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To cite this article: Balaram Gajra, Ravi R. Patel & Chintan Dalwadi (2015): Formulation, optimization and characterization of cationic polymeric nanoparticles of mast cell stabilizing agent using the Box–Behnken experimental design, Drug Development and Industrial Pharmacy, DOI: [10.3109/03639045.2015.1093496](https://doi.org/10.3109/03639045.2015.1093496)

To link to this article: <http://dx.doi.org/10.3109/03639045.2015.1093496>



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Published online: 11 Nov 2015.



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RESEARCH ARTICLE

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, morphological, *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 $62.68 \pm 2.4\%$ 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

History

Received 23 April 2015

Revised 25 July 2015

Accepted 4 September 2015

Published online 11 November 2015

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 constraints, 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,

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paracellular absorption and transport via M cells of Peyer's Patches (PP) in the intestinal mucosa⁵.

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 mucoadhesivity⁶. 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 GIT⁷.

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 representation⁸. 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 models⁹.

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.¹⁰. 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, redispersed 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

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

Independent variables	Coded levels of variables		
	Low	Medium	High
X_1 = concentration of chitosan (%w/v)	–1	0	1
X_2 = concentration of TPP (%w/v)	–1	0	1
X_3 = drug/polymer ratio (w/w)	–1	0	1
Dependent variables (Responses)	Constraints		
Y_1 = Particle size	Minimize		
Y_2 = % Entrapment efficiency	Maximize		

Concentration range of each variable was chosen on the basis of preliminary experiments and literature survey.

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 (X_1), concentration of TPP (X_2) and drug/polymer ratio (X_3) were selected and evaluated at three different levels high, medium and low. The physicochemical properties of CSNPs studied as response were particle size (Y_1) and % drug entrapment efficiency (%EE) (Y_2) 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:

$Y = A_0 + A_1X_1 + A_2X_2 + A_3X_3 + A_4X_1X_2 + A_5X_2X_3 + A_6X_1X_3 + A_7X_1^2 + A_8X_2^2 + A_9X_3^2 + E$, in which Y is the measured response associated with each factor-level combination; A_0 is an intercept; A_1 – A_9 are the regression coefficients; A_1 – A_3 are the main effect of X_1 – X_3 ; A_4 – A_9 are the interaction of the main factors, and E is the error term¹¹.

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 responses¹². 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 field¹³. 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 interference¹⁴. The %EE was

determined in triplicate. The %EE of SCG-CSNPs was calculated by the following equation.

$$\% \text{ Drug entrapment efficiency} = \frac{S_a - S_b}{S_a} \times 100$$

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 supernatant 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 triplicate¹⁵.

% Drug loading

$$= \frac{\text{Amount of SCG encapsulated into nanoparticles}}{\text{Amount of SCG added} + \text{Amount of excipients added}} \times 100$$

$$\% \text{ Yield} = \frac{\text{Weight of freeze dried nanoparticles}}{\text{Weight of starting materials}} \times 100$$

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 4000 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° (2 θ) 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% phosphotungstic 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 37 ± 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 equation¹⁶.

% Hemolysis

$$= \frac{(\text{sample absorbance} - \text{negative control absorbance})}{(\text{positive control absorbance} - \text{negative control absorbance})} \times 100$$

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 37 ± 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 Korsmeyer-Peppas model¹⁷.

Ex-vivo permeation study

The study protocol was approved by the Institutional Animal Ethics Committee (Protocol No: RPCP/IAEC/2011–2012/MPH-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 materials¹⁸. Study was conducted in triplicate. Apparent permeability coefficients (P_{app} in $\text{cm/s} \times 10^{-6}$) were calculated from following formula.

$$P_{app} = \frac{\partial Q}{\partial t} \times \frac{1}{ACo}$$

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.

$$\text{Permeability enhancement ratio} = \frac{P_{app} \text{ of nanoparticles}}{P_{app} \text{ of drug solution}}$$

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 $37 \pm 0.5^\circ\text{C}$. The $5\ \mu\text{m}$ 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, respectively¹⁹.

Stability study

The stability study of SCG-CSNPs was carried out at room temperature ($25 \pm 2^\circ\text{C}$), refrigerated condition ($4 \pm 1^\circ\text{C}$), and accelerated condition ($40 \pm 2^\circ\text{C}/75 \pm 5\% \text{ RH}$) 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 storage²¹.

Statistical analysis

All experiments were performed in triplicate. The results were given as mean $\pm$ 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 converse effect¹¹. 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 size²². 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.	Independent variables			Dependent variables		
	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 $\pm$ S.D; $n = 3$.

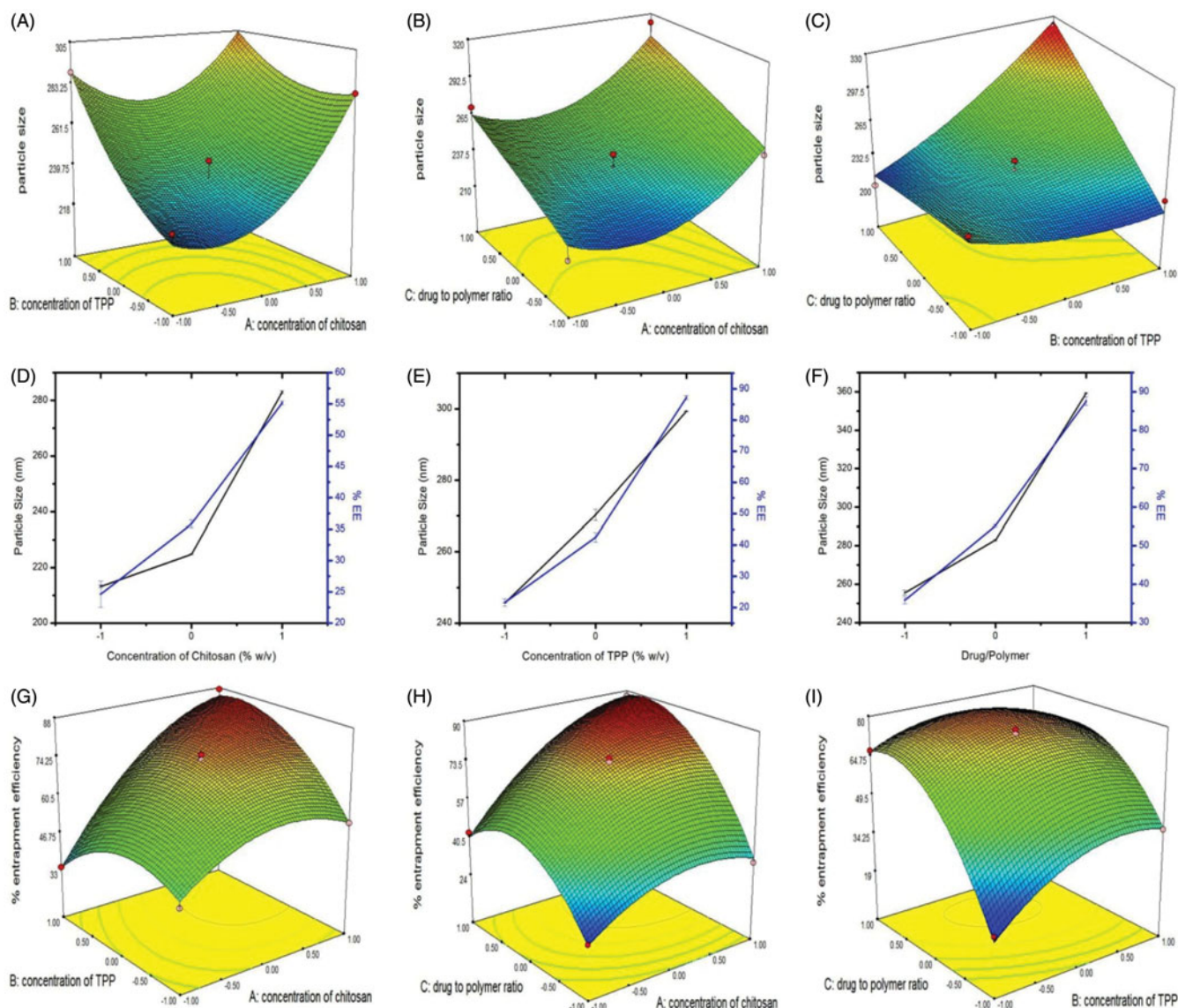


Figure 1. Three-dimensional response surface plots showing the effect of variables on response: (A) effect of concentration of chitosan and concentration of TPP on particle size (actual constant drug to polymer ratio = 0); (B) effect of concentration of chitosan and drug to polymer ratio on particle size (actual constant concentration TPP = 0); (C) effect of concentration of TPP and drug to polymer ratio on particle size (actual constant concentration of chitosan = 0); (G) effect of concentration of chitosan and concentration of TPP on % entrapment efficiency (actual constant drug to polymer ratio = 0); (H) effect of concentration of chitosan and drug to polymer ratio on % entrapment efficiency (actual constant concentration TPP = 0); (I) effect of concentration of TPP and drug to polymer ratio on % entrapment efficiency (actual constant concentration of chitosan = 0). D, E and F show overall effect of concentration of chitosan, concentration of TPP and drug/polymer ratio on response variables particle size and % drug entrapment efficiency, respectively.

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 chains¹⁰. 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 size²².

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).

$$Y_2 = 75.70 + 15.12X_1 + 4.24X_2 + 16.56X_3 + 10.51X_1X_2 + 8.47X_1X_3 - 6.95X_2X_3 - 10.15X_1^2 - 10.53X_2^2 - 17.90X_3^2$$

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 1G, H and I).

Table 3. Release kinetic parameters for optimized SCG-CSNPs in phosphate buffer pH 7.4.

Batch	Zero order	First order	Higuchi model	Hixon–Crowel Model	Korsemeyer–Peppas model
SCG-CSNPs	$R^2 = 0.831$	$R^2 = 0.618$	$R^2 = 0.931$	$R^2 = 0.695$	$R^2 = 0.925$ $n = 0.5696$

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 %EE²³. 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 %EE²⁴. 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 SCG²⁵. 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 -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 $62.68 \pm 2.4\%$, 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 $8.66 \pm 0.6\%$ and $85.84 \pm 2.7\%$, 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 NPs²⁶. The zeta potential of the optimized SCG-CSNPs was found to be +32 mV indicating the formation of stable nanodispersion due to sufficient electrostatic repulsion²⁷. Moreover, positive surface charge would impart mucoadhesive characteristics by electrostatic interaction with the negatively charged mucin of mucous; thereby increasing the residence time and absorption²⁸.

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 ($1400\text{--}600 \text{ cm}^{-1}$) contains a large number of characteristic bands due to vibration within the molecule²⁹. The FTIR spectrum of chitosan showed a characteristic peak at 3421 cm^{-1} (combined peaks of primary -NH₂ 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 $2934\text{--}3421 \text{ cm}^{-1}$ owing to hydrogen bonding between -OH and -NH₂ groups³⁰ (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 modification⁴.

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 ³¹. Pure SCG showed single endothermic peak at 264°C indicating crystalline nature of drug³². 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 CSNPs³³.

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 SCG³². The crystal peaks of SCG were also present in the physical mixture indicating presence of SCG in crystalline form due to absence of any

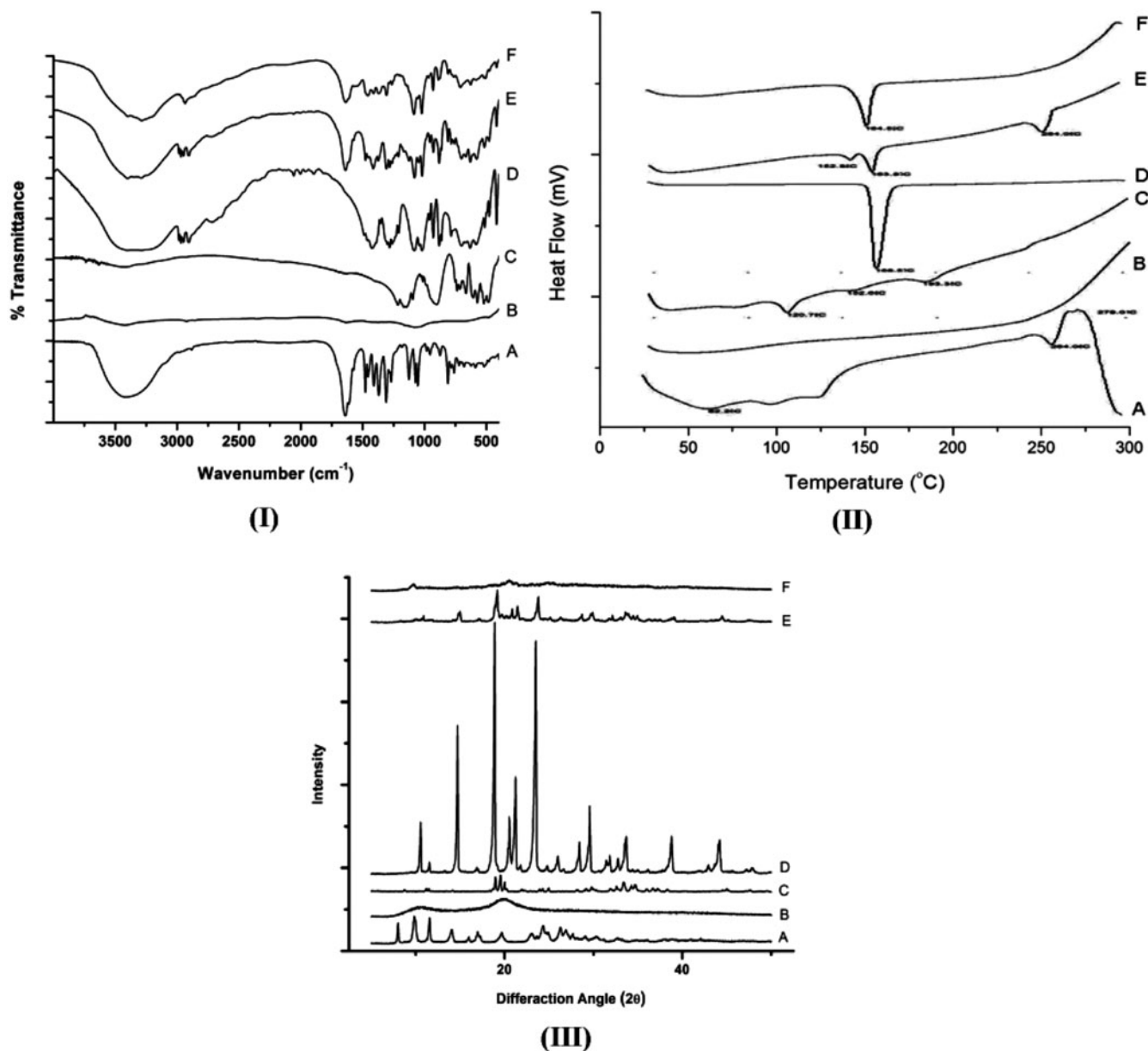


Figure 2. Overlay (I) FTIR spectra (II) DSC thermograms and (III) XRD pattern of (A) SCG (B) chitosan (C) TPP (D) mannitol (E) physical mixture of SCG with excipients (1:1) and (F) SCG-CSNPs.

interaction³³. 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 interactions³⁴ that ultimately imparts insoluble characteristic to SCG-CSNPs in acid, neutral, and alkaline conditions³⁵.

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 regions³⁶.

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 little³⁷.

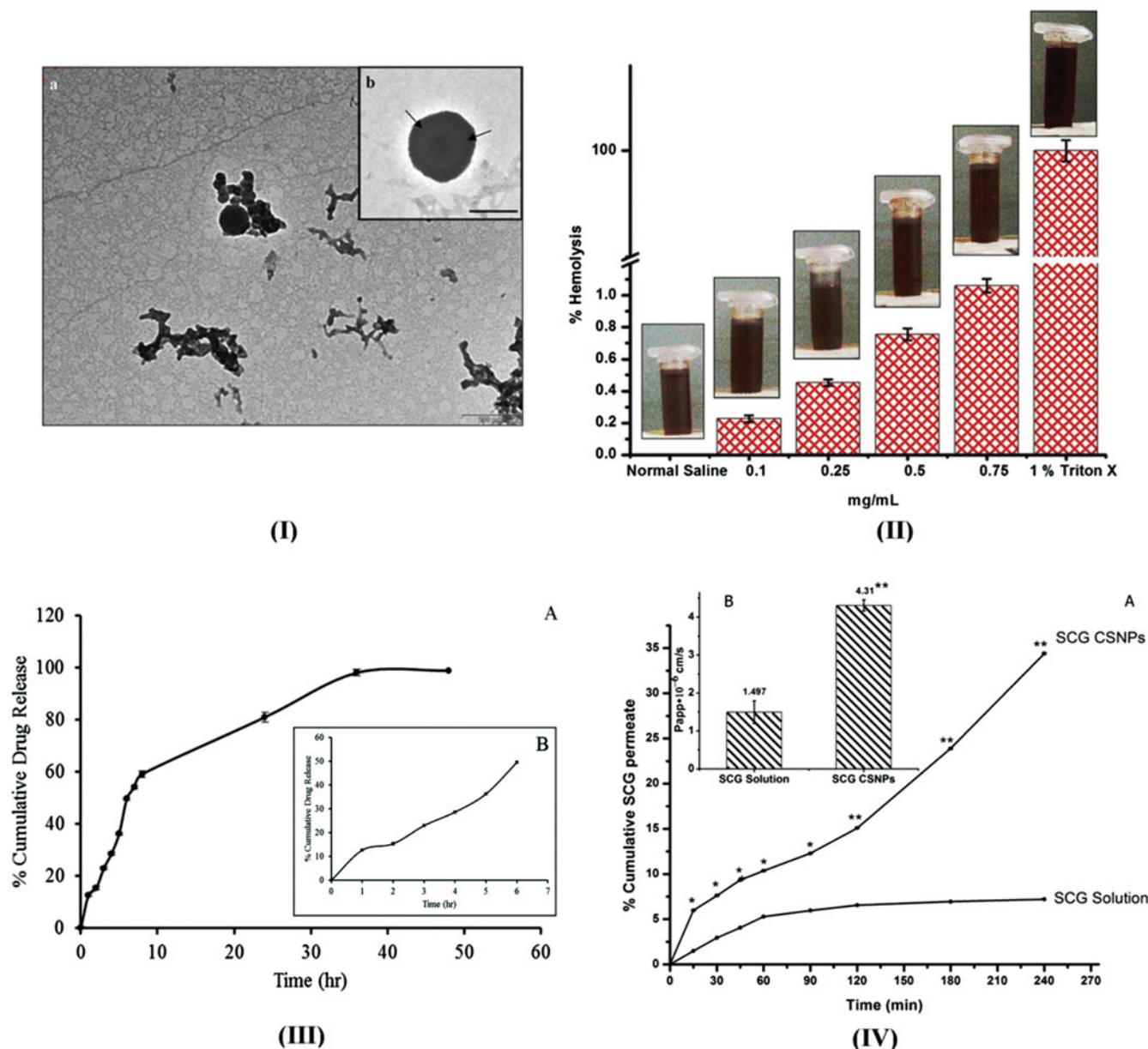


Figure 3. (I) Transmission electron micrograph of SCG-CSNPs (A) under normal view (B) The inset image shows enlarge view of single SCG-CSNP (scale 200 nm) (II) % Hemolysis after 48 h incubation of freeze-dried SCG-CSNPs with rat blood. (III) *In-vitro* drug release profile of the SCG-CSNPs in phosphate buffer pH 7.4 (B) The inset image shows initial release profile of SCG-CSNPs (IV) *Ex-vivo* permeability study of SCG-CSNPs (A) SCG transport and (B) apparent permeability coefficients (Papp) for from SCG-CSNPs and SCG solution. *Significant values at $p < 0.05$, **significant values at $p < 0.001$ compared with SCG solution. (means $\pm$ S.D; $n = 3$).

In-vitro drug release study

The optimized SCG-CSNPs showed biphasic release in which nearby 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)³⁸ 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 time³⁰.

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 enhancement³⁹. 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.

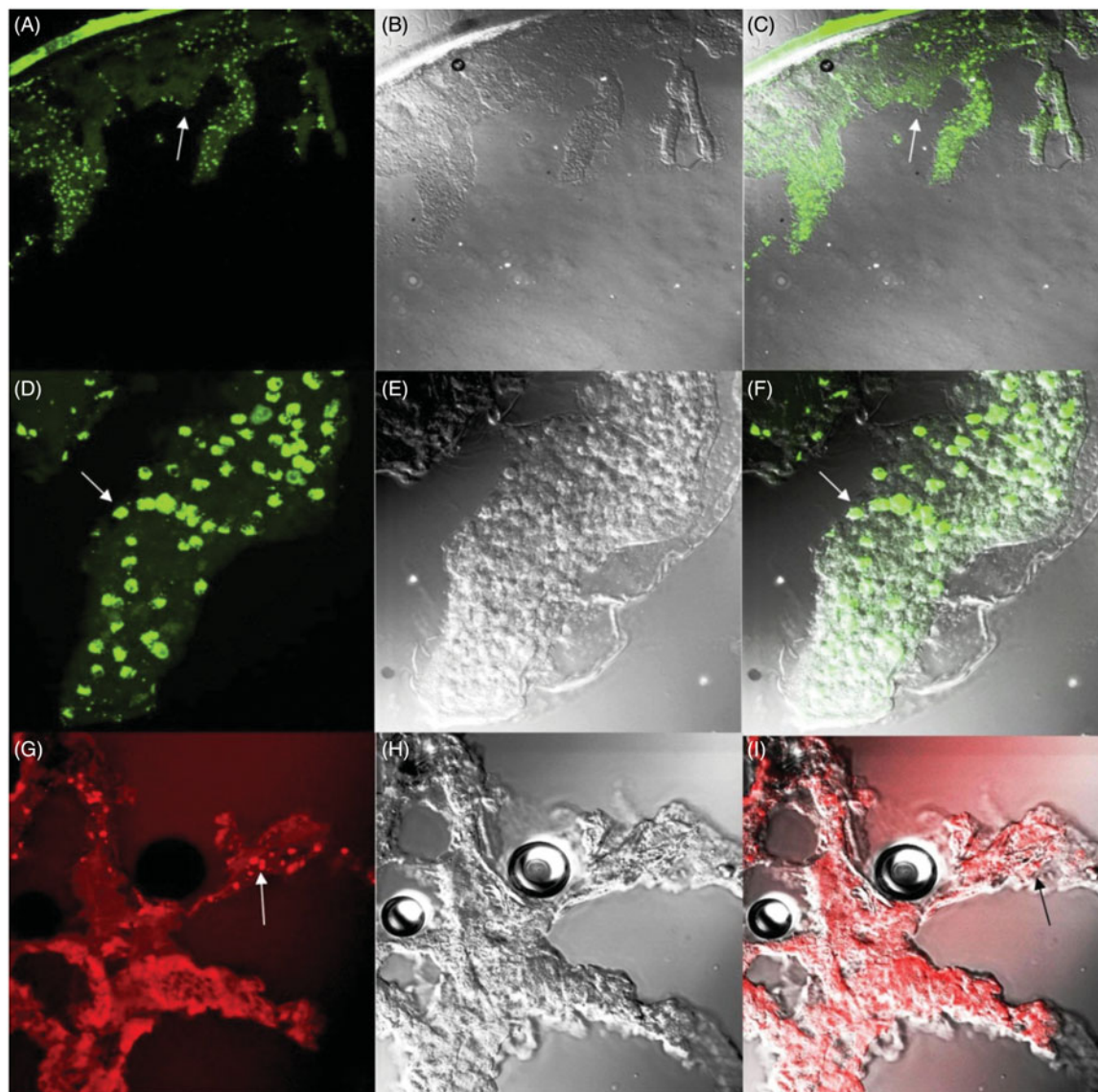


Figure 4. 3D combined CLSM images of rat intestine showing uptake of FITC-SCG-CSNPs and transported into the tissues underlying the absorptive cells. (A) Fluorescent micrograph showing absorption of SCG-CSNPs from the M cells of Peyer's patches (white arrow) and transport across the intestinal wall, (B) DIC image, (C) Merge of fluorescent and DIC image. (D) Fluorescent micrograph showing absorption of SCG-CSNPs from the enterocyte of the intestinal villi (white arrow), (E) DIC image, (F) Merge of fluorescent and DIC image. (G) Fluorescent micrograph showing absorption of rhodamine-B-SCG-CSNPs from the enterocyte of the intestinal villi (white arrow), (H) DIC image, (I) Merge of fluorescent and DIC image. Scanning was performed at 10 $\times$ plain (A, B, C) and at 40 $\times$ plain using emersion oil objective.

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 jejunum 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^{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 cells¹⁹. Moreover, simultaneous transcellular endocytosis through enterocytes and the direct

uptake of CSNPs by M-cells overlaying the PP can contribute to an enhancement of permeability⁴¹. 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 study⁴² 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

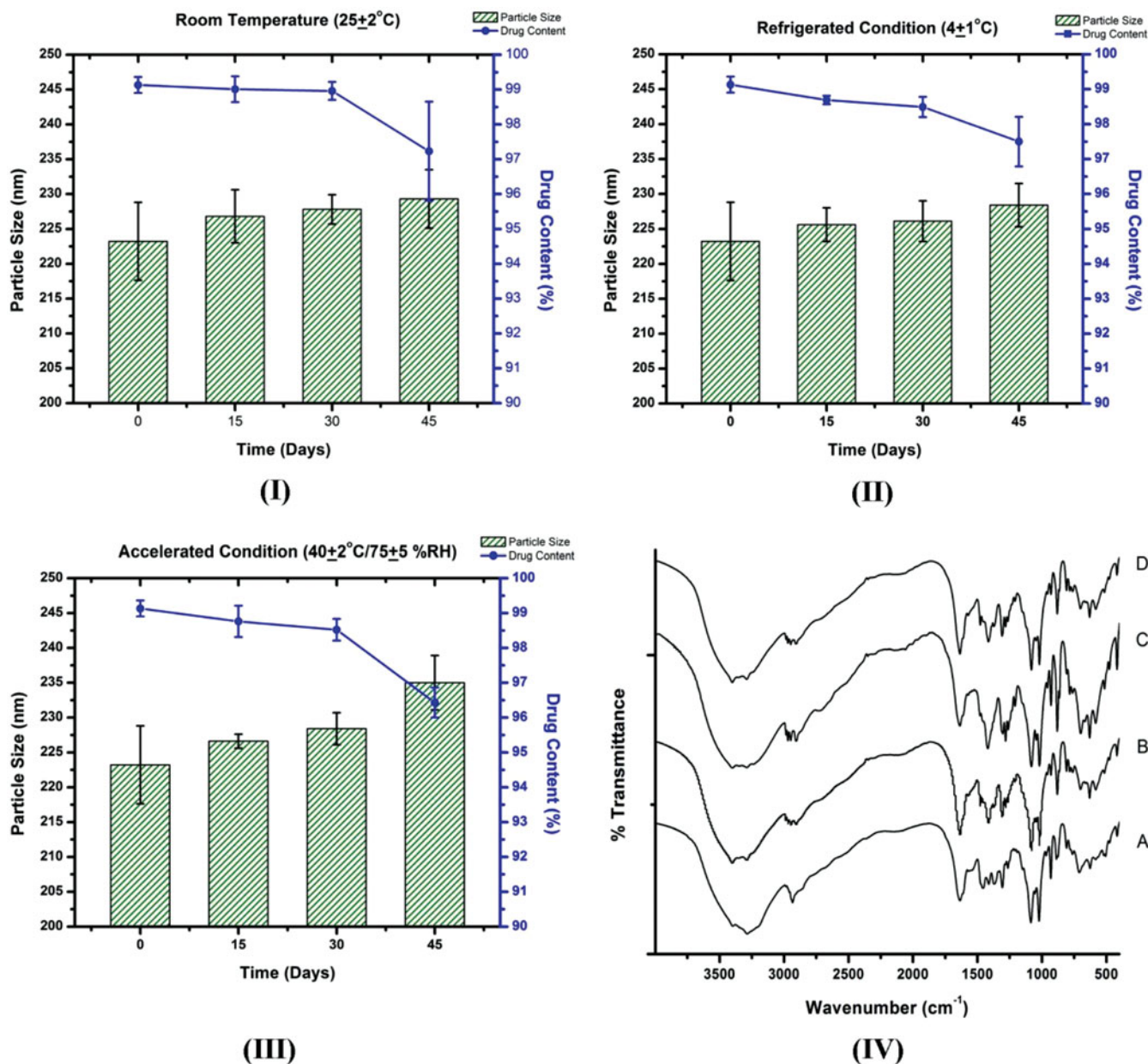


Figure 5. (I), (II), (III) Particle size and drug content (%) of SCG-CSNPs at different time interval and in different environmental conditions during stability study. (Mean $\pm$ S.D; $n = 3$); *significant values at $p < 0.05$ compared with properties of SCG-CSNPs on 0 day. The comparison was made by employing the unpaired Student t -test between the results of SCG-CSNPs on 0 day and 45th day. (IV) FTIR spectra of SCG-CSNPs during stability study on (A) 0 day (B) stored at $25 \pm 2^\circ\text{C}$ after 45 days (C) stored at $4 \pm 1^\circ\text{C}$ after 45 days (D) stored at $40 \pm 2^\circ\text{C}$ and $75 \pm 5\% \text{RH}$ after 45 days.

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.

Acknowledgements

The authors acknowledge the help of Cognis GmbH, Germany, and Entod Pharmaceutical Ltd, Mumbai, for kindly providing gratis sample of chitosan and sodium cromoglicate, respectively.

Declaration of interest

The authors state no conflicts of interest.

References

1. Sukumaran TU. Allergic rhinitis and co-morbidities training module. Indian Pediatr 2011;48:511–13.

2. Budavari S. The merck index: an encyclopedia of chemicals, drugs, and biologicals (Merck Index). Rahway (NJ): MERCK & CO., Inc.; 1989.
3. Sweetman S. Martindale the complete drug reference. London: Pharmaceutical Press; 2005.
4. Ding X, Rath P, Angelo R, et al. Oral absorption enhancement of cromolyn sodium through noncovalent complexation. *Pharm Res* 2004;21:2196–206.
5. Gupta R. Nanoparticle technology for drug delivery. New York, USA: Taylor & Francis Group; 2006.
6. Kaur SP, Rao R, Hussain A, et al. Preparation and characterization of rivastigmine loaded chitosan nanoparticles. *J Pharm Sci Res* 2011;3:1227–32.
7. Saha P, Goyal A, Rath G. Formulation and evaluation of chitosan-based ampicillin trihydrate nanoparticles. *Trop J Pharm Res* 2010;9: 483–8.
8. Patel RR, Kumar N, Khan G, et al. Investigation of critical variables of core-shell polymeric lipid hybrid nanoparticles by using plackett-burman screening design. *Adv Sci Lett* 2014;20:923–32.
9. Box GEP, Behnken DW. Some new three level designs for the study of quantitative variables. *Technometrics* 1960;2:455–76.
10. Calvo P, Remunan-Lopez C, Vila-Jato JL, et al. Chitosan and chitosan/ethylene oxide-propylene oxide block copolymer nanoparticles as novel carriers for proteins and vaccines. *Pharm Res* 1997; 14:1431–6.
11. Gajra B, Dalwadi C, Patel RR. Formulation and optimization of itraconazole polymeric lipid hybrid nanoparticles (lipomer) using Box–Behnken design. *Daru* 2015;23:3
12. Solanki AB, Parikh JR, Parikh RH. Formulation and optimization of piroxicam proniosomes by 3-factor, 3-level Box–Behnken design. *AAPS Pharm Sci Tech* 2007;8:E1–7.
13. Chaubey P, Patel RR, Mishra B. Development and optimization of curcumin-loaded mannosylated chitosan nanoparticles using response surface methodology in the treatment of visceral leishmaniasis. *Expert Opin Drug Deliv* 2014;11:1163–81.
14. Guhagarkar S, Gaikwad R, Samad A, et al. Polyethylene sebacate-doxorubicin nanoparticles for hepatic targeting. *Int J Pharm* 2010; 401:113–22.
15. Subedi RK, Kang KW, Choi HK. Preparation and characterization of solid lipid nanoparticles loaded with doxorubicin. *Eur J Pharm Sci* 2009;37:508–13.
16. Lin YS, Haynes CL. Synthesis and characterization of biocompatible and size-tunable multifunctional porous silica nanoparticles. *Chem Mater* 2009;21:3979–86.
17. Chaurasia S, Kumar N, Patel RR, et al. Optimization of parameters for the fabrication of curcumin loaded polymeric nanoparticles using Taguchi robust design. *Adv Sci Lett* 2014;20:1028–38.
18. Barthe L, Woodley JF, Kenworthy S, et al. An improved everted gut sac as a simple and accurate technique to measure paracellular transport across the small intestine. *Eur J Drug Metab Pharmacokinet* 1998;23:313–23.
19. Woitiski CB, Sarmiento B, Carvalho RA, et al. Facilitated nanoscale delivery of insulin across intestinal membrane models. *Int J Pharm* 2011;412:123–31.
20. Katas H, Wen CM. Preparation and characterization of highly loaded fluorescent chitosan nanoparticles. *ISRN Pharm* 2011;1–5.
21. Wilson B, Samanta MK, Santhi K, et al. Chitosan nanoparticles as a new delivery system for the anti-Alzheimer drug tacrine. *Nanomedicine* 2010;6:144–52.
22. Meng J, Sturgis T, Youan B. Engineering tenofovir loaded chitosan nanoparticles to maximize microbicide mucoadhesion. *Eur J Pharm Sci* 2011;44:57–67.
23. Avadi MR, Sadeghi AM, Mohammadpour N, et al. Preparation and characterization of insulin nanoparticles using chitosan and Arabic gum with ionic gelation method. *Nanomedicine* 2010;6:58–63.
24. Thagele R, Mishra A, Pathak A. Formulation and characterization of clarithromycin based nanoparticulate drug delivery system. *Int J Pharm Life Sci* 2011;2:510–15.
25. Ko JA, Park HJ, Hwang SJ, et al. Preparation and characterization of chitosan microparticles intended for controlled drug delivery. *Int J Pharm* 2002;249:165–74.
26. Hans M, Lowman A. Biodegradable nanoparticles for drug delivery and targeting. *Cur Opin Sol Stat Mater Sci* 2002;6:319–27.
27. Muller R, Jacobs C, Kayser O. Nanosuspensions as particulate drug formulations in therapy rationale for development and what we can expect for the future. *Adv Drug Deliv Rev* 2001;47:3–19.
28. Wang J, Tauchi Y, Deguchi Y. Positively charged gelatin microspheres as gastric mucoadhesive drug delivery system for eradication of *H. pylori*. *Drug Deliv* 2000;7:237–43.
29. Alani AWG, Robinson JR. Mechanistic understanding of oral drug absorption enhancement of cromolyn sodium by an amino acid derivative. *Pharm Res* 2008;25:48–54.
30. Rao N, Kulkarni U, Deshmukh A, et al. Preparation and characterization of ionotropic cross-linked chitosan microparticles for controlled release of aceclofenac. *Int J Pharm Sci Drug Res* 2010; 2:107–11.
31. Liao S, Hung C. A kinetic study of thermal degradation of chitosan/polycaprolactam blends. *Macromol Res* 2004;12:466–73.
32. Nolan LM, Li J, Tajber L, et al. Particle engineering of materials for oral inhalation by dry powder inhalers. II-sodium cromoglicate. *Int J Pharm* 2011;405:36–46.
33. Kumar N, Chaurasia S, Patel RR, et al. Development and optimization of atorvastatin calcium loaded oral biodegradable polymeric nanoparticles using central composite design. *Adv Sci Lett* 2014;20:984–93.
34. Yoksan R, Jirawutthiwongchai J, Arpo K. Encapsulation of ascorbyl palmitate in chitosan nanoparticles by oil-in-water emulsion and ionic gelation processes. *Colloids Surf B Biointerfaces* 2010;76: 292–97.
35. Zhao A, Wang T, Yao M, et al. Effects of chitosan-TPP nanoparticles on hepatic tissue after severe bleeding. *J Med Coll PLA* 2011;26:283–92.
36. Fan W, Yan W, Xu Z, et al. Formation mechanism of monodisperse, low molecular weight chitosan nanoparticles by ionic gelation technique. *Colloids Surf B Biointerfaces* 2012;90:21–27.
37. Rejinold NS, Muthunayanan M, Divyarani VV, et al. Curcumin-loaded biocompatible thermoresponsive polymeric nanoparticles for cancer drug delivery. *J Colloid Interface Sci* 2011;360:39–51.
38. Chaurasia S, Patel RR, Chaubey P, et al. Lipopolysaccharide based oral nanocarriers for the improvement of bioavailability and anticancer efficacy of curcumin. *Carbohydr Polym* 2015;130:9–17.
39. Schipper N, Olsson S, Hoogstraate J, et al. Chitosans as absorption enhancers for poorly absorbable drugs 2: mechanism of absorption enhancement. *Pharm Res* 1997;14:923–9.
40. Huang M, Ma Z, Khor E, et al. Uptake of FITC-chitosan nanoparticles by A549 cells. *Pharm Res* 2002;19:1488–94.
41. Sarmiento B, Ribeiro A, Veiga F, et al. Alginate/chitosan nanoparticles are effective for oral insulin delivery. *Pharm Res* 2007;24:2198–206.
42. Morris G, Castile J, Smith A, et al. The effect of prolonged storage at different temperatures on the particle size distribution of tripolyphosphate (TPP) – chitosan nanoparticles. *Carbohydr Polym* 2011;84:1430–4.

Appendix A
Supplementary data

Table A.1 Statistical analysis of particle size (Y_1) and % entrapment efficiency (Y_2) in the Box-Behnken design

Term	Particle Size (Y_1)		%Entrapment Efficiency (Y_2)	
	Coefficient	<i>p</i> -value	Coefficient	<i>p</i> -value
Constant	+238.13	-	+75.70	-
X_1	18.92	0.0064*	15.12	<0.0001***
X_2	21.94	0.0034*	4.24	0.0042**
X_3	20.64	0.0045*	16.56	<0.0001***
X_1X_2	-11.15	0.1199	10.51	0.0003**
X_1X_3	0.2	0.9745	8.47	0.0009**
X_2X_3	35.02	0.0020*	-6.95	0.0022*
X_1^2	25.97	0.0086*	-10.15	0.0005**
X_2^2	10.60	0.1479	-10.53	0.0004**
X_3^2	-0.95	0.8836	-17.90	<0.0001***

*significant values at $p < 0.05$, **significant values at $p < 0.001$, *** significant values at $p < 0.0001$

Table A.2 Model summary statistics of the quadratic response surface models for particle size (Y_1) and % entrapment efficiency (Y_2)

Response Variable	Quadratic Model							
	F-value	Prob>F ^s	R ²	Adj. R ²	Pred. R ²	Adeq. Prec.	C.V. (%)	Lack of Fit
Y_1	14.38	0.0045**	0.9628	0.8959	0.4607	11.722	4.63	0.1591 [#]
Y_2	130.14	<0.0001***	0.9957	0.9881	0.9366	34.635	4.38	0.1159 [#]

Adj. R², Adjusted R²; Pred. R², Predicted R²; Adeq. Prec., Adequate Precision; C.V., Coefficient of Variation

^{\$}Prob>F is the significance level, *significant values at p<0.05, **significant values at p<0.001, *** significant values at p<0.0001, # not significant

Table A.3 Actual and predicted values of particle size and % EE for check point analysis

Check point Batch code	X ₁	X ₂	X ₃	Predicted value		Experimental Value		% Bias*	
				Particle size (nm)	%EE	Particle size (nm)	%EE		
CP 1	-0.5	-0.5	-0.5	221.06	48.99	205.2±4.7	45.57±4.2	7.17	6.98
CP 2	0.5	0.5	0.5	243.06	60.34	223.6±13.05	61.59±1.38	8.00	2.07
CP 3	-0.66	0.67	-0.69	292.64	84.28	299.3±3.1	89.57±0.431	-2.27	-6.27

*Bias was calculated as [(predicted value-experimental value)/predicted value] X 100.

All data are shown as mean ±S.D; n=3

Figures

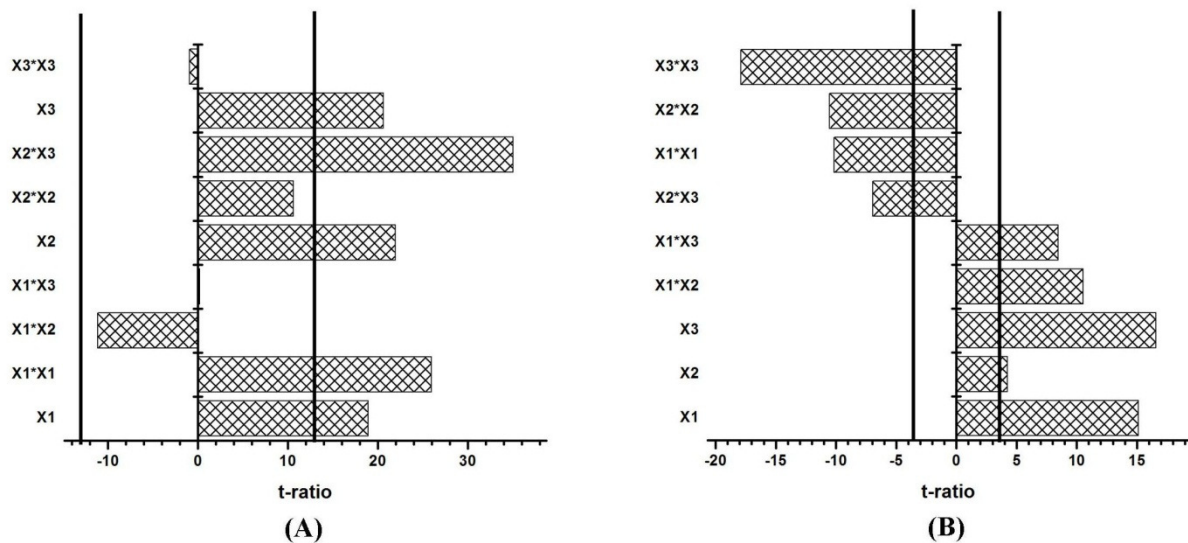


Fig. A.1 Pareto chart showing the quantitative effect of different formulation variables and their interaction on particle size (A) and % drug entrapment efficiency (B). The x-axis shows the standardized effect (t-ratio) of the variables; bars extending beyond the vertical line indicate values reaching statistical significance ($p < 0.05$)

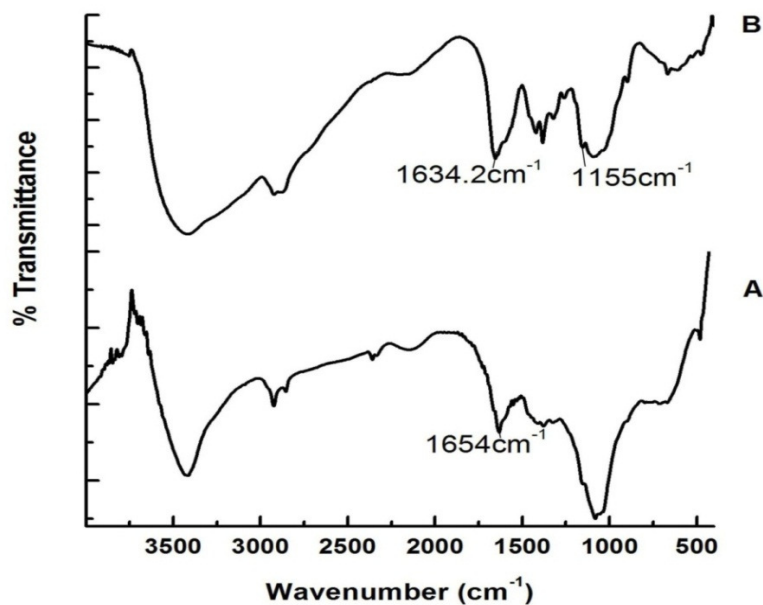


Fig. A.2 FTIR spectra of (A) chitosan and (B) crosslinked chitosan

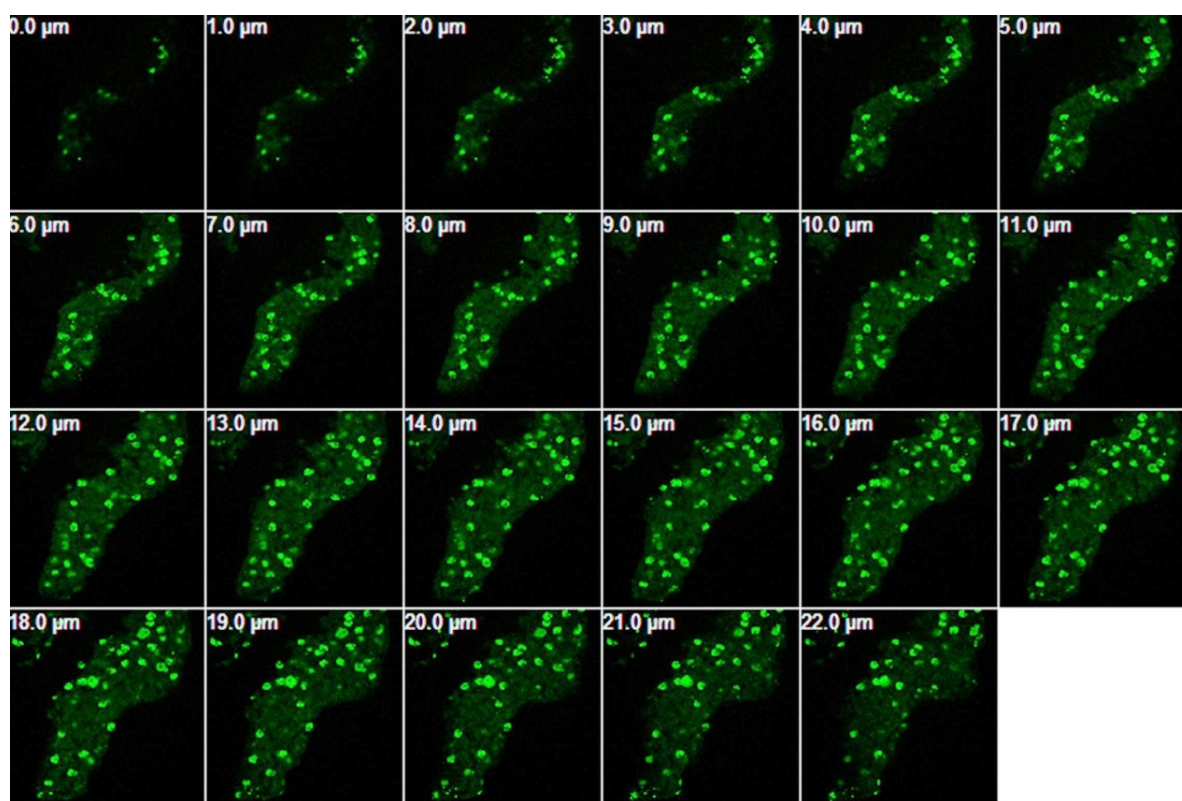


Fig. A.3 Three dimensional (3D) confocal images optically sectioned in the x-y plane at regularly spaced distances along the z-axis showing internalization of FITC-SCG-CSNPs in the intestinal villi. The top images represent sections closest to the apical membrane of cells, and the bottom images are the intracellular space toward basolateral side.

Lyophilization of SCG-CSNPs

Lyophilization of SCG-CSNPs was carried out on benchtop K freeze dryer (Virtis, 4KBTZL/105, USA). The nanoparticle dispersion with 1% mannitol as cryoprotectant was frozen at -60°C for 3 hr in a deep freezer and then subjected to freeze drying. Sublimation lasted for 24 hr at a vacuum pressure of 100 milli torque without heating at room temperature, with the condenser surface temperature maintained at -100°C. Freeze dried nanoparticles were reconstituted by double distilled water followed by sonication for period of 1 min.

Result

Different properties of lyophilized SCG-CSNPs like physical appearance, reconstitution nature and size ratio (before and after freeze drying) were analyzed and given in Table 7. The freeze dried SCG-CSNPs were found to be soft, white, amorphous, fluffy, free flowing in nature and were easily redispersed to form SCG-CSNPs by mere normal manual shaking. There was no significant difference in particle size and %EE of SCG-CSNPs after freeze drying as compared to before freeze drying ($p>0.05$). Moreover, Sa/Sb (ratio of particle size after and before freeze drying) remained almost unity (1.089). The % yield of optimized batch was found to be 85.84%. The % drug loading was checked by direct method, by measuring the actual amount of SCG within the CSNPs and it was found to be $8.66 \pm 0.6\%$.

Table A.4. Particle size, PDI and % EE after and before freeze drying

Before Freeze drying			After freeze drying			Ratio (Sa/Sb)
Particle size (nm)	PDI	%EE	Particle size (nm)	PDI	%EE	1.089
204.9 ± 2.3	0.096 ± 0.04	63.68 ± 2.03	223 ± 5.6	0.206 ± 0.1	62.8 ± 2.67	

All data are shown as mean $\pm$ S.D; n=3

Conductivity study and pH study

The conductivity study and pH study were performed to monitor the cross-linking reaction between chitosan and TPP. The change in conductivity and pH was measured after each ml of addition of TPP solution to the chitosan solution on the conductivity meter (CM 183, Elico) and the pH meter (EI 120, Elico), respectively.

Result

When chitosan is dissolved in acidic environment, the amino group becomes positively charged. TPP provides both hydroxyl and phosphoric ions in presence of water that additionally depends on pH of solution. As crosslinking reaction involves interaction between cations and anions, pH of the TPP solution significantly affect the extent and kind of cross-linking. At pH 3 of TPP solution only phosphoric ions were present whereas at pH 9 both phosphoric as well as hydroxyl ions were present which compete with each other for interaction with positively charged amino group of chitosan. The conductivity study was performed to investigate the nature of crosslinking with the change in the ionic species relative to change in pH. At lower pH (pH 3), conductivity decreased up to 5 ml due to interaction between amino group and phosphoric ion. Further, addition of TPP solution, conductivity increases after equivalence point attributable to saturation of amino group to chitosan and also due to increase of H^+ ions. Hence, the process was confirmed as preponderantly ionic crosslinking. Whereas at higher pH of TPP solution (pH 9), the decrease in conductance was seen up to 9 mL due to presence of both hydroxyl and phosphoric ions which compete with each other for positive amino group. The hydroxyl radical interacts with amino group by deprotonation (Fig. S.2A). Moreover, the results were additionally well explained by pH measurement. At higher pH (pH 9), gradual increase in pH was observed whereas at lower pH (pH 3), the pH of the system remains unchanged throughout crosslinking (Fig. S.2B). From the study it had been confirmed that at lower pH crosslinking

reaction proceed by ionic crosslinking whereas higher pH deprotonation and ionic crosslinking, each mechanisms exists.

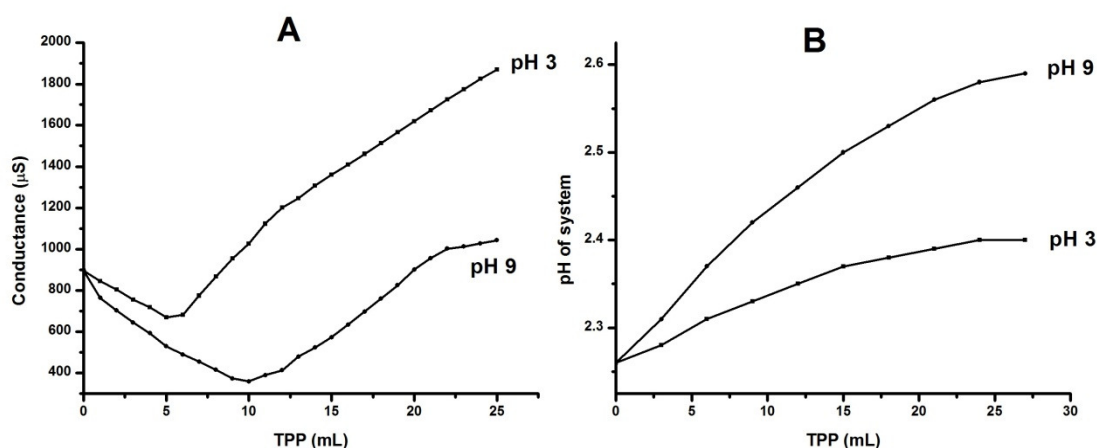


Fig. A.4. Effect of pH of TPP on (A) conductivity and (B) pH of the system during crosslinking of chitosan with TPP

Swelling index study

200 mg of freeze dried SCG-CSNPs were dispersed in 250 ml phosphate buffer (pH 6.8) for a period of 6 hr at room temperature. The swollen NPs were collected by centrifugation and the wet weight of the swollen CSNPs was recorded by first blotting with filter paper to remove excess water on the surface and then weighing immediately on electronic balance at predetermined time period (0.5, 1, 2, 3, 4, 5 and 6 h). Swelling index (SI) was then calculated using the following formula.

$$\text{Swelling index} = \frac{W_s - W_i}{W_i} \times 100$$

Where, W_i was the initial weight, W_s was the swollen weight

Result

Swelling study was carried out to assess the crosslinking density of the SCG-CSNPs as it directly affects the physical properties of SCG-CSNPs and thereby permeability and drug release behaviour. From the study it was found that swelling of the SCG-CSNPs was time dependent in phosphate buffer pH 6.8 as shown in Fig. S.4. A contrary relation observed between the swelling capacity and the crosslinking density of the CSNPs. The decrease in

swelling capacity with increase in the crosslinking density could be attributed to the more tightly crosslinked chitosan matrix that does not swell as compared to loosely packed chitosan matrix. At lower volume of crosslinking agent, the chitosan network loosely crosslinked and had high hydrodynamic free volume to accommodate more solvent molecules thereby inducing CSNPs matrix swelling. The water uptake and thereby swelling in hydrogels depends upon the extent of hydrodynamic free volume and accessibility of hydrophilic functional groups with which water can establish hydrogen bonds. As SCG-CSNPs shows lesser swelling compared to other available literature indicates that there was better crosslinking density in the SCG-CSNPs with tightly crosslinked matrix which provides structure rigidity and presence of less number of free hydrophilic groups in CSNPs that are responsible for swelling. Moreover, the obtained results also correlate that at neutral and basic pH, CSNPs formed by deprotonation mechanism and fewer amounts of phosphate groups involves in crosslinking process. As phosphate group imparts water holding capacity and thereby swelling property, due to presence of fewer amounts of phosphate groups in crosslinked SCG-CSNPs, it shows lesser swelling behaviour.

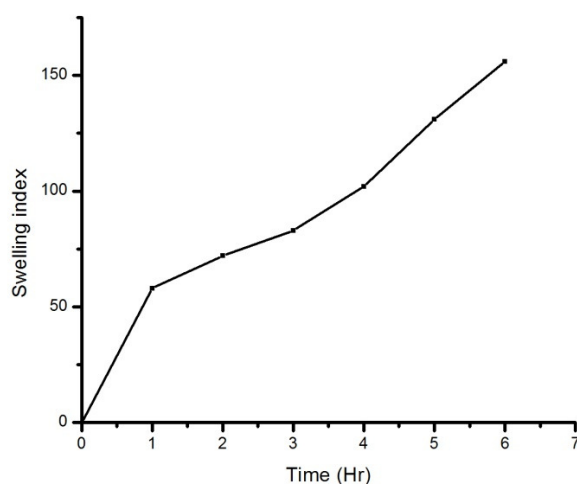


Fig. A.5. Swelling behaviour of SCG-CSNPs in phosphate buffer pH 6.8