Formulation, optimization and characterization of cationic polymeric nanoparticles of mast cell stabilizing agent using the Box–Behnken experimental design

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Abstract

Objective: The present research work was intended to develop and optimize sustained release of biodegradable chitosan nanoparticles (CSNPs) as delivery vehicle for sodium cromoglicate (SCG) using the circumscribed Box–Behnken experimental design (BBD) and evaluate its potential for oral permeability enhancement.

Methods: The 3-factor, 3-level BBD was employed to investigate the combined influence of formulation variables on particle size and entrapment efficiency (%EE) of SCG-CSNPs prepared by ionic gelation method. The generated polynomial equation was validated and desirability function was utilized for optimization. Optimized SCG-CSNPs were evaluated for physicochemical and in-vitro characterizations and permeability enhancement potential by ex-vivo and uptake study using CLSM.

Results: SCG-CSNPs exhibited particle size of 200.4 ± 4.06 nm and %EE of 62.68 ± 2.4% with unimodal size distribution having cationic, spherical, smooth surface. Physicochemical and in-vitro characterization revealed existence of SCG in amorphous form inside CSNPs without interaction and showed sustained release profile. Ex-vivo and uptake study showed the permeability enhancement potential of CSNPs.

Conclusions: The developed SCG-CSNPs can be considered as promising delivery strategy with respect to improved permeability and sustained drug release, proving importance of CSNPs as potential oral delivery system for treatment of allergic rhinitis. Hence, further studies should be performed for establishing the pharmacokinetic potential of the CSNPs.

Keywords

Allergic rhinitis, Box–Behnken experimental design, chitosan nanoparticles, permeability enhancement, sodium cromoglicate

Introduction

Allergic rhinitis (AR) is one of the most commonly diagnosed respiratory disorders in the globe which affects about 40% of population worldwide (nearly 600 million people) as per WHO. Among them, 30% of the children having AR are prone to develop asthma in their life¹. Sodium cromoglicate (SCG) is the safest and widely employed mast cell stabilizing agent for the prevention and treatment of allergic conditions. However, high hydrophilicity and consequently poor permeability through the gastrointestinal tract (GIT) is one of its major concerns². Further, SCG easily clears off owing to its shorter half-life, which ultimately increases dosing frequency and develops hurdles toward compliant pharmacotherapy³. In order to defeat the constrains, an alternative formulation strategy is highly desirable which could easily achieve therapeutically-active concentration followed by oral administration as well as improve patient compliance by reducing dosing frequency. Moreover, novel formulation indeed should be able to eliminate the obstacles related to dose-delivery variability and transient irritation which are commonly found associated with local dosing of marketed intranasal and pulmonary delivery systems. Various formulation strategies including pro-drugs and liposomes have been implied but limited success has been achieved due to their erratic and unpredictable absorption profiles as well as their inability to retain its integrity at absorption site⁴.

Nanoparticulate-carrier system can circumvent drawbacks encountered during the oral delivery of SCG. Polymeric nanoparticles (NPs) are colloidal submicron size entities ranging from 10–1000 nm in diameter, and are assembled from a wide variety of biodegradable and non-biodegradable polymers. The beneficial features such as protection of drugs from degradation, improvement in permeation of the drugs across GIT, controlled release of the encapsulated or adsorbed drug, reduction in dosing frequency etc. may aid in resolving the obstacles of non-adherence to the prescribed oral SCG therapy. The utility of NPs, particularly for oral drug delivery arises due to the particulate uptake mechanisms that exist in the GIT, especially transcellular absorption through the epithelial cells lining,
paracellular absorption and transport via M cells of Peyer’s Patches (PP) in the intestinal mucosa.

Chitosan is widely employed as a polymeric carrier for engineering of NPs due to its ideal properties such as biocompatibility, bioactivity, biodegradability, non-toxicity, low cost and mucoadhesivity. It also possesses a positive charge and exhibits an absorption enhancing effect. Moreover, chitosan NPs can transit directly and/or adhere to the mucosa, which is a prerequisite step before the translocation of particles across GIT.

Optimization of formulation by using statistical design of experiment is a powerful, efficient and logistic approach for the development of pharmaceutical dosage forms. The experimental design depicts the disparity of dependent variables as a function of independent variables in a well-designed experiment with the lowest number of runs in a systematic manner. It also allows the establishment of optimal parameters by the mode of suitable mathematical and graphical representation. Box–Behnken experimental design (BBD) is a class of rotatable second-order designs based on three-level incomplete factorial design that can efficiently envisage non-linear response models as compared to two level designs which limit prediction up to linear response models.

Thus, this study was intended to design and optimize biodegradable SCG encapsulated chitosan nanoparticles (SCG-CSNPs), using the Box–Behnken experimental design for oral permeability enhancement with the hypothesis that if the drug is entrapped in polymeric nanoparticles, there would be an enhancement in GI permeability by the likelihood of transcellular pathway through enterocytes of intestine and by paracellular pathway as well as uptake by M-cells of PP and hence, increased therapeutic efficacy of drug could be anticipated.

Materials and methods

Materials

Chitosan (MW 50 – 1000 kDa and Degree of deacetylation >85%) and SCG were kindly supplied as a gift sample from Cognis Gmbh, Germany and Entod Pharmaceutical Ltd., Mumbai, India, respectively. Sodium tripolyphosphate (TPP), Fluorescein isothiocyanate (FITC) and Rhodamine B were purchased from Sigma Aldrich (Bangalore, India). Glacial acetic acid was purchased from Loba chemie, Mumbai, India. All the chemicals used in the study were of analytical grade.

Preparation of sodium cromoglicate-encapsulated chitosan nanoparticles

The SCG-encapsulated chitosan nanoparticles (SCG-CSNPs) were prepared by ionic gelation method as previously reported by Calvo et al.: Chitosan was dissolved in 20 ml of 0.2% v/v aqueous glacial acetic acid solution under stirring at room temperature. Subsequently, TPP and SCG in predefined ratio were dissolved in 10 ml of distilled water. Later, SCG containing TPP solution was added into chitosan solution at constant flow rate of 0.5 ml/min under stirring at 600 rpm at room temperature and allowed to crosslink for 1 h. The dispersion was centrifuged at 10 000 rpm for 20 min at 20 °C using cooling centrifuge (Remi, Mumbai, India). Sediment was washed, dispersed in distilled water containing 1% mannitol as cryoprotectant, lyophilized and stored till further use.

Box–Behnken experimental design

A BBD with statistical model incorporating interactive and polynomial terms was utilized to optimize and assess the responses. In this study, BBD with three factors and three levels was employed to generate quadratic response surface and second order polynomial models to quantify and thereby to optimize the properties of CSNPs. Based on preliminary studies, concentration of chitosan (X₁), concentration of TPP (X₂) and drug/polymer ratio (X₃) were selected and evaluated at three different levels high, medium and low. The physicochemical properties of CSNPs studied as response were particle size (Y₁) and % drug entrapment efficiency (%EE) (Y₂) with applied constraints as described in Table 1. Fifteen runs for the BBD were generated using the Design Expert® 8.0.6 software (State Ease, Inc., Minneapolis, MN). All experiments were performed in randomized manner to eradicate possible sources of bias.

The non-linear quadratic model generated by the BBD is of the following form:

\[ Y = A₀ + A₁X₁ + A₂X₂ + A₃X₃ + A₄X₁X₂ + A₅X₂X₃ + A₆X₁X₃ + A₇X₁² + A₈X₂² + A₉X₃² + E, \]

in which \( Y \) is the measured response associated with each factor-level combination; \( A₀ \) is an intercept; \( A₁ \)–\( A₉ \) are the regression coefficients; \( A₁A₃ \) are the main effect of \( X₁X₃ \); \( A₃A₇ \) are the interaction of the main factors, and \( E \) is the error term.

The multiple linear regressions using ANOVA was carried out for deciding the significance and influence of each individual factor as well as their interactions on response variables. A checkpoint analysis was performed to confirm the role of the derived polynomial equation and contour plots in predicting the responses. Optimization was performed by using desirability approach.

Physicochemical characterization

Particle size, polydispersity index and zeta potential analysis

The particle size and polydispersity index (PDI) of the SCG-CSNPs was determined by dynamic light scattering technique (DLS) using Malvern Zetasizer nano-s90 (Malvern Instruments Ltd., Worcestershire, UK). The zeta potential of nanoparticulate dispersion was determined by measuring the electrophoretic mobility of charged particles under the influence of an applied electric field. All measurements were taken at 25 °C in triplicate and light scattering was measured with an angle of 90°.

Determination of % drug-entrapment efficiency, % drug loading and % yield

The %EE of SCG-CSNPs was determined by indirect method. The nanoparticulate dispersion was centrifuged at 10 000 rpm for 20 min at 20 °C using cooling centrifuge. The free drug content in supernatant was measured by Ultraviolet-Visible spectroscopy at 239 nm (Shimadzu UV 1800, Japan) against blank sample to avoid any possible excipient interference. The %EE was

Table 1. Independent variables with their levels and dependent variables in the Box–Behnken design.

| Independent variables | Low | Medium | High |
|-----------------------|-----|--------|------|
| X₁ = concentration of chitosan (%w/v) | 0.15 | 0.3 | 0.45 |
| X₂ = concentration of TPP (%w/v) | 0.1 | 0.2 | 0.3 |
| X₃ = drug/polymer ratio (w/w) | 0.25 | 0.5 | 0.75 |
| Dependent variables (Responses) | Constraints | Minimize | Maximize |
| Y₁ = Particle size | | | |
| Y₂ = % Entrapment efficiency | | | |

Concentration range of each variable was chosen on the basis of preliminary experiments and literature survey.
determined in triplicate. The %EE of SCG-CSNPs was calculated by the following equation.

\[
\% \text{ Drug entrapment efficiency} = \left( \frac{S_a - S_b}{S_a} \right) \times 100
\]

where, \(S_a\) is the total amount of SCG added, \(S_b\) is the amount of SCG in supernatant after centrifugation.

The % drug loading was measured by direct method. Twenty-five mg of freeze-dried formulation was taken in 10 ml of methanol and sonicated for 30 min. The nanoparticulate dispersion was stored for 48 hr. The resultant mixture was centrifuged at 10000 rpm for 10 min at 20 °C. The free drug content in supernant was measured by Ultraviolet-Visible spectroscopy at 239 nm against blank sample to avoid any possible excipient interference. The % drug loading and % yield was calculated by following formula in triplicate\(^{15}\).

\[
\% \text{ Drug loading} = \left( \frac{\text{Amount of SCG encapsulated into nanoparticles}}{\text{Amount of SCG added + Amount of excipients added}} \right) \times 100
\]

\[
\% \text{ Yield} = \left( \frac{\text{Weight of freeze dried nanoparticles}}{\text{Weight of starting materials}} \right) \times 100
\]

Solid state characterization

Fourier-transform infrared spectroscopy. The Fourier-transform infrared (FTIR) study was performed in order to confirm the cross-linking reaction, to evaluate interaction, if any, between drug and excipients and to ensure the SCG encapsulation into the CSNPs. FTIR spectra were recorded on a FTIR spectrophotometer (NICOLET 6700, Thermoscientific, Waltham, MA). The compressed KBr pellets of the samples were placed in the IR light path and the spectra were scanned over the wave number range of 1000 to 400 cm\(^{-1}\) with resolution of 4 cm\(^{-1}\).

Differential scanning calorimetry study. The Differential scanning calorimetry (DSC) study was performed in order to check the compatibility of drug with excipients and to characterize the physical state of SCG in CSNPs by using Differential scanning calorimeter (DSC-60, Shimadzu Corporation, Kyoto, Japan). About 5 mg of sample was weighed, crimped into an aluminum pan and measurement were recorded in the scanning temperature range from 50 to 300 °C at a heating rate of 10 °C/min against an empty aluminum pan as a reference.

Powder X-ray diffraction study. The Powder X-ray diffraction (PXRD) pattern were recorded at room temperature using X-ray diffractometer (D2 PHASER, Bruker, GmbH, Germany), with a voltage of 30 kV, 5 mA current, 4°/min scanning speed. The samples were scanned from 5° to 50° (2θ) range with a step size 0.01° and a step interval of 0.1 Sec.

Morphological study

The high-resolution transmission electron microscopy (HR-TEM) was employed to assess the surface morphology of SCG-CSNPs. To obtain the specimens, drop of SCG-CSNPs was placed on a copper grid with a drop of 2% phosphotungastic acid for negative staining and air dried. The NPs were viewed under HRTEM (Holland Technai 20, Philips, Holland) at 200 kV accelerating voltage having magnification of 0.23 nm. Images were visualized and collected by soft imaging software.

In-vitro hemolytic assay

Blood compatibility of SCG-CSNPs was evaluated by in-vitro hemolytic assay. To 0.5 ml of fresh rat blood sample, 0.5 ml of different concentration of SCG-CSNPs were added and incubated for 48 h in an incubator shaker at 3 7 ± 1 °C. After incubation, the plasma were collected by centrifugation and analyzed by Ultraviolet-Visible spectrophotometer at 540 nm for released hemoglobin content. The results were compared with that of positive control (blood + 1% triton X) and negative control (blood + saline). The % hemolysis was calculated in triplicate by following equation\(^{16}\).

\[
\% \text{ Hemolysis} = \left( \frac{\text{sample absorbance} - \text{negative control absorbance}}{\text{positive control absorbance} - \text{negative control absorbance}} \right) \times 100
\]

In-vitro drug release study

A 3 ml of SCG-CSNPs dispersion equivalent to 25 mg of SCG was filled in dialysis bag (MWCO 12–14 kDa) with two ends tied and immersed in 150 ml of phosphate buffer pH 7.4 at 3 7 ± 0.5 °C under constant stirring at 100 rpm. At pre-determined time intervals, 5 ml of samples were withdrawn with replacement of equal volume of fresh media to maintain sink conditions. The samples were filtered through 0.2 μ syringe filters and analyzed spectrophotometrically for drug content against blank sample taken at 0 h, to avoid any possible excipient interference. The in-vitro release study was conducted in triplicate. The evaluation of drug release data was conducted using model dependent approach by fitting different kinetic models: zero-order, first-order, Higuchi model, Hixon-Crowell model and Korsemeyer–Peppas model\(^{17}\).

Ex-vivo permeation study

The study protocol was approved by the Institutional Animal Ethics Committee (Protocol No: RPCP/IAEC/2011–2012/MPP– PT-10). The wistar rats were sacrificed. The small intestine was immediately excised and placed into ice-cold, bubbled (carbogen, 95:5 O2/CO2) Ringer buffer. The jejunum, 20–30 cm distal from the pyloric sphincter was rinsed and cut into segments. The SCG-CSNPs and pure SCG were dispersed in 1 ml of phosphate buffer pH 6.8 to obtain 10 mg/ml concentration. The intestine was tied after filling 1 ml of sample and placed in a 40 ml of phosphate-buffer pH 7.4 containing receiver chamber at 37 ± 0.5 °C with continuous aeration. At predetermined time interval samples were withdrawn from receiver compartment and replaced with an equal volume of fresh buffer. The samples were analyzed for drug content by spectrophotometrically at 239 nm against blank sample taken at 0 hr, to avoid any possible interference by tissue materials\(^18\). Study was conducted in triplicate. Apparent permeability coefficients (Papp in cm/s × 10\(^{-6}\)) were calculated from following formula.

\[
P_{\text{app}} = \frac{\partial Q}{\partial t} \times \frac{1}{A \times Co}
\]

where \(\partial Q/\partial t\) is the steady-state appearance rate on the acceptor side of the tissue, A is the exposed area (cm\(^2\)) and Co is the initial concentration of the drug in the donor compartment. Permeability enhancement ratio was calculated from following formula.

\[
\text{Permeability enhancement ratio} = \frac{P_{\text{app}} \text{ of nanoparticles}}{P_{\text{app}} \text{ of drug solution}}
\]
In-vitro cellular uptake study in small intestinal cells

For uptake study, FITC and Rhodamine B-labeled SCG-CSNPs were prepared by previously reported methods. \(^{19,20}\) This dispersion was filled to 3–5 cm of isolated segments of jejunum and ileum of wistar rats and incubated for 2 h in phosphate buffer pH 7.4 at 37 ± 0.5 °C. The 5 μm cross-sections were sliced using cryo-microtome (Leica CM1850) and uptake in the intestinal mucosa was observed under a LSM 510 META confocal microscope (Carl Zeiss Inc., Thornwood, NY) at 488 nm and 524 nm excitation wavelengths for FITC and Rhodamine B, respectively. \(^{19}\)

Stability study

The stability study of SCG-CSNPs was carried out at room temperature (25 ± 2 °C), refrigerated condition (4 ± 1 °C), and accelerated condition (40 ± 2 °C/75 ± 5% RH) over a period of 45 days in triplicate. Samples were evaluated at 15th, 30th and 45th day for their drug content and particle size as an indicator for physical stability. Chemical stability during the storage was checked by FTIR study after 45 days of storage. \(^{21}\)

Statistical analysis

All experiments were performed in triplicate. The results were given as mean ± standard deviation (SD). Statistical comparisons of the results to control were made with simple analysis of variance (ANOVA) and independent student t-tests. The level of significance was taken as \(p < 0.05\).

Results and discussion

Experimental design

In the present experimental design, the effect of independent variables, i.e., concentration of chitosan \(X_1\), concentration of TPP \(X_2\) and drug/polymer ratio \(X_3\) was studied on physico-chemical characteristics of SCG-CSNPs (dependent variables) such as particle size \(Y_1\) and % EE \(Y_2\) (Table 1). Transformed values of all the 15 batches along with their results are shown in Table 2.

Coefficients with one factor in quadratic polynomial equations are attributed to the main effect of that particular factor, while the coefficients with more than one factor are attributed to the interaction between those variants. A positive coefficient of the factor indicates the existence of direct relationship between the factor and the response whereas negative coefficient indicates inverse effect. \(^{11}\) Response surface graphs were generated using polynomial equations, which represent simultaneous effect of any two variables on response parameters by taking one variable at a constant level. The significance of the variants and their interactions with quantitative effect are shown in Pareto chart (Figure S1).

Effect on particle size

The particle size of SCG-CSNPs was varied in the range of 200.7 nm to 319.9 nm as consequence of the change in the independent variables. The SCG-CSNPs exhibited narrow particle size distribution, as indicated by relatively low PDI values. Low PDI values also indicate the relatively homogenous nature of the dispersion. The polynomial equation with the coefficients of the model estimated by multiple linear regressions, showing the effect of independent variables was as follows.

\[
Y_1 = 238.13 + 18.92X_1 + 21.94X_2 + 20.64X_3 - 11.15X_1X_2
+ 0.20X_1X_3 + 35.02X_2X_3 + 25.97X_1^2 + 10.60X_2^2 - 0.95X_3^2
\]

The quadratic model having \(p\) values of 0.0045 and \(F\) ratio of 14.38 implies that it is significant for particle size. The goodness of fit between predicted values and experimental values was substantiated by the value of correlation coefficient \((R^2)\) which was found to be 0.9628, indicating a good fit. The \(p\) values for lack of fit of model was 0.1591 indicates that the current model had no lack of fit and provided a satisfactory fit to the data \((p > 0.05)\). Therefore, this model can be used to navigate the design space (Tables S1 and S2).

The response surface 3D plots indicating the effect of independent parameters on particle size are shown in Figure 1 (A, B and C). It was observed that all the independent parameters \(X_1, X_2\) and \(X_3\) significantly affect the particle size (Figure 1D, E and F). The increase in the particle size with \(X_1\) could be attributed to the fact that viscosity is far higher at higher concentrations of chitosan. This leads to an increase in the viscous forces which pose resistance to droplet break down by stirring and hence, lead to increase in particle size. \(X_2\) holds a direct

| Table 2. Box–Behnken experimental design showing various runs with independent variables and their measured responses: particle size \((Y_1)\), % entrapment efficiency \((Y_2)\), and polydispersity index (PDI) of SCG-CSNPs. |
| Run No. | \(X_1\) | \(X_2\) | \(X_3\) | \(Y_1\) | \(Y_2\) | PDI |
|---------|--------|--------|--------|--------|--------|------|
| Factorial points |
| 1 | −1 | −1 | 0 | 227.8 ± 0.3 | 43.88 ± 0.92 | 0.165 ± 0.09 |
| 2 | 1 | −1 | 0 | 283 ± 0.5 | 55.19 ± 0.31 | 0.134 ± 0.015 |
| 3 | −1 | 1 | 0 | 288.7 ± 0.04 | 33.82 ± 0.56 | 0.146 ± 0.13 |
| 4 | 1 | 1 | 0 | 299.3 ± 0.09 | 87.17 ± 0.67 | 0.066 ± 0.069 |
| 5 | −1 | 0 | −1 | 213.2 ± 0.54 | 24.63 ± 2.1 | 0.191 ± 0.093 |
| 6 | 1 | 0 | −1 | 255.6 ± 1.2 | 35.84 ± 0.91 | 0.111 ± 0.16 |
| 7 | −1 | 0 | 1 | 270.3 ± 1.6 | 42.52 ± 1.5 | 0.212 ± 0.19 |
| 8 | 1 | 0 | 1 | 313.5 ± 0.31 | 87.6 ± 1.1 | 0.164 ± 0.11 |
| 9 | 0 | −1 | −1 | 245.7 ± 0.03 | 21.6 ± 1.23 | 0.266 ± 0.04 |
| 10 | 0 | 1 | −1 | 224.8 ± 0.16 | 35.82 ± 0.61 | 0.082 ± 0.098 |
| 11 | 0 | −1 | 1 | 200.7 ± 0.54 | 66.92 ± 0.87 | 0.167 ± 0.087 |
| 12 | 0 | 1 | 1 | 319.9 ± 2.1 | 56.68 ± 1.9 | 0.122 ± 0.032 |
| Centre points |
| 13 | 0 | 0 | 0 | 234.2 ± 0.89 | 71.25 ± 0.56 | 0.096 ± 0.014 |
| 14 | 0 | 0 | 0 | 245.3 ± 0.42 | 72.92 ± 1.1 | 0.054 ± 0.011 |
| 15 | 0 | 0 | 0 | 234.9 ± 0.48 | 71.41 ± 0.68 | 0.082 ± 0.026 |

All data are shown as mean ± S.D; \(n = 3\).
relationship with the particle size. The increase in particle size with increase in the $X_2$ could be due to the stiffness of cross-linkage between TPP and chitosan. As a result of increased $X_2$, there would be more tripolyphosphoric ions to cross-link with amino groups on chitosan chains. Similarly, the $X_3$ showed significant positive effect on particle size due to the presence of more amount of drug in polymeric solution which decreases shear stress to break down the NPs that ultimately result in more drug entrapment and finally, increases size of NPs. It might be additionally due to the increasing cross-linking density as a result of anionic nature of drug. However, the mechanism by which $X_2X_3$ contributed to particle size increment was not clear. It might have happened due to the reason that TPP and SCG (both were in anionic form in the solution) competitively interacted with the same chemical group of chitosan. Therefore, $X_2X_3$ interaction would have possibly had an effect on the cross-linking efficiency resulting in loosely packed polymeric chains inside the CSNPs with raised particle size.

**Effect on % entrapment efficiency**

The %EE of SCG-CSNPs was found in the range of 21.6% to 87.6%. The following polynomial equation showing the effect of the independent variables was obtained (Table 3).

$$Y_2 = 75.70 + 15.12X_1 + 4.24X_2 + 16.56X_3 + 10.51X_1X_2 + 8.47X_1X_3 + 95X_2X_3 - 15X_1^2 - 10.53X_2^2 - 17.90X_3^2$$

The quadratic model having $p$ values of 0.0001 and $F$ ratio of 130.14 implies that the model is significant for %EE. The value of $R^2$ for polynomial equation was found to be 0.9957, indicating a good fit. The model showed absence of lack of fit having $p$ values of 0.1159 ($p > 0.05$). Hence, this model can be used to navigate the design space (Tables S1 and S2). All the independent parameters: $X_1$, $X_2$ and $X_3$ and their interactive effects significantly affected the %EE which can be seen in the response surface 3D plot of %EE (Figure 1 G, H and I).
Table 3. Release kinetic parameters for optimized SCG-CSNPs in phosphate buffer pH 7.4.

| Batch        | Zero order $R^2$ | First order $R^2$ | Higuchi model $R^2$ | Hixon–Crowel Model $R^2$ | Korsemeyer–Peppas model $R^2$ |
|--------------|------------------|-------------------|---------------------|--------------------------|-------------------------------|
| SCG-CSNPs    | 0.831            | 0.618             | 0.931               | 0.695                    | 0.925                         |

Increase in the %EE with increase of $X_1$ could be attributed to various facts, them being: higher amount of chitosan has higher ability of ionic gel formation which increases viscosity of the solution thereby increase in the diffusional resistance of the SCG to move into the external phase through the polymer droplet, results in increase in the %EE\(^2\). Moreover, the time required for polymer precipitation decreases at higher polymer concentration and rapidly precipitates on the surface of dispersed phase, which prevents SCG molecules to diffuse out of CSNPs across the phase boundary, resulting in an increase in %EE\(^3\). The %EE increase with corresponding increase in $X_2$ might be due to better cross-linking density of chitosan matrix as well as more chitosan molecules can participate in the ionic gelation process to form CSNPs which can reconcile more SCG\(^2\). The $X_3$ had direct correlation with the %EE. By increasing the relative amount of drug to polymer in the solution, more SCG can interact with the chitosan through electrostatic forces, thereby higher % of SCG can be entrapped in the CSNPs. The interaction factor $X_1X_2$ and $X_1X_3$ are positively related to the %EE owing to the above phenomena. However, the reason behind contrary relation of $X_2X_3$ with %EE was not clear but it might be due to the anionic nature of both TPP and SCG that competitively interact with the similar chemical group of chitosan, thereby affecting the cross-linking efficiency. It resulted in loosely-packed polymeric chain inside the CSNPs from which SCG molecule can simply diffuse out in the external media and thus reduce the %EE.

**Check point analysis**

A check point analysis was performed to confirm the prediction. There was an excellent agreement between the measured responses and predicted responses. The experimental values were very close to the predicted values, with low percentage bias, suggesting that the mathematical model were reliable and hence, the proposed model can be used to navigate the design space (Table S3).

**Optimization using desirability approach**

The numerical optimization technique based on desirability approach was utilized for optimization of SCG-CSNPs with an objective of achieving minimal particle size while keeping entrapment maximum and the levels of the variables that give the optimum response were determined. The level of $X_1$, $X_2$, and $X_3$ was found to be 0.14, 1.0, and 0.92, respectively having predicted 209.17 nm particle size and 65.46% EE with overall desirability of 0.82. The prepared optimized SCG-CSNPs using respective level of variables showed particle size of 200.4 ± 4.06 nm and %EE of 62.68 ± 2.4%, which were very close to the predicted values with low percentage of bias. The % drug loading and % yield of optimized SCG-CSNPs were found to be 8.66 ± 0.6% and 85.84 ± 2.7%, respectively.

**Characterization**

**Zeta potential**

The zeta potential measures the surface charge of particles which can greatly influence particle stability in suspension through the electrostatic repulsion between particles likewise in-vivo interaction of NPs\(^2\). The zeta potential of the optimized SCG-CSNPs was found to be ±32 mV indicating the formation of stable nanodispersion due to sufficient electrostatic repulsion\(^7\). Moreover, positive surface charge would impart mucoadhesive characteristics by electrostatic interaction with the negatively charged mucin of mucus; thereby increasing the residence time and absorption\(^8\).

**Solid state characterization**

**FTIR spectroscopy.** FTIR spectra of chitosan, SCG, TPP, mannitol, physical mixture and freeze dried SCG-CSNPs are shown in the Figure 2(I). FTIR spectrum of SCG showed characteristic peak at 1639.54 cm\(^{-1}\) (C = O), 1573 cm\(^{-1}\) (asymmetric COO\(^-\)), 1410 cm\(^{-1}\) (symmetric COO\(^-\)), 3416 cm\(^{-1}\) (O-H). The fingerprint region (1400–600 cm\(^{-1}\)) contains a large number of characteristic bands due to vibration within the molecule\(^9\). The FTIR spectrum of chitosan showed a characteristic peak at 3421 cm\(^{-1}\) (combined peaks of primary -NH\(_2\) and -OH), 1654.27 cm\(^{-1}\) (C = O stretching of amide I). In physical mixture, all the above characteristic peaks of SCG and chitosan are retained, showing no interaction between them and are compatible with each other. The peaks of 1654.27 cm\(^{-1}\) disappear in the cross-linked CSNPs and two new peaks at 1634 cm\(^{-1}\) and 1556 cm\(^{-1}\) appears. The disappearance of the peak could be attributed to the linkage between the phosphoric groups of the TPP and ammonium groups of chitosan. The cross-linked CSNPs also showed a peak at 1155 cm\(^{-1}\) (P = O) and broader peak between 2934–3421 cm\(^{-1}\) owing to hydrogen bonding between -OH and -NH\(_2\) groups\(^3\) (Figure S2). Moreover, all the characteristic peaks of SCG seem at the same wave number in SCG-CSNPs as present in pure form, indicate successful encapsulation of SCG into CSNPs without any modification\(^3\).

**DSC study.** The overlay DSC thermograms corresponding to SCG, chitosan, TPP, mannitol, physical mixture and freeze dried SCG-CSNPs are depicted in Figure 2(II). Under the experimental conditions no degradation endothermic peak was ascertained for chitosan that normally occurs at 280°C\(^3\). Pure SCG showed single endothermic peak at 264°C indicating crystalline nature of drug\(^3\). The presence of endothermic peak of SCG along with other excipients in the thermogram of physical mixture clearly indicates the compatibility with each other. The peak of SCG disappeared in SCG-CSNPs indicates absence of crystallinity whereas, the peak at 164°C in the SCG-CSNPs formulation corresponds to the mannitol. The disappearance of endothermic peak of SCG confirmed the entrapment of SCG in the CSNPs and existence of SCG in an amorphous or disordered-crystalline drug phase of a molecular dispersion inside the polymeric matrix of CSNPs\(^3\).

**PXRD study.** The X-ray diffractograms of SCG, chitosan, TPP, mannitol, physical mixture and SCG-CSNPs are presented in Figure 2(III). The XRD pattern of chitosan shows two prominent crystalline peaks at 11° and 20° (2θ) with high intensity. The 2θ value of SCG at 8°, 9.83°, 11.5°, 14°, 16.9°, 19.7°, 24.3°, 26.6° shows high degree of crystalline nature of SCG\(^3\). The crystal peaks of SCG were also present in the physical mixture indicating presence of SCG in crystalline form due to absence of any...
interaction. Whereas XRD pattern of SCG-CSNPs revealed marked differences in the molecular state of SCG compared to pure SCG. The characteristic intense crystalline peaks of SCG were absent and only broad, diffuse peaks were observed in the CSNPs formulation which suggests that SCG have been incorporated in the polymer matrix as amorphous nanodispersion or molecular dispersion form. Moreover, a shift of peak positions, reduction of peak intensity, and broadness of peaks, reflecting the destruction of the native chitosan packing structure owing to a modification in the arrangement of molecules in the crystal lattice induced by ionic interactions that ultimately imparts insoluble characteristic to SCG-CSNPs in acid, neutral, and alkaline conditions.

Morphological study

The TEM micrographs Figure 3(I) showed smooth, spherical shaped particles with uniform size distribution without any rough pores. A good correlation was obtained for the particle size measured by DLS and TEM. The part of aggregation might be due to hydrogen bonding interactions between SCG-CSNPs gradually become dominant in the freeze drying process. It conjointly can be noticed that these SCG-CSNPs have a deeper color in the core and surface, indicating that these regions have higher electron density distribution. As TPP contains phosphorus element, which has a higher electron density than those elements of chitosan, therefore it can be inferred that chitosan has higher degree of cross-linking density with TPP in those regions.

In-vitro hemolytic assay

Hemolytic assay was carried out to evaluate the blood compatibility of SCG-CSNPs as shown in Figure 3(II). The blood compatibility is a significant index for biomaterials because the materials might be expose in blood environment and damage the erythrocytes in certain degree or cause the formation of thrombus. The results indicate that the % hemolysis of the samples was within the range of less than 5%, the critical safe hemolytic ratio for biomaterials according to ISO guideline (ISO/TR 7406), which indicates that the damage of samples on the erythrocytes was very little.
In-vitro drug release study

The optimized SCG-CSNPs showed biphasic release in which nearly 10 to 12% of the drug released within 1 h and after that sustained drug release up to 48 h as depicted in Figure 3(III). This shows uniform distribution of SCG in CSNPs with some localized drug on the surface. The slow and sustained release of the encapsulated SCG from the polymer matrix indicates efficient encapsulation and immobilization of SCG. By fitting different kinetic models to the drug-release profile, the Higuchi model showed highest $R^2$ value (Table 3) which indicates that the drug release from SCG-CSNPs occurred through the diffusion controlled process based on Fick’s law, in which diffusion coefficient depends upon both the concentration and time.

Ex-vivo permeation study

Measurement of the $P_{app}$ and the permeability enhancement ratio of SCG-CSNPs indicated that the permeability of SCG was improved due to formulation effect Figure 3(IV). The permeability enhancement ratio was found to be 2.87 for SCG-CSNPs compared to SCG solution. Being a paracellularly transported molecule, enhancement of SCG permeability might be due to transport through the M cells of PP and manipulation of intercellular tight junctions by the CSNPs via interacting with the cell membrane, thereby inducing structural reorganization of tight junction-associated proteins resulted in permeability enhancement. Moreover, the marked similarity can be seen in the results of in-vitro drug release and ex-vivo study Figure 3(III) & (IV). The nearly 10–12% drug was released from the SCG-CSNPs within 1 h, followed by release of 30–35% release at 4 h, by adopting similar release pattern across dialysis membrane as well as rat intestinal tissue. The close resemblance between the results across the artificial membrane and natural membrane indicates that SCG-CSNPs will show similar permeation and release behavior upon in-vivo administration, for sustaining the SCG activity.
In-vitro cellular uptake study in small intestinal cells

In-vitro SCG-CSNPs uptake study was conducted to demonstrate the permeation of SCG-CSNPs across the intestinal barrier and to evaluate the absorption mechanism of SCG-CSNPs. The CLSM images containing FITC and Rhodamine-B-labeled SCG-CSNPs internalized and distributed in the small intestinal mucosal cross-sections consisting of jejunal villi and M cell of PP of are shown in Figure 4 via green and red colored fluorescence, respectively. The green fluorescent FITC-SCG-CSNPs below the surface layer of the enterocytes, inside the intestinal wall in the 3D section suggesting that the SCG-CSNPs was at various stages of internalization in the enterocytes of the intestinal villi (Figure S3). This sign indicates that endocytosis took place effectively inside the enterocytes\textsuperscript{40,41}. The results effectively demonstrate that no single mechanism appears dominant in SCG-CSNPs uptake. CSNPs may facilitate SCG permeation by potentially opening the tight junction between enterocytes without affecting the viability of intestinal cells\textsuperscript{19}. Moreover, simultaneous transcellular endocytosis through enterocytes and the direct uptake of CSNPs by M-cells overlaying the PP can contribute to an enhancement of permeability\textsuperscript{41}. As SCG appears to remain associated with the nanostructures while permeating through the intestinal mucosa, indicating that CSNPs play an important role in facilitating SCG permeation through the intestinal cells and ultimately enhances the permeability of SCG.

Stability study

Physical and chemical stability study was performed at different environmental conditions with an objective to determine the intactness of SCG-CSNPs. There were no significant changes ($p > 0.05$) in the physical as well as chemical characteristics of the formulation in terms of particle size and drug content, during stability study\textsuperscript{42} Figure 5(I, II, III). Chemical interaction between the drug and polymer, if any, during the stability study was determined by FTIR spectroscopy which revealed no any significant changes over 45 days by comparing the FTIR spectra of SCG-CSNPs at zero time and after 45 days of storage, at various conditions Figure 5(IV). These all results jointly indicate...
that the developed SCG-CSNPs are physically and chemically stable and retain their pharmaceutical properties at various environmental conditions.

**Conclusion**

Present study conclusively demonstrates the feasibility of forming SCG-CSNPs engineered by ionotropic gelation method and optimized using box–behnken experimental design. The stable, smaller sized, spherical shaped, non-hemolytic CSNPs with favorable positive surface charge were important to achieve for oral permeability enhancement. The sustained release pattern and enhanced permeation of SCG across GIT by various mechanisms as studied by ex-vivo and uptake study using CLSM study makes SCG-CSNPs as promising delivery system for oral treatment of allergic rhinitis. Further, an understanding of the clinical relevance of the in-vitro data will be valuable for future in-vivo experiments to establish SCG-CSNPs as an effective oral delivery system.

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**Declaration of interest**

The authors state no conflicts of interest.

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Supplementary material available online
Supplementary Table S1-S4 and Figures S1-S5