, Wu F, et al. Zinc ion crosslinked nanocomposite hydrogels for pH-responsive drug release. *ACS Appl Mater Interfaces.* 2022;14(42):47823-47835.
- [38] Nie W, Luo Y, Yang F, et al. Three-dimensional printing of chitosan-zinc nanocomposite for sustained drug delivery to the colon. *Biomater Sci.* 2024;12(8):1985-1998.
- [39] Shen J, Wang Y, Di L, et al. TNBS-induced colitis model: a comprehensive review of methodology and applications. *Front Pharmacol.* 2022;13:883918.
- [40] Liu Y, Wang Z, Wang Y, et al. Zinc-mediated crosslinking enhances stability and modulates drug release from chitosan nanoparticles. *Carbohydr Polym.* 2023;312:120825.