Chapter 7
Experimental –Nitrendipine loaded Alginate Microspheres

7.1 MATERIALS AND METHODS

7.1.1 Materials

- Nitrendipine was a gift sample from USV Limited, Mumbai, India.
- Sodium alginate, liquid paraffin (light), calcium chloride, Span 80® were procured from S. D. Fine Chemicals, Mumbai, India.
- Potassium phosphate monobasic (KH_2PO_4), Sodium hydroxide, Methanol, Isopropyl alcohol (IPA) and Hydrochloric acid were procured from SD Fine chemicals, Mumbai, India.
- A dialysis membrane (cut-off Mw 12000) was procured from Hi Media, India.
- All other chemicals and reagents used in the study were of analytical grade.

7.1.2 Equipments

- Eurostar high speed stirrer (IKA Labortechnik, Germany)
- Remi high speed magnetic stirrer (Remi, MS500, Remi equipments, Mumbai)
- Malvern particle size analyzer (Malvern Mastersizer 2000, Malvern Instruments, UK)
- UV-VIS spectrophotometer (Shimadzu UV1610, Japan)
- Light transmission microscope (Olympus Optical Co. Ltd., Japan)
- Scanning electron microscopy (JSM 5610 LV, Jeol Datum Ltd., Japan)
- Differential Scanning Calorimeter (Mettler Toledo DSC 822e, Japan)
- X-ray diffractometer (Bruker AXS D8 Advance, with X-ray source of Cu, Wavelength 1.5406 A° and Si(Li) PSD detector)

7.2 Preparation of Alginate microspheres

The emulsification method was utilized for the preparation of microspheres followed by cross linking with calcium chloride (Rastogi et al., 2007). Nitrendipine (NTD) was dispersed in an aqueous solution containing 3% w/v sodium alginate. The aqueous phase was emulsified in light liquid paraffin containing 2% (v/v) Span 80 in the ratio 1:10 using a Eurostar (IKA Labortechnik, Germany) high speed stirrer at 1200 rpm for 60 min. Calcium chloride solution (2%) in a mixture of methanol and isopropyl alcohol (2:3) was added drop wise and the dispersion was stirred for another 10 min.

Microspheres were collected by filtration in vacuum, washed with isopropyl alcohol thrice and finally air dried at room temperature. Various variables like drug: polymer ratio, concentration of cross linking agent and time of cross linking were considered in the optimization of the formulation.

7.3 Experimental design

Various batches of alginate microspheres were prepared based on the 2^3 factorial design. The independent variables were drug: polymer weight ratio (X_1), calcium chloride concentration (X_2) and cross linking time (X_3). The independent variables and their levels are shown in Table 7.1. Particle size of the microspheres (Y1) and *in vitro* mucoadhesion (Y2) were taken as response parameters as the dependent variables. Table 7.2 shows the independent and dependent variables.

Table 7.1 Factorial design parameters and experimental conditions.

Factors	Levels used, Actual (coded)	
	Low (-1)	High (+1)
X_1=Drug: polymer weight ratio	0.5:1	1:1
X_2=Concentration of $CaCl_2$ (%)	2	4
X_3=Cross linking time (min)	10	20

7.4 Characterization of the microspheres

Characterization of the alginate microspheres was carried out as per the methods described in **Section 4.5** for following studies:

7.4.1. Particle size measurements

7.4.2. Surface morphology

7.4.3 Flow Properties

7.4.4 Determination of encapsulation efficiency

The microspheres (5 mg) loaded with nitrendipine were crushed and added in a mixture of 10 ml phosphate buffer pH 6.2 containing 1.0 % Tween 80 under stirring. The mixture was filtered and the amount of nitrendipine was determined spectrophotometrically at 355 nm on UV spectrophotometer (Shimadzu UV1610, Japan). It was confirmed from preliminary UV studies that the presence of dissolved polymers did not interfere with the absorbance of the nitrendipine at 355 nm. The percentage encapsulation efficiency was calculated using equation (7.1).

$$\% \text{ Encapsulation efficiency} = \frac{\text{Actual loading}}{\text{Theoretical loading}} \times 100 \qquad \text{..... 7.1}$$

7.4.5 Measurement of in vitro mucoadhesion

7.4.6 Differential scanning calorimetry (DSC) analysis

7.4.7 X-ray diffraction (XRD) studies

7.4.8 In vitro drug release

The drug release profiles of NTD loaded alginate microspheres were evaluated using same method described in **Section 4.5.8** except the media used was phosphate buffer pH 6.2 containing 1.0 % Tween 80.

7.5 Optimization data analysis and model-validation

ANOVA was used to establish the statistical validation of the polynomial equations generated by Design Expert® software (version 7.1.3, Stat-Ease Inc, Minneapolis, MN). Fitting a multiple linear regression model to a 2^3 factorial design gave a predictor equation which was a first-order polynomial, having the form:

$$Y=b_0+b_1X_1+b_2X_2+b_3X_3+b_{12}X_1X_2+b_{13}X_1X_3+b_{23}X_2X_3+b_{123}X_1X_2X_3 \qquad \text{..... 7.2}$$

where Y is the measured response associated with each factor level combination; b_0 is an intercept representing the arithmetic average of all quantitative outcomes of 8 runs; b_1 to b_{123} are regression coefficients computed from the observed experimental values of Y; and X_1, X_2 and X_3 are the coded levels of independent variables. The terms X_1X_2, X_2X_3 and X_1X_3 represent the interaction terms. The main effects (X_1, X_2 and

X_3) represent the average result of changing one factor at a time from its low to high value. The interaction terms show how the response changes when two factors are changed simultaneously. The polynomial equation was used to draw conclusions after considering the magnitude of coefficients and the mathematical sign it carries, i.e., positive or negative. A positive sign signifies a synergistic effect, whereas a negative sign stands for an antagonistic effect (Dhiman et al., 2008).

In the model analysis, the responses: the particle size of the microspheres and in vitro mucoadhesion of all model formulations were treated by Design-Expert® software. The best fitting mathematical model was selected based on the comparisons of several statistical parameters including the coefficient of variation (CV), the multiple correlation coefficient (R^2), adjusted multiple correlation coefficient (adjusted R^2), and the predicted residual sum of square (PRESS), proved by Design- Expert® software. Among them, PRESS indicates how well the model fits the data, and for the chosen model it should be small relative to the other models under consideration. Level of significance was considered at $P<0.05$. Three dimensional response surface plots and two dimensional contour plots resulting from equations were obtained by the Design Expert® software. Subsequently, the desirability approach was used to generate the optimum settings for the formulations (Huang et al., 2005; Narendra et al., 2005)

2FI (interaction) model:

$$Y = b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 \quad \text{..... 7.3}$$

Linear model:

$$Y = b_1X_1 + b_2X_2 + b_3X_3 \quad \text{..... 7.4}$$

7.6 Histology studies

The study carried out as per the method described in Section **4.5.11.**

7.7 Stability study

Stability studies of alginate microspheres of NTD were carried out as per method described in **Section 4.5.12.**

7.8 RESULTS AND DISCUSSION

7.8.1 Particle size

The size of all the eight batches of microspheres prepared in this study was in the range of 22.68 – 48.95 μm (Table 7.2), which is favorable for intranasal absorption.

Table 7.2 Formulation of the microspheres utilizing 2^3 factorial design.

Formulation Code	X1	X2	X3	Y1*	Y2*
ALNT1	0.5:1	2	10	22.68 ±1.95	83.67 ±2.46
ALNT2	1:1	2	10	40.46 ±2.68	74.91 ±2.35
ALNT3	0.5:1	4	10	29.35 ±1.98	78.48 ±2.54
ALNT4	1:1	4	10	43.32 ±2.52	73.64 ±2.13
ALNT5	0.5:1	2	20	32.98 ±2.34	80.36 ±1.86
ALNT 6	1:1	2	20	48.95 ±2.24	69.24 ±2.67
ALNT7	0.5:1	4	20	33.82 ±2.28	74.56 ±2.47
ALNT8	1:1	4	20	45.65 ±1.59	70.98 ±1.96

*Values are expressed as mean ± SD. Y1 and Y2 are particle size and in vitro mucoadhesion respectively.

Preliminary studies showed that as the concentration of polymer was increased, the particle size also proportionally increased. Low alginate concentrations (1%, w/v and 2%, w/v) resulted in clumping of microspheres, whereas high sodium alginate concentration (4%, w/v) resulted in formation of discrete larger microspheres (70 - 80 μm). This could be attributed to an increase in the relative viscosity at higher concentration of polymer and formation of larger particles during emulsification. Hence the optimized concentration of 3%, w/v was selected for preparing the different batches of the microspheres. The high shearing rate required for emulsification caused the breakdown of the viscous aqueous alginate solution to fine globules resulting in small microspheres.

The size of the microspheres was increased with an increase in drug loading. This can be attributed to the corresponding increase in viscosity of drug-polymer dispersion comprising the internal phase of the emulsion. The viscosity increase within the internal phase results in the generation of a coarser emulsion with larger droplets, leading eventually to the formation of larger microspheres (Bhardwaj et al., 1995).

Similar increase in the size of microspheres was also observed with increase in calcium chloride concentration as well as cross-linking time. The addition of higher amount of Ca^{2+} will result in relatively more cross-linking of the guluronic acid units of sodium alginate, thereby leading to formation of larger microspheres. Similarly, increasing the cross linking time will increase the extent of cross linking and thereby increase the particle size. Alginate molecules are linear block co-polymers of β-D-mannuronic (M) and α-L-guluronic acids (G) with a variation in composition and sequential arrangements (Fig. 7.1). Up to now, it was assumed that the G-blocks are the only molecules in alginate that bind divalent ions cooperatively and are, therefore, the main structural feature contributing to gel formation. Recent findings, however, show that not only G-blocks but also blocks of alternating M and G (MG-blocks) can form cross links with calcium. Hence, calcium junctions of GG–GG, MG–GG and MG–MG must be hold responsible for gel formation (Donati et al., 2005).

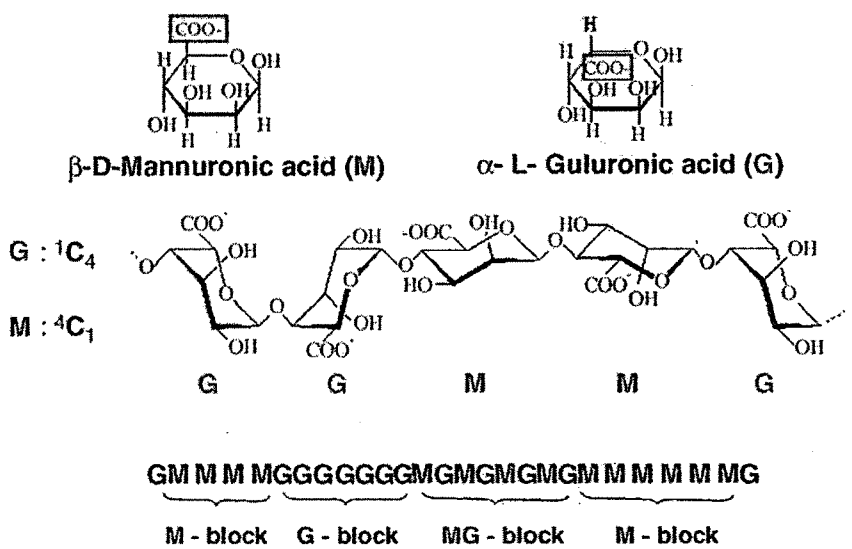

Fig. 7.1 The structure of alginate. Alginate molecules are linear block copolymers of β-D-mannuronic (M) and α-L-guluronic acids (G) with a variation in composition and sequential arrangements.

7.8.2 Surface morphology

Scanning electron microcopy (SEM) was used to investigate the physical appearance of the NTD loaded alginate microparticles. SEM observation revealed that the microspheres were spherical in shape (Fig. 7.2 and 7.3). The drug may be present in the bulk of the microspheres and not surface associated.

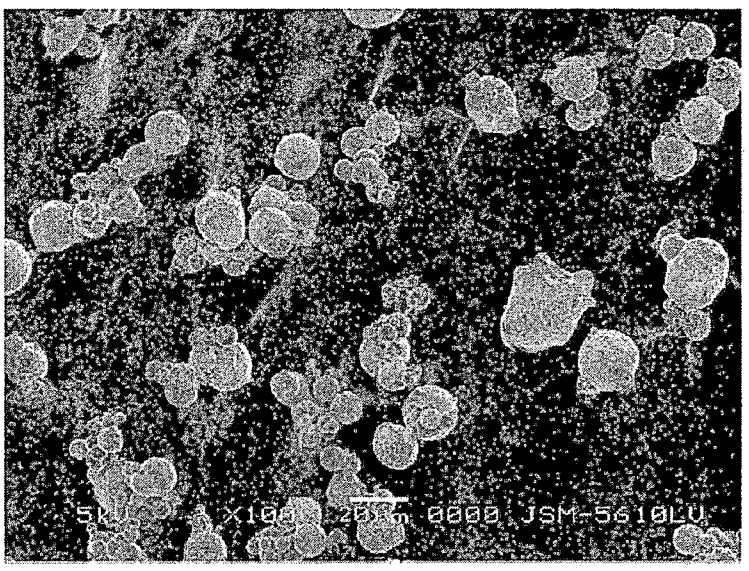

Fig. 7.2 SEM Photograph NTD loaded alginate microspheres at 2000X

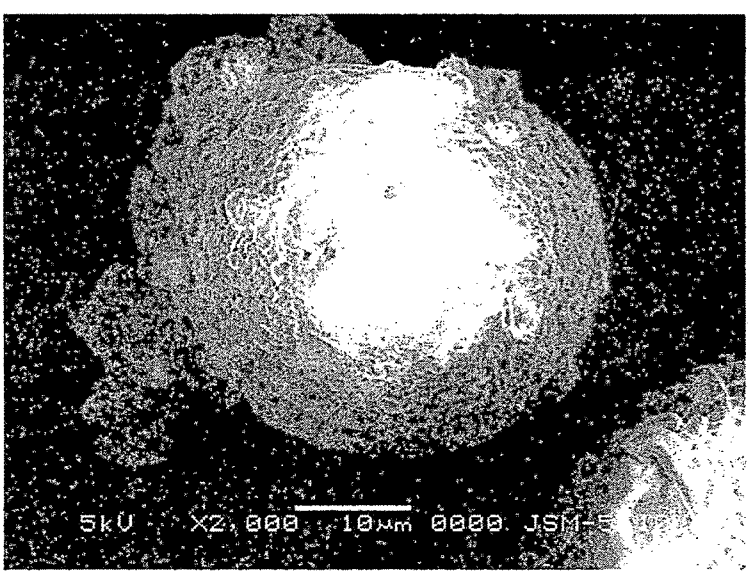

Fig. 7.3 SEM Photograph of NTD loaded alginate microspheres at 100X

7.8.3 Flow Properties

The results of flow properties measurements are shown in Table 7.3. The values of angle of repose were in the range of 25.41^0 to 35.62^0 which is within the normal acceptable range of 20^0 – 40^0 (Wells and Aulton, 1988). The microspheres thus showed reasonably good flow potential. The values of compressibility index were in the range 15.12±2.65 to 20.18±1.36, also indicating good flow characteristics of the microspheres.

Table 7.3 Characteristics of prepared NTD loaded Alginate microspheres

Formulation Code	Angle of repose (θ)	Compressibility Index (%)	% Encapsulation Efficiency
ALNT1	25.41±2.13	15.12±2.65	47.32±2.46
ALNT2	30.24±2.35	17.34±2.48	63.42±1.82
ALNT3	28.27±1.86	16.49±1.94	44.32±2.86
ALNT4	34.56±2.47	18.44±2.13	57.12±2.98
ALNT5	30.84±2.43	15.82±2.34	46.57±2.45
ALNT 6	24.96±1.84	16.22±1.85	58.42±2.12
ALNT7	35.62±1.98	19.28±2.64	42.12±2.34
ALNT8	32.78±2.42	20.18±1.36	55.86±3.14

7.8.4 Encapsulation efficiency (EE)

The % EE was found to be in the range between 42.12 and 63.42. The % EE showed a dependence on drug loading, amount of cross linking agent and time of cross linking. The formulations loaded with higher amount of drug exhibited higher encapsulation efficiencies. The encapsulation efficiency, however, showed an inverse relationship with increasing calcium chloride concentration and cross linking time. Both these factors lead to an increase in cross link density, which will reduce the free volume spaces within the polymer matrix and hence, a reduction in encapsulation efficiency is observed.

7.8.5 In vitro mucoadhesion

The results of in vitro mucoadhesion (Table 7.2) showed that all the batches of microspheres had satisfactory mucoadhesive property ranging from 69.24 to 83.67% (Fig. 7.4) and could adequately adhere on nasal mucosa. The results also showed that with increasing polymer ratio, higher mucoadhesion percentages were obtained. This could be attributed to the availability of higher amount of polymer for interaction with mucus. Increase in calcium chloride concentration and cross linking time decreased the mucoadhesive property of the microspheres.

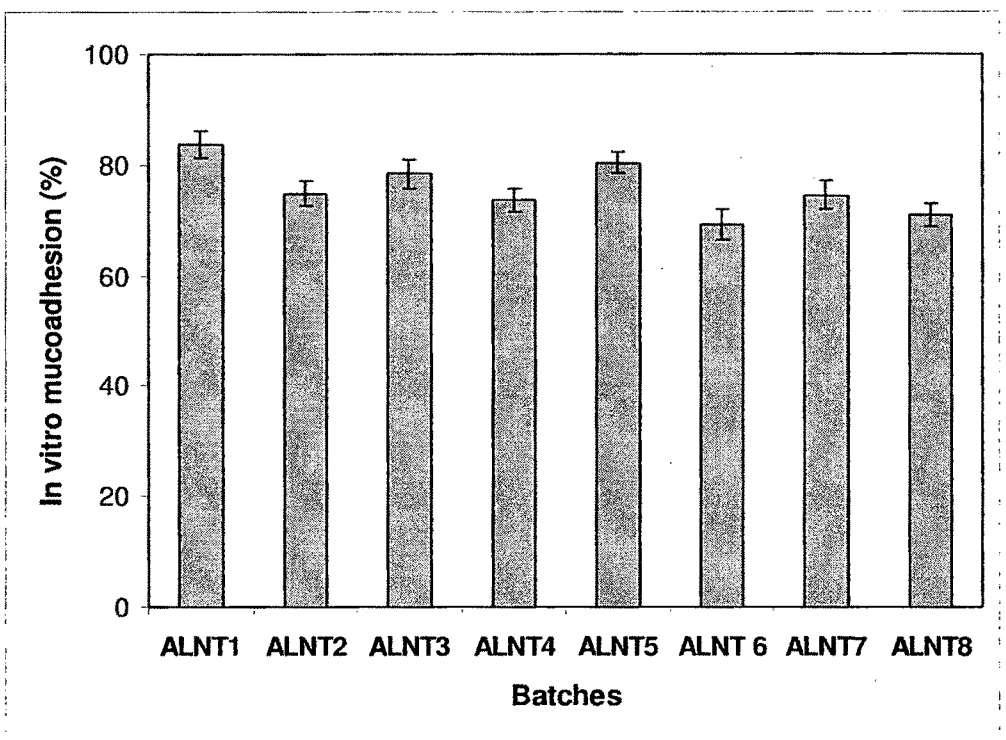

Fig. 7.4 Percentage in vitro mucoadhesion for different batches of microspheres

7.8.6 Differential scanning calorimetry (DSC) analysis

DSC thermograms of pure nitrendipine, placebo alginate microspheres and nitrendipine loaded alginate microspheres are displayed in Fig. 7.5. Thermogram of Nitrendipine showed a sharp peak at 159 ^{0}C due to melting of the drug, but in case of nitrendipine loaded microspheres, no characteristic peak was observed at 159 ^{0}C, implying that there are no free Nitrendipine crystals in the system i.e. the microspheres exhibited thermic behaviour characteristic of amorphous substances.

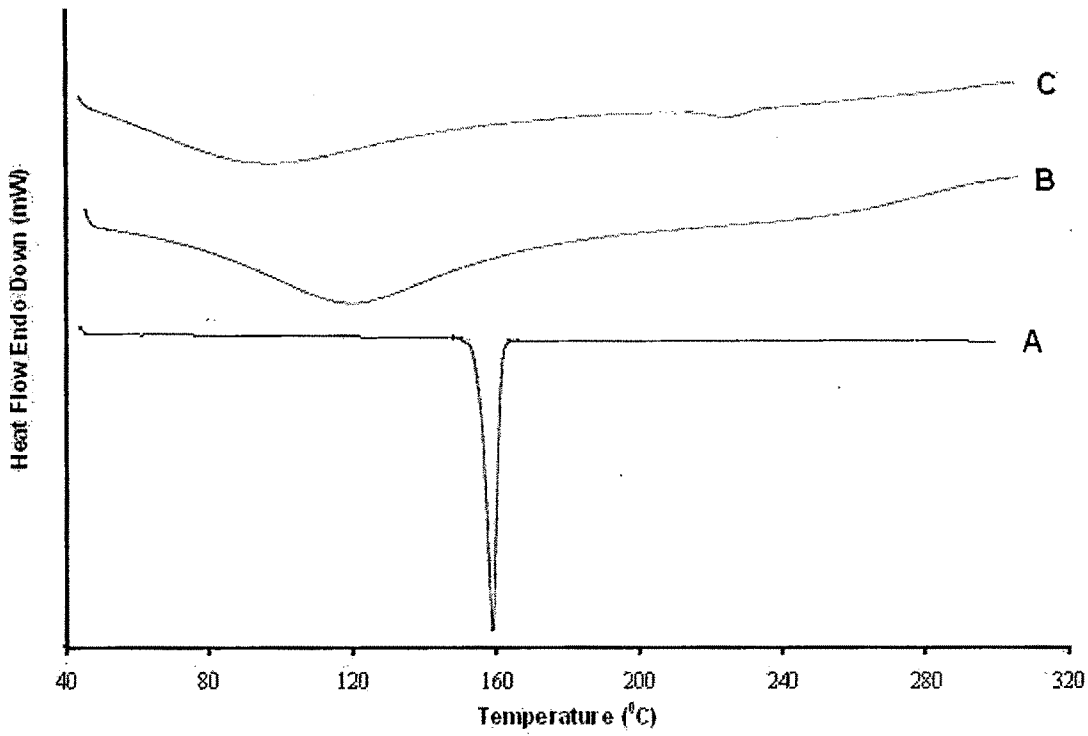

Fig. 7.5 DSC thermograms of (A) pure NTD; (B) placebo microspheres; (C) drug loaded microspheres.

7.8.7 X-ray diffraction (XRD) studies

The XRD spectra recorded for plain NTD (A), placebo alginate microspheres (B) and NTD loaded alginate microspheres (C) are presented in Fig. 7.6. NTD showed characteristic intense peaks at 2θ of 9.99^0, 11.33^0, 13.09^0, 23.80^0, 24.30^0, 27.45^0 and 28.70^0. However, these peaks were not observed in the NTD loaded microspheres. This indicates that drug particles are dispersed at molecular level in the polymer matrix.

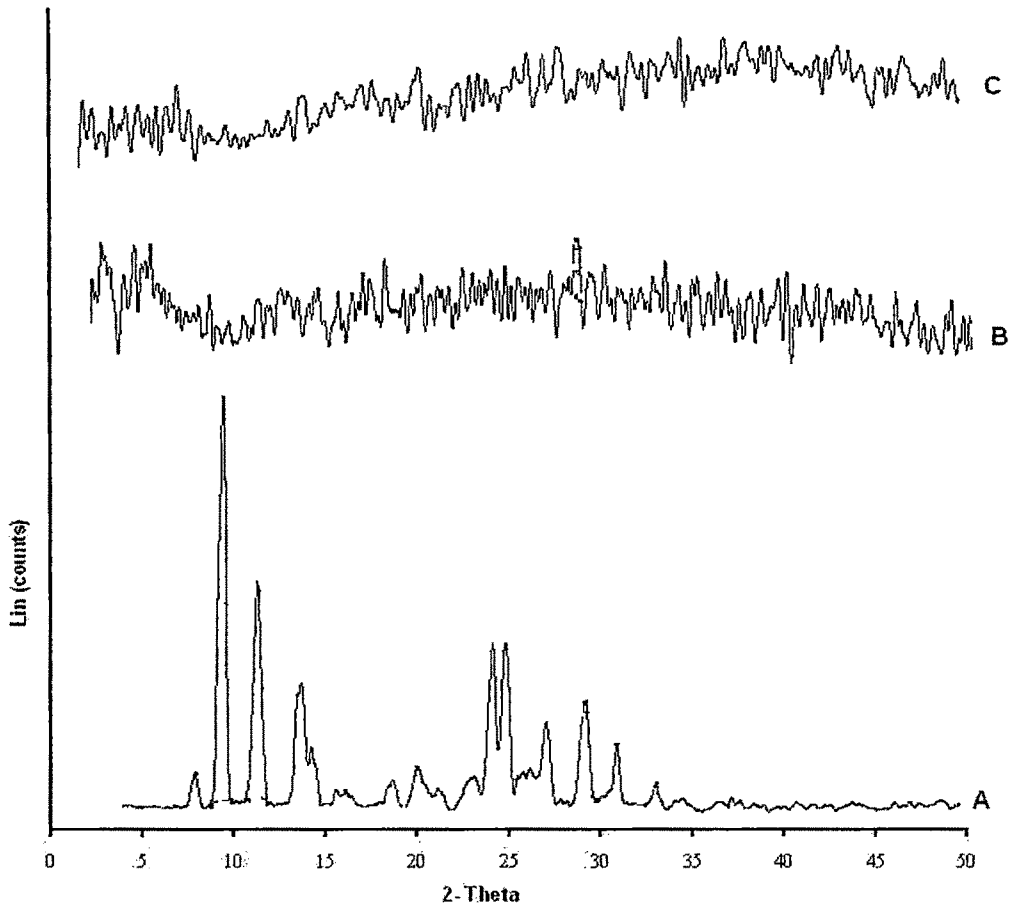

Fig. 7.6 Powder X-ray diffraction patterns of (A) pure NTD; (B) placebo microspheres; (C) NTD loaded microspheres.

7.8.8 In vitro drug release

The in vitro drug release profile of NTD from the alginate microspheres is shown in Fig. 7.7 and Fig. 7.8. The release pattern showed a slow and controlled release phase resulting from the controlled diffusion of entrapped drug. *In vitro* drug release proportionally increased with increasing the drug concentration. As expected (Thanoo et al., 1992), with an increase in the cross linking agent concentration, a respective decrease in the rate and extent of drug release was observed.

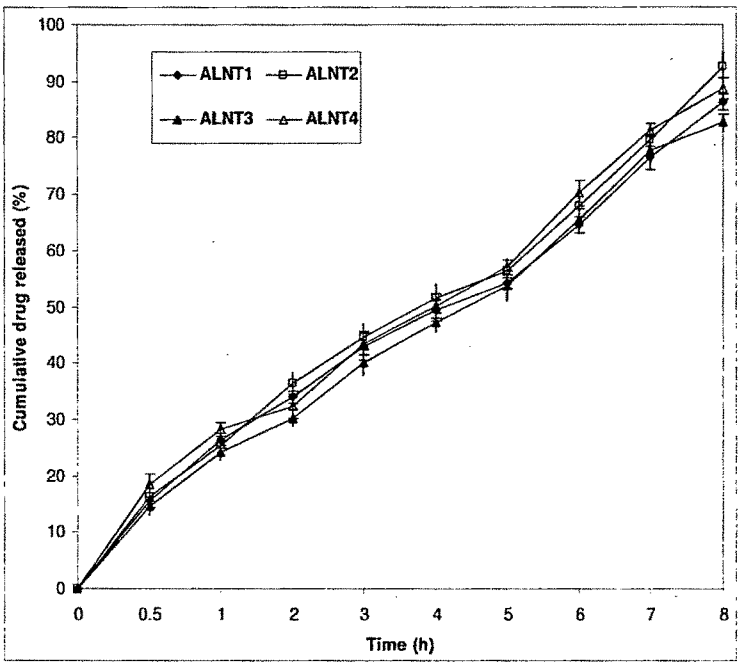

Fig. 7.7 In *vitro* drug release profile of alginate microspheres of NTD (Batches ALNT1 to ALNT4). The values are mean ± SD (n = 3).

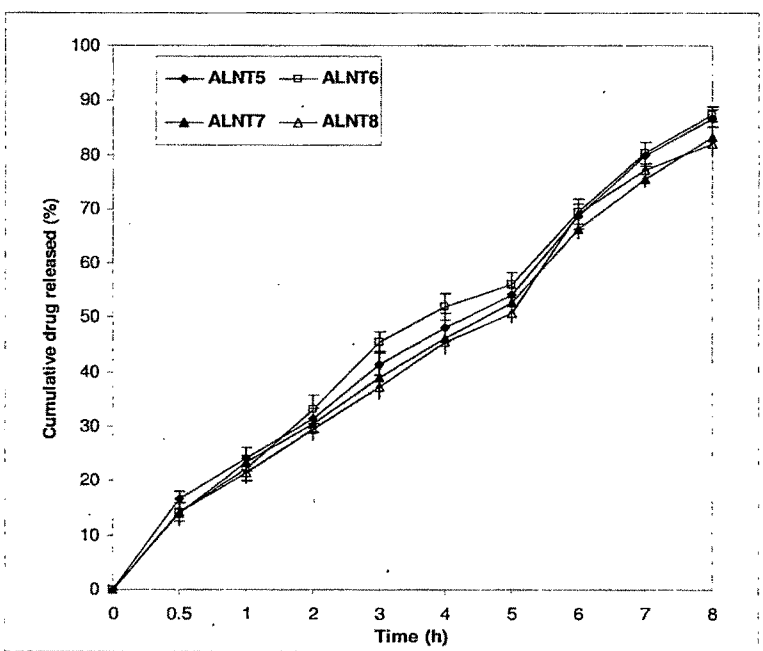

Fig. 7.8 *In vitro* drug release profile of alginate microspheres of NTD (Batches ALNT5 to ALNT8). The values are mean ± SD (n = 3).

Mathematical modeling of release kinetics

In order to understand the mechanism and kinetics of drug release, the data was fitted to various kinetic equations. Three kinetic models including the zero-order release equation ($Q_t=K_1t$), first-order equation ($Q_t=Q_0e^{-K_2t}$), and Higuchi equation ($Q_t=K_3t^{1/2}$) were applied to process the in vitro data to find the equation with the best fit as explained in **Section 5.8.8.**

The linear regression analyses are summarized in Table 7.3. A model with the greatest regression coefficient (r^2) was chosen as the dominant model.

Table 7.3 Kinetic model of Nitrendipine release from Alginate microspheres

Formulation code	Regression coefficient (r^2)			Release kinetics
	Zero-order	First-order	Higuchi model	
ALNT1	0.9681	0.8742	0.9636	Zero-order
ALNT2	0.9681	0.7428	0.9604	Zero-order
ALNT3	0.9781	0.9133	0.9661	Zero-order
ALNT4	0.9710	0.8713	0.9554	Zero-order
ALNT5	0.9781	0.8681	0.9586	Zero-order
ALNT6	0.9772	0.8615	0.9797	Higuchi
ALNT7	0.9806	0.8789	0.9653	Zero-order
ALNT8	0.9802	0.9046	0.9548	Zero-order

In order to further investigate the release mechanism, the data were analyzed using the Peppas equation as explained in **Section 5.8.8.**

Using the least squares procedure, the values of n, k and correlation coefficient (r^2) were estimated (Table 7.4). In spherical matrices, if $n \leq 0.43$, a Fickian diffusion (case-I), $0.43 \leq n < 0.85$, anomalous or non-Fickian transport and $n \geq 0.85$, a case-II transport (zero order) drug release mechanism dominates. The values of n for all the batches

ranged from 0.56 to 0.73 with correlation coefficient close to 0.99, indicating non-Fickian or anomalous type of transport controlled both by diffusion and relaxation of the polymer (Mundargi et al., 2008).

Table 7.4 Release kinetics parameters and mechanisms of different formulations

Batch code	n	k	Correlation coefficient, r^2	Release mechanism
ALNT1	0.60	0.337	0.9823	Non-Fickian
ALNT2	0.63	0.321	0.9957	Non-Fickian
ALNT3	0.59	0.342	0.9867	Non-Fickian
ALNT4	0.56	0.361	0.9657	Non-Fickian
ALNT5	0.58	0.353	0.9836	Non-Fickian
ALNT6	0.67	0.299	0.9986	Non-Fickian
ALNT7	0.63	0.322	0.9719	Non-Fickian
ALNT8	0.73	0.268	0.9912	Non-Fickian

7.9 Optimization data analysis and model-validation

7.9.1 Fitting of data to the model

The three factors with lower and upper design points in coded and uncoded values are shown in Table 7.1. The ranges of responses Y1 and Y2 were 22.68 - 48.95 µm and 69.24 - 83.67% respectively. All the responses observed for eight formulations prepared were fitted to various models using Design- Expert® software. It was observed that the best-fitted models were interactive (2FI) and linear. The values of R^2, adjusted R^2, predicted R^2, SD and % CV are given in Table 7.5 along with the regression equation generated for each response. The results of ANOVA in Table 7.6 for the dependent variables demonstrate that the model was significant ($P< 0.05$) for both the response variables.

Table 7.5 Summary of results of regression analysis for responses Y1 and Y2

Models	R^2	Adjusted R^2	Predicted R^2	SD	% CV	P
Response (Y1)						
Interactive model	1.0000	0.9998	0.9984	0.12	0.31	0.0093
Response (Y2)						
Linear model	0.8840	0.7970	0.5359	2.18	2.87	0.0242

Regression equations of the fitted interactive and linear model

$Y1 = 37.15 + 7.44X_1 + 0.88X_2 + 3.20X_3 - 0.99X_1X_2 - 0.49X_1X_3 - 1.50X_2X_3$

$Y2 = 75.73 - 3.54X_1 - 1.31X_2 - 1.95X_3$

Table 7.6 Results of analysis of variance for measured response

Parameters	DF*	SS*	MS*	F*	Significance F
Particle size					
Model	6	559.2	93.2	6846.65	0.0093 significant
Residual	1	0.014	0.014	---	---
Total	7	559.21	---	---	---
In vitro mucoadhesion					
Model	3	144.21	48.07	10.16	0.0242 significant
Residual	4	18.93	4.73	---	---
Total	7	163.14	---	---	---

*DF indicates degrees of freedom; SS sum of square; MS mean sum of square and F is Fischer's ratio.

It was observed that all the three independent variables viz. X_1 (drug: polymer weight ratio), X_2 (concentration of $CaCl_2$) and X_3 (cross linking time) had positive effect on particle size (Y1), but, negative effect on *in vitro* mucoadhesion (Y2). The effect of drug: polymer weight ratio was eight times greater than $CaCl_2$ concentration and two fold more than the cross linking time on the particle size of the microspheres. The coefficients with more than one factor term in the regression equation represent interaction terms. It also shows that the relationship between factors and responses is not always linear. When more than one factor are changed simultaneously and used at different levels in a formulation, a factor can produce different degree of response. The interaction effect of X_1X_2; X_1X_3 and X_2X_3 was unfavorable (negative) for response Y1 i.e. particle size. The effect of drug: polymer weight ratio was approximately thrice more than $CaCl_2$ concentration and two fold more than the cross linking time on in vitro mucoadhesion of the microspheres. But all these factors had negative effect on in vitro mucoadhesion of the microspheres.

5.9.2 Contour plots and response surface analysis

Three dimensional response surface plots generated by the Design Expert® software are presented in Fig. 7.9-7.11 and Fig. 7.15-7.17 while two dimensional contour plots are presented in Fig. 7.12-5.14 and Fig. 7.18-7.20 for the studied responses i.e. particle size and in vitro mucoadhesion. In all the presented figures, the third factor was kept at a constant level. Fig. 7.9 and Figure 7.12 depicts response surface, contour plots of the effects of drug: polymer ratio (X_1) and $CaCl_2$ concentration (X_2) on particle size which indicate a linear effect on particle size of the microspheres. The combined effects of $CaCl_2$ concentration (X_2) and cross linking time (X_3) and drug: polymer ratio (X_1) and cross linking time (X_3) on particle size as shown in Fig. 7.10, 7.11 and Fig. 7.13, 7.14 are also linear. This explains that higher the amount of $CaCl_2$ or higher the time of cross linking, more will be the cross-linking of the guluronic acid units of alginate leading to formation of microspheres with large size.

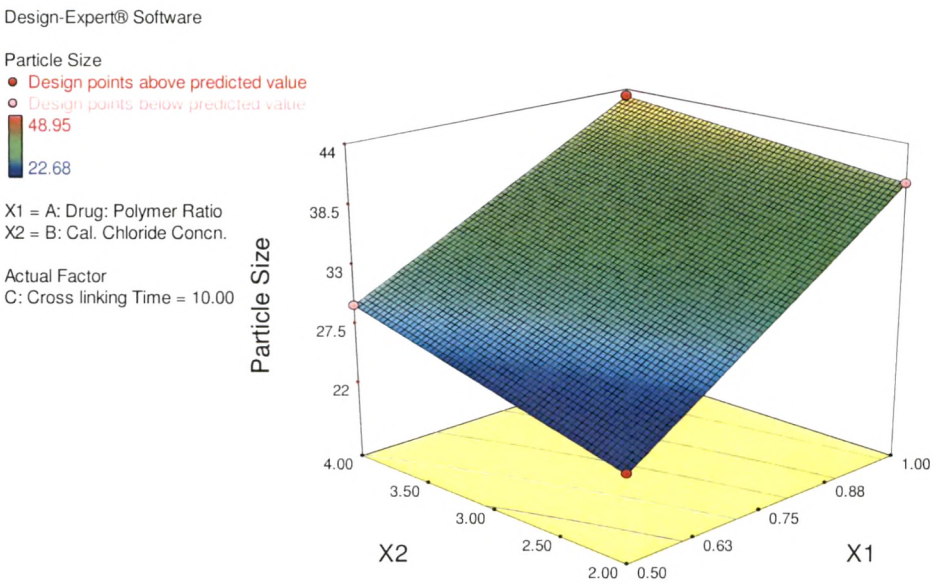

Fig. 7.9 Response surface plots for the (a) effects of drug: polymer ratio (X1) and $CaCl_2$ concentration (X2) on particle size (Y1).

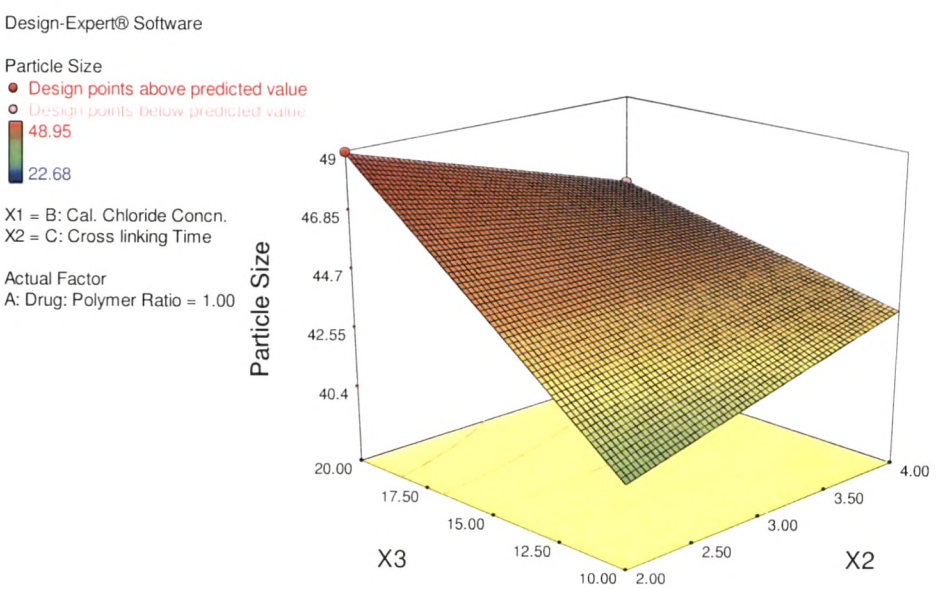

Fig. 7.10 Response surface plots for the effects of $CaCl_2$ concentration (X2) and cross linking time (X3) on particle size (Y1).

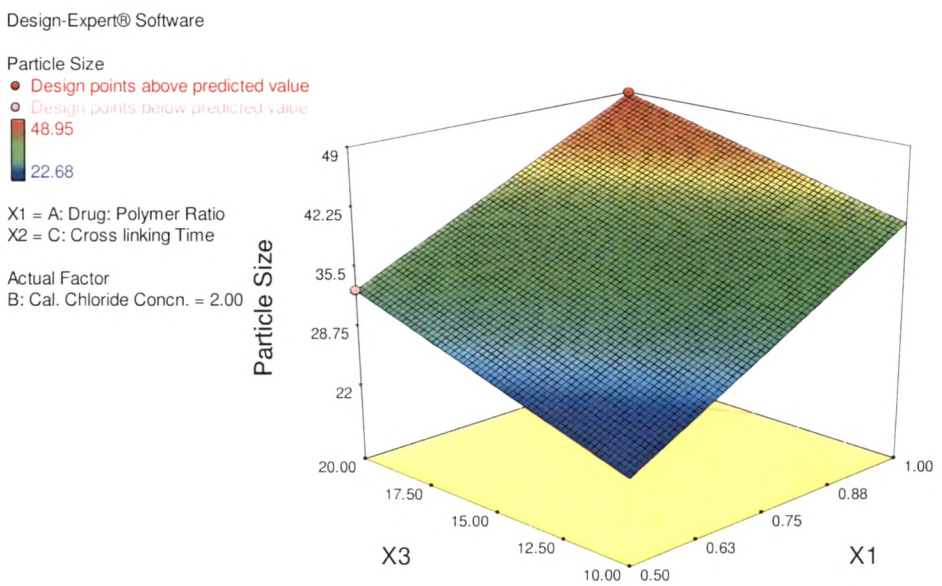

Fig. 7.11 Response surface plots for the effects of drug: polymer ratio (X1) and cross linking time (X3) on particle size (Y1).

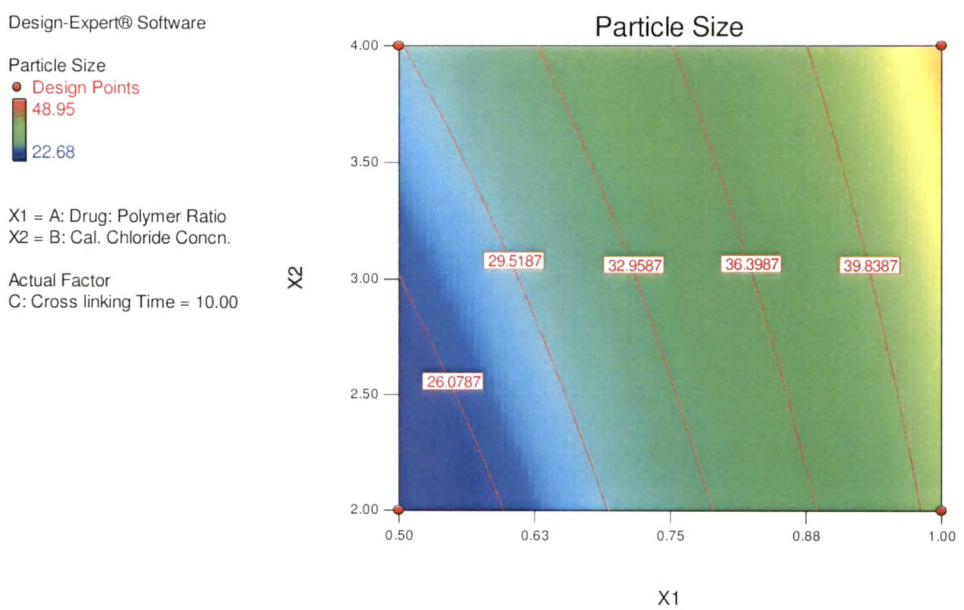

Fig. 7.12 Contour plots for the effects of drug: polymer ratio (X1) and $CaCl_2$ concentration (X2) on particle size (Y1).

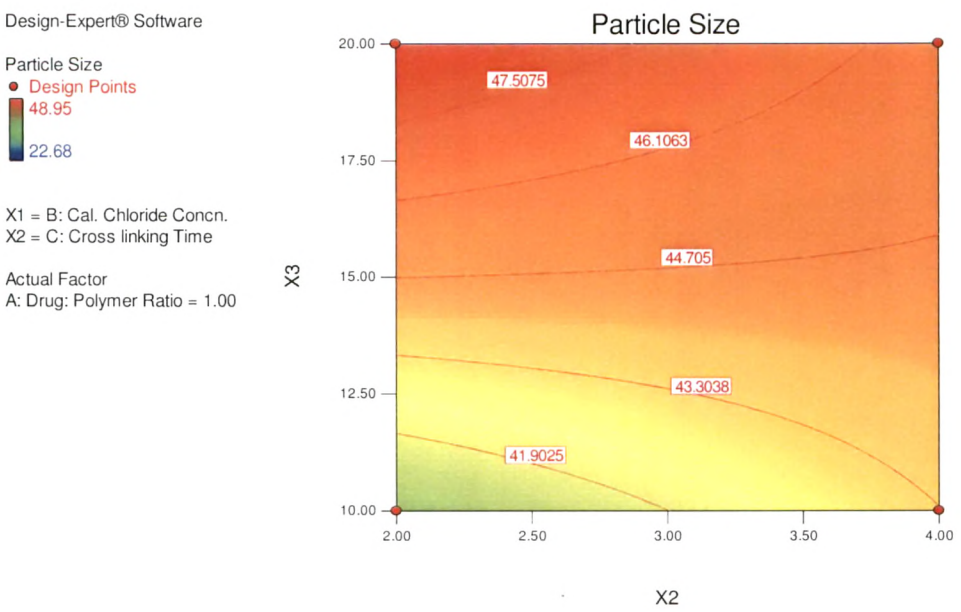

Fig. 7.13 Contour plots for the effects of $CaCl_2$ concentration (X2) and cross linking time (X3) on particle size (Y1).

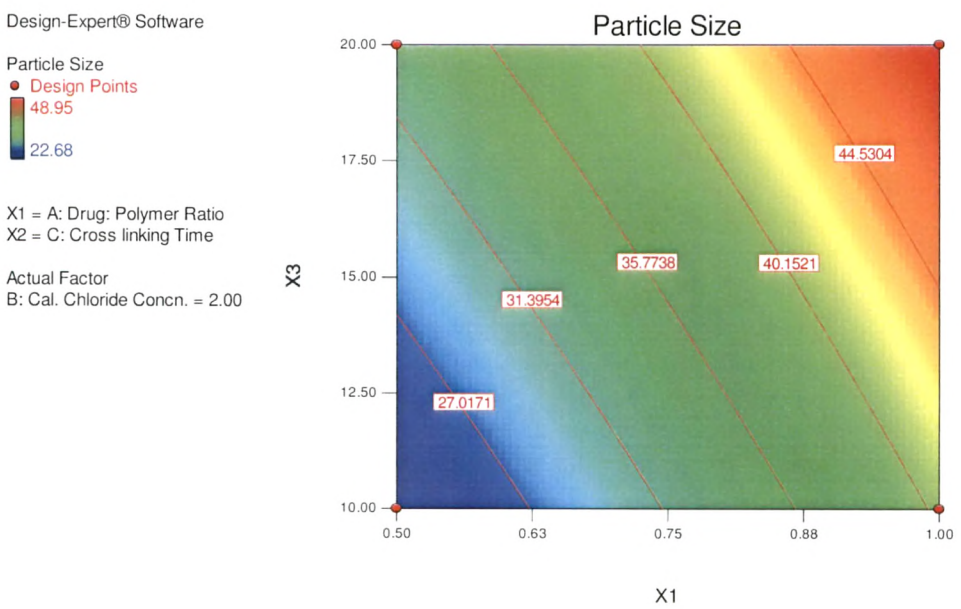

Fig. 7.14 Contour plots for the effects of drug: polymer ratio (X1) and cross linking time (X3) on particle size (Y1).

The combined effect of X_1 and X_2 on in vitro mucoadhesion of the microspheres was observed to be non linear as in Fig. 7.13 and 7.16. At low value of drug: polymer ratio and $CaCl_2$ concentration, higher value for in vitro mucoadhesion was observed. Similar effects were observed for factors X_2, X_3 and X_1, X_3 as shown in Fig. 7.14, 7.15 and Fig. 7.17, 7.18 respectively. As the $CaCl_2$ concentration and cross linking time increased from low to high, the value for in vitro mucoadhesion of the microspheres was decreased. Most of the studies showed that the prerequisite for a good mucoadhesiveness of a polymer is its high flexibility of its polymer backbone structure and of its polar functional groups. Such a flexibility of the polymer chains, however, is slightly reduced if the polymer molecules are cross linked either with each other or with coagulation agents like calcium ions.

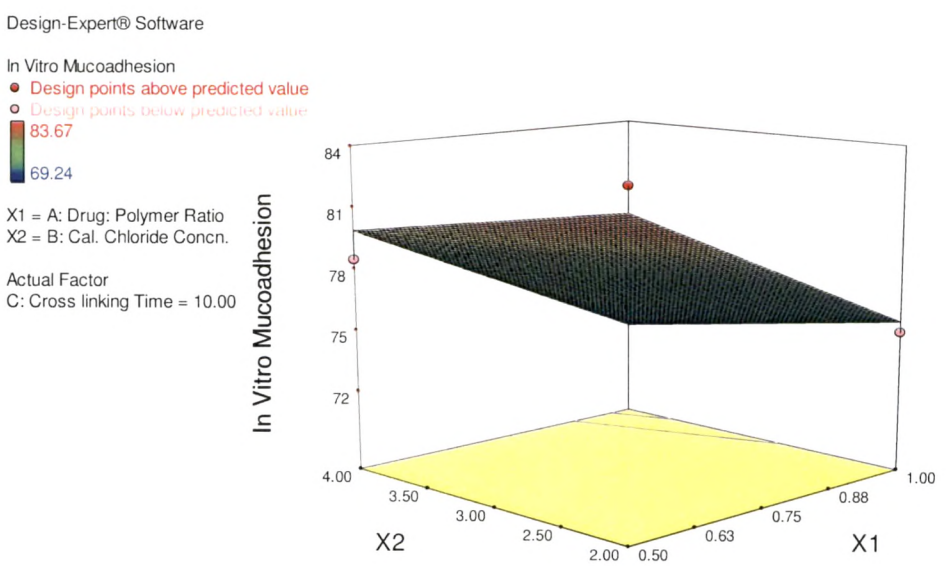

Fig. 7.15 Response surface plots for the effects of drug: polymer ratio (X1) and $CaCl_2$ concentration (X2) on in vitro mucoadhesion (Y2).

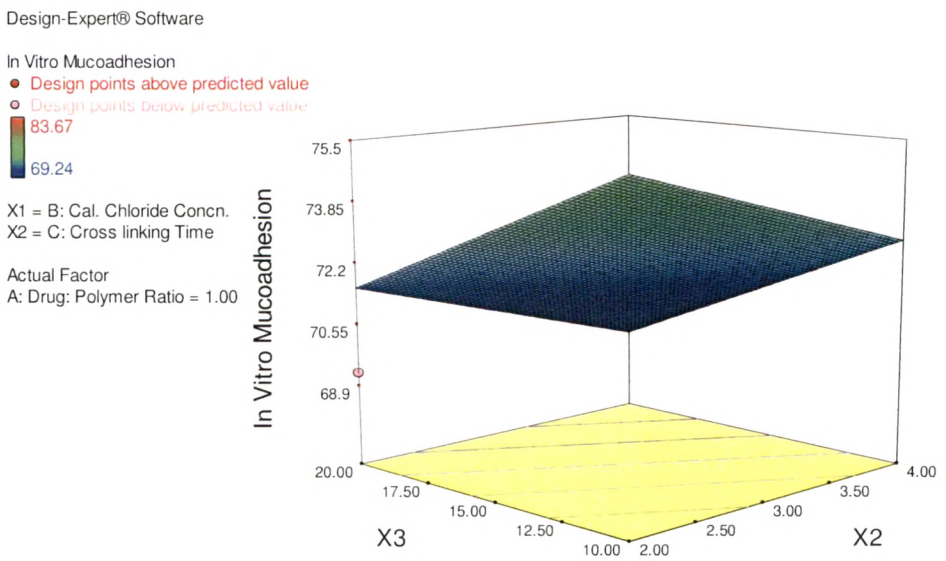

Fig. 7.16 Response surface plots for the effects of $CaCl_2$ concentration (X2) and cross linking time (X3) on in vitro mucoadhesion (Y2).

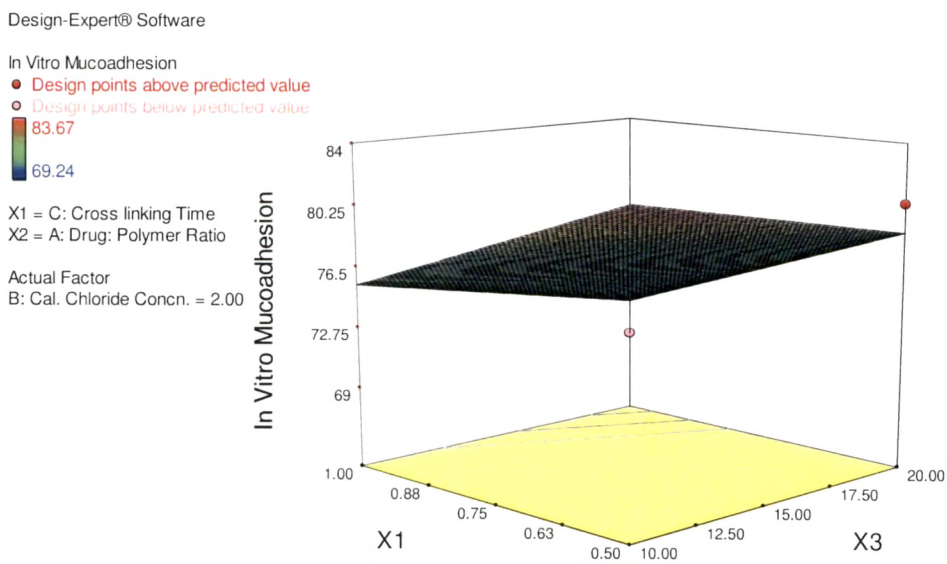

Fig. 7.17 Response surface plots for the effects of drug: polymer ratio (X1) and cross linking time (X3) on in vitro mucoadhesion (Y2).

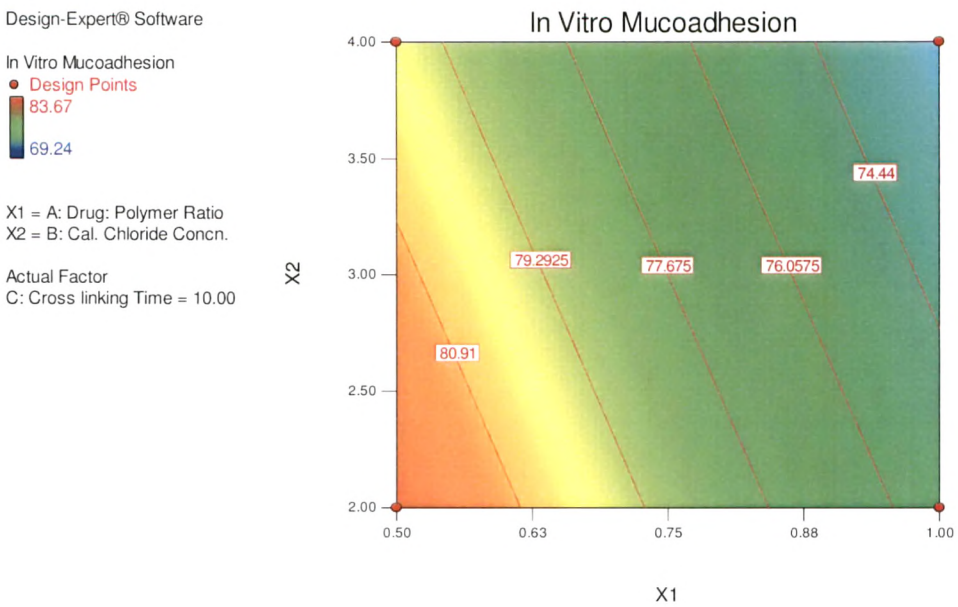

Fig. 7.18 Contour plots for the effects of drug: polymer ratio (X1) and $CaCl_2$ concentration (X2) on in vitro mucoadhesion (Y2).

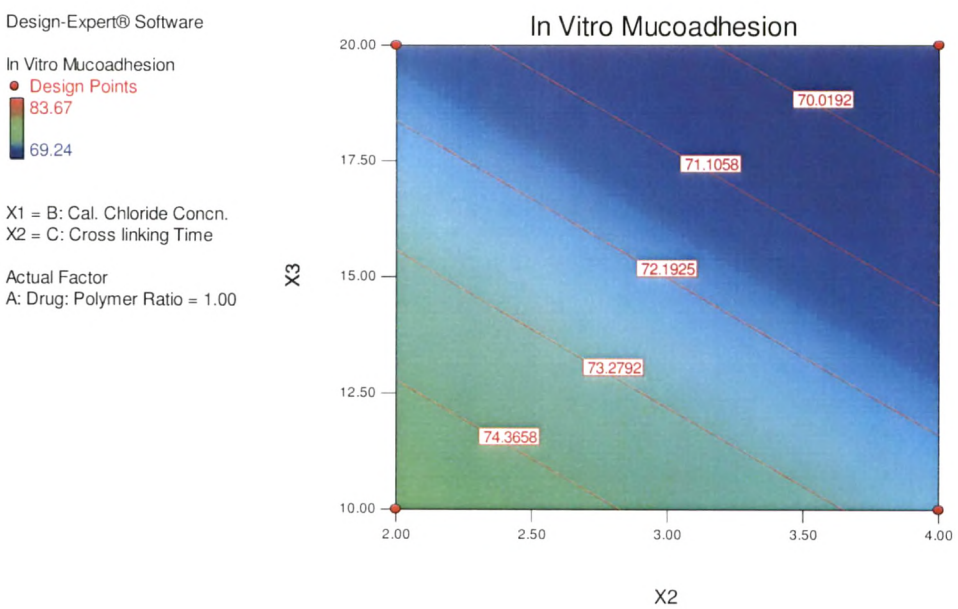

Fig. 7.19 Contour plots for the effects of $CaCl_2$ concentration (X2) and cross linking time (X3) on in vitro mucoadhesion (Y2).

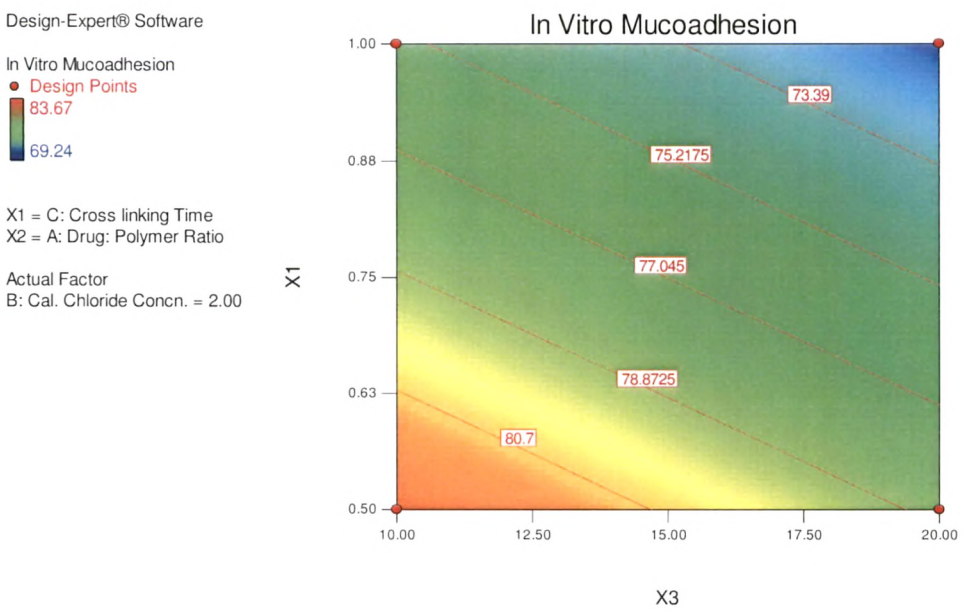

Fig. 7.20 Contour plots for the effects of drug: polymer ratio (X1) and cross linking time (X3) on in vitro mucoadhesion (Y2).

7.9.3 Optimization and validation

A numerical optimization technique by the desirability approach was used to generate the optimum settings for the formulation. The process was optimized for the dependent (response) variables Y1 and Y2. The optimum formulation was selected based on the criteria of attaining the value of particle size and in vitro mucoadhesion in the range. Formulation ALNT2 having drug: polymer ratio (1:1), $CaCl_2$ concentration (2%) and cross linking time (10 min) fulfilled all the criteria set from desirability search. To gainsay the reliability of the response surface model, new optimized formulation (as per formula ALNT2) was prepared according to the predicted model and evaluated for the responses.

The result in Table 7.7 illustrates the comparison between the observed and predicted values of both the responses Y1 and Y2 for all the formulations is presented. It can be seen that in all cases there was a reasonable agreement between the predicted and the experimental values as prediction error was found to vary between -3.24% and +2.97%. For this reason it can be concluded that the equations describe adequately the influence of the selected independent variables on the responses under study. This

indicates that the optimization technique was appropriate for optimizing the alginate microsphere formulation. The linear correlation plots drawn between the predicted and experimental values for all the batches of the microspheres are shown in Fig. 7.21 and Fig. 7.22 which demonstrated high values of R^2 (1 and 0.884). Thus the low magnitudes of error as well as the significant values of R^2 in the present investigation prove the high prognostic ability of the optimization technique by factorial design.

Table 7.7 The predicted and observed response variables of the sodium alginate microspheres.

Responses	Formulation code	Predicted value	Observed value	Prediction error* (%)
Y1	ALNT1	22.64	22.68	0.17
	ALNT2	40.50	40.46	-0.09
	ALNT3	29.39	29.35	-0.13
	ALNT4	43.28	43.32	0.09
	ALNT5	33.02	32.98	-0.12
	ALNT6	48.91	48.95	0.08
	ALNT7	33.78	33.82	-0.21
	ALNT8	45.69	45.65	0.11
Y2	ALNT1	82.52	83.67	1.13
	ALNT2	75.45	74.91	-0.71
	ALNT3	79.89	78.48	-1..76
	ALNT4	72.82	73.64	1.12
	ALNT5	78.63	80.36	2.20
	ALNT6	71.56	69.24	-3.24
	ALNT7	76.00	74.56	-1.89
	ALNT8	68.93	70.98	2.97

*Prediction error (%) = (Observed value – Predicted value)/ Predicted value x100%
Y1 and Y2 are particle size and in vitro mucoadhesion respectively.

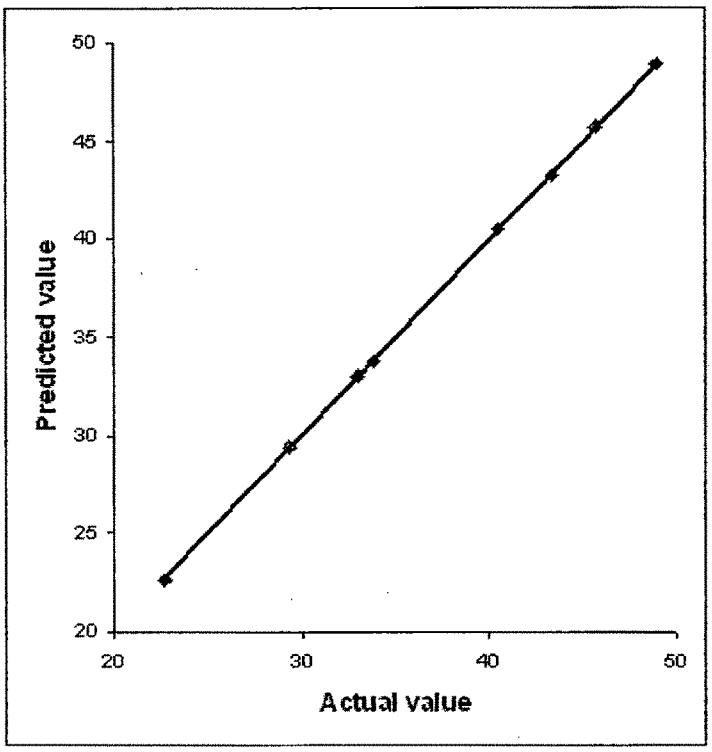

Fig. 7.21 Correlation between actual and predicted values for particle size

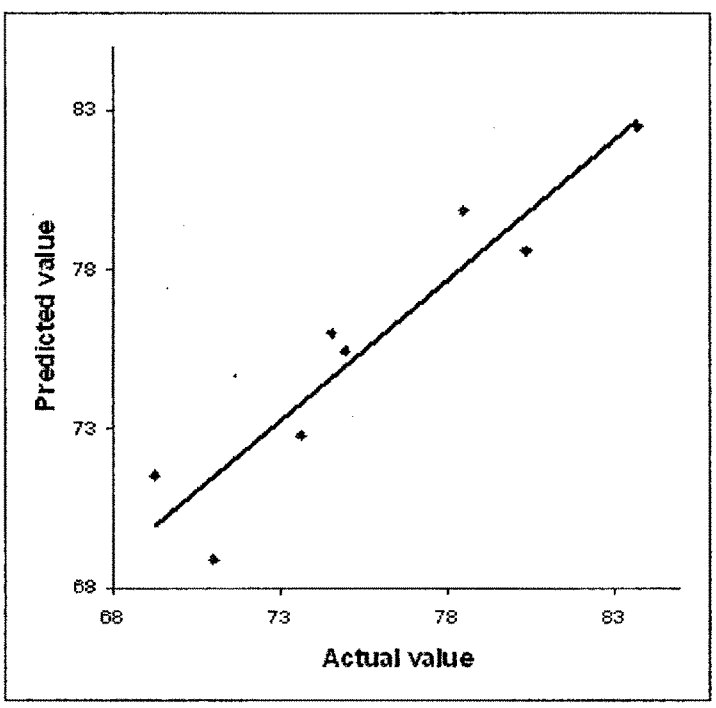

Fig. 7.22 Correlation between actual and predicted values for in vitro mucoadhesion

7.10 Histology studies

The histology of nasal mucosa for control and treated with optimized batch of NTD loaded alginate microspheres after 8 hours is shown in Fig. 7.23. The microscopic observations indicated that the optimized formulation had no significant effect on the microscopic structure of sheep nasal mucosa. The surface epithelium lining and the granular cellular structure of the nasal mucosa were totally intact. No major changes in the ultra structure of mucosa morphology could be seen and the epithelial cells appeared mostly unchanged. Neither cell necrosis nor removal of the epithelium from the nasal mucosa was observed after diffusion study as compared with control mucosa treated with phosphate buffer pH 6.2 Thus, the microsphere formulation seems to be safe with respect to nasal administration.

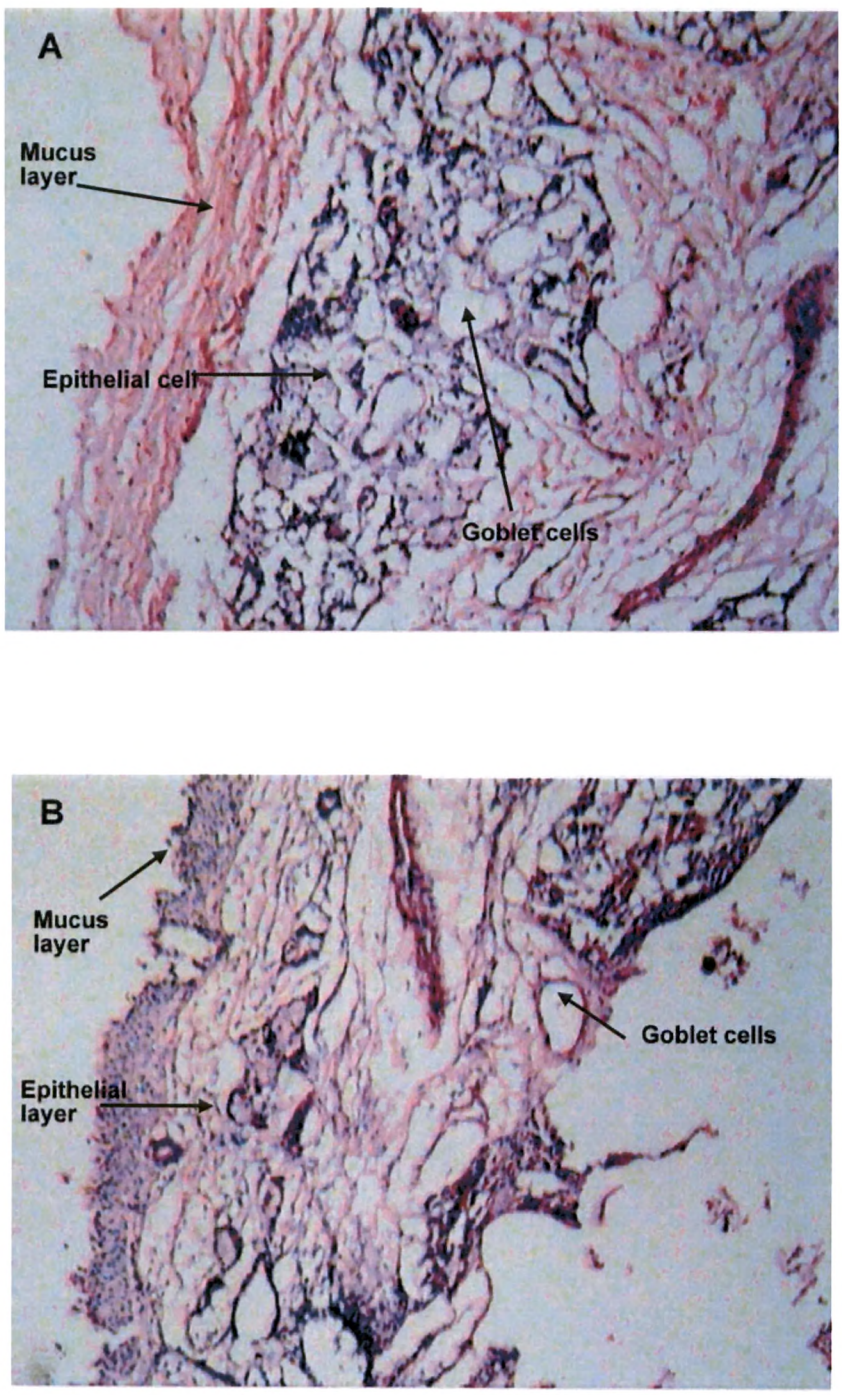

Fig. 7.23 Histology evaluations of sections of sheep nasal mucosa. (A) Control mucosa after incubation with phosphate buffer pH 6.2 in diffusion chamber; (B) Mucosa after incubation in diffusion chamber with microsphere formulation.

7.11 Stability studies

The stability data of the NTD loaded alginate microspheres is shown in Table 7.8. No physical changes in the formulation were observed during storage. The results obtained in the stability test showed that the particle size and drug content from the system stored at a temperature of 40 ^{0}C and a relative humidity of 75% was unchanged during a 3-month period of accelerated storage conditions. Particle size and drug content values after 1, 2 and 3 months showed no significant differences ($p>0.05$). This indicated that the alginate microsphere formulation of NTD was stable and do not require special storage conditions.

Table 7.8 Stability study results for NTD loaded Alginate microspheres under accelerated condition

Time/months	Appearance	Particle size* (μm)	Drug content* (%)
0	White	40.46±2.68	100.0±1.98
1	White	39.67±2.14	99.12±2.64
2	White	38.56±2.35	98.24±2.52
3	White	38.12±2.36	97.36±2.48

*n = 3; Mean ± SD

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