ORIGINAL ARTICLE

Optimization of nifedipine loaded gastroretentive microcapsules for biliary colic

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The aim of this research work was to formulate and systematically evaluate *in vitro* performance of gastroretentive microcapsules of nifedipine for biliary colic. Cross-linked reinforced alginate-chitosan microcapsules were prepared by ionotropic gelation method using calcium chloride ($CaCl_2$) as a cross-linking agent. The microcapsules were evaluated for physical characteristics such as particle size, particle shape and surface morphology by scanning electron microscopy, drug entrapment efficiency, *in vitro* drug release and *in vitro* bioadhesion studies. Results of preliminary trials indicated that the polymer concentration, cross-linking agent and chitosan had a noticeable effect on size and surface morphology. A Box-Behnken design was employed to study the effect of independent variables, polymer concentration (X_1), $CaCl_2$ concentration (X_2), chitosan (X_3) and pH of encapsulation medium (X_4) on dependent variables, drug entrapment efficiency and percentage drug release respectively. The entrapment efficiency varied from 6.14 to 79.21% depending upon the independent variables. The release can be sustained for more than 7 hours for all batches. It was observed that polymer and cross-linker concentration had a more significant effect on the dependent variables. Validation of optimization study, performed using 6 confirmatory runs, indicated very high degree of prognostic ability of response surface methodology, with mean percentage error (± SD) as −0.85 ± 4.39% and 2.83 ± 2.91% for drug entrapment and drug release. Optimization was done on the basis of maximum entrapment (82.26%) which was predicted using 6% alginate, 8.11% $CaCl_2$, 2% chitosan at a pH of 3.55 of encapsulation medium. The optimized formulation depicted a release of 57.17% at 7 hours. Point prediction tool of design expert software shows 101.91% and 96.82% validity of the predicted model for drug entrapment and percent drug release. The release follow Higuchi kinetics followed by non-fickian diffusion process. *In vitro* wash off test showed 71% bioadhesion after 1 hour.

Key words: *Biliary colic, bioadhesion, ionotropic gelation*

INTRODUCTION

Biliary colic is an extremely severe pain in the upper right-hand part of the abdomen. Sweating and vomiting often accompany the pain which comes and goes. The triggering events may be different but the final reason for the pain is functional obstruction of the biliary tract and sphincter smooth muscle hypertrophy.[1] In these conditions, sphincter of Oddi located at the junction of bile duct and duodenum fails to relax appropriately that can cause delayed emptying and abdominal pain. In this case anti-spasmodic and smooth muscle relaxants have a theoretic role in the management of these patients.[2] All these medications act by systemic route. These drugs do not provide predictably uniform results in all patients. All the drugs used in the conditions do carry some side effects like constipation, heart failure, nausea, dry mouth with contraindications. In the prior art certain trials have been carried out using nifedipine in bilary colic. The mechanism involves is the relaxant effect of nifedipine on sphincter of oddi.[3] The controlled drug delivery can be attained using biodegradable polymers such as alginate and chitosan. Alginates are block co-polymers of L-guluronic acid (G) and D-mannuronic acid (M) residues connected by 1:4 glycosidic linkages. Poly (L-guluronic acid) sequences of alginate are rigid and buckle shaped. Allignment of two such sequences forms an array of cavities simulating the "egg-box" which have carboxylate and oxygen atom.[4,5] Monovalent cations and Mg^{2+} donot induce gelation (Sutherland 1991), while Ba^{2+} and

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Sr^{2+} ions produce stronger alginate gels than Ca^{2+}.[6] Sodium alginate gel microspheres formed when contacted with calcium ions in solution by cross linking between the carboxylate anion of alginate guluronate units and the calcium ions.[7,8] The fact that the alginate beads can be formed very easily in aqueous solution at room temperature and get dissolved at physiological condition makes the alginate gel very useful in delivery of bioactives, peptides and proteins.[9]

Moreover, alginates possess a bioadhesive property[10] and may be useful in increasing drug residence time at the site of absorption, thereby improving overall drug effectiveness and bioavailability. Alginate gel microspheres have been conventionally prepared by dropping an alginate solution through spray gun into a $CaCl_2$ solution.[11] One of the biggest advantage is associated with minimum use of organic solvents that results in obtaining microparticles with low cost and safety. However, this approach has been associated with certain drawback, namely, poor entrapment efficiency that is because of high porosity of alginate gel that result in loss of entrapped molecule during hardening and washing process; low stability of beads as Ca^{2+} can be removed by the chelating agent or high concentration of ions such as Na^+, Mg^{2+}. As Ca^{2+} are removed from gels, cross-linking diminishes and the gels are destabilizes leading to leakage and loss of entrapped materials[12] and gel erosion that accelerates the release of drug.[13] These disadvantages can be overcome by incorporation of polycations such as chitosan in the encapsulation medium, which strengthen alginate beads and forms permanent membrane.[14,15] Moreover, the CS-alginate complex was noted to be stable to pH values ranging from 3.7 to 4.7.[16] Therefore, it is expected that the interaction of alginate with chitosan could result in a stronger material that could be more stable in both acidic media and in media containing calcium chelators. It was reported that chitosan incorporation suppressed the erosion profile of alginate beads with improvement in loading capacity of dextran.[17] Similar results reported for nitrofurantoin loaded alginate-chitosan microcapsule.[18]

Calcium channel blockers have a dilation effect on smooth muscles. This effect can be utilized particularly for relieving the pain in patients suffering from biliary colic due to reduction in congestion of sphincter of oddi. Since the bilary colic usually starts abruptly and gradually continued with nausea and vomiting over 2-6 hours, a sustained effect is presumed to be beneficial. Mucoadhesive drug delivery system has been formulated to obtain sustained release over prolonged period of time. The formulation originally designed act as pylorus relaxant by providing local action (slow release high density formulation) of drug on the duodenum that is expected to be successful in relieving biliary pain due to reduction in spasm of sphincter of oddi. This positioned gastric release is useful to produce a lasting local action of calcium channel blockers into the duodenum over desired period. In association with relieving the congestion, such formulation is also expected to dilate pylorus and result in passage of undigested food from stomach through pylorus to duodenum. No such treatment modality however, exists presently for symptomatic relief of pain in biliary colic.

Our attempt is to develop high-density mucoadhesive gastroretentive system for the treatment of biliary colic as the high density targeted to the sphincter of odii, relieve congestion and provide sustained release for prolonged period of time. Since the action is purely local, on physical contact with the effect or organ, gastric pylorus or duodenum, the dose chosen is very low (10% or less of pharmacological dose) which seems to beneficial in two aspects. Firstly, a reduction in systemic effects; secondly, nifedipine will not encounter bioavailability problem due to its poor solubility. In addition the same formulation can be useful in varieties of disorder like pylorospsam due to gastritis, duodenal ulcers, duodeno-gastric reflux, and dyspepsia.

In the present study, alginate-chitosan microcapsules were prepared by ionotropic gelation using nifedipine as a core material, which is a calcium channel blocker of dihydropyridine class. A Box-Behnken statistical design was employed to evaluate the combined effects of the selected variables on drug entrapment and percent drug release.

MATERIALS AND METHODS

Nifedipine (average M.W. 346.34) was purchased from Sigma Aldrich, India. Medium viscosity sodium alginate was purchased from Merck, India (2% w/v solution 5000 cp). Chitosan (75% deacetylated, M_r 460.000Da) was obtained as a gift sample from Ranbaxy Laboratory Ltd, India. Calcium chloride ($CaCl_2.2H_2O$) and tween 80 was purchased from Central drug house, India. All other chemicals and reagents were of analytical grade.

Preparation of microcapsules

Alginate chitosan microcapsules were prepared by ionotropic gelation method using a simple one-step process similar to previously reported process with modification.[19]

The mechanism involved in the formation of microcapsules was the cation-induced gelation of alginate with simultaneous encapsulation of nifedipine [Figure 1]. Drug (2 mg) was dissolved in methylene chloride (1 mL) due to its water insoluble behavior and then mixed with aqueous phase, consisting of 3 ml of different alginate concentrations [2%, 4%, 6%w/v; Table 1] with continuous stirring on magnetic stirrer (Remi stirrer, Mumbai, India) for 20 minutes. The ratio of organic and aqueous phase was 2:10. After thorough mixing, the solution was kept for 20 to 30 minutes in order to make the solution bubble-free. This primary emulsion was dropped into encapsulation medium through spray gun (1 mm nozzle) connected to a pump (20 Hg pressure), which gives the possibility of regulating the size of the droplets.

The encapsulation medium contained an aqueous solution of cross-linker solution of $CaCl_2$ (5 to 15% w/v) and chitosan (0.5 to 2% w/v) in the ratio of 1:1 maintained at different pH conditions from 2.95 to 4.10 (25°C). In order to produce discrete and freely flowing microparticles, tween 80 (0.5%) was added in encapsulation medium. The gelation medium was maintained under constant stirring (500 rpm) for 1hour using magnetic stirrer in order to prevent sticking of the droplets and to ensure uniform cross-linking process. A dropping height of 10 cm was used to ensure that spherical droplets were formed. Once the polymer solution had been dropped in gelation bath, a capsular membrane formed instantaneously around each droplet due to cross-linking of the interfacial alginate molecules by calcium cations. The microcapsules obtained were filtered using Whatman filter paper (No. 542, pore size 2.7 µm) and washed several time with 1% calcium chloride solution instead of water for avoiding any loss of calcium. The collected micro particles were allowed to dry in vacuum desiccator (RAC exporters, India) for 24 hours at room temperature. All experiments were done in a dark room to avoid photo decomposition of nifedipine.

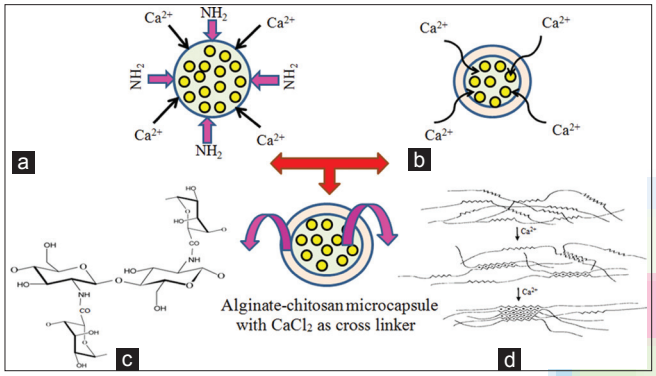


Figure 1: Mechanism of microcapsules formation by ionotropic gelation. (a) Polyelectrolyte complex formation between carboxylate ion of alginate and protonated amino group of chitosan to form an outer membrane. (b) Calcium mediated gelation of alginate in which calcium slowly diffused through cross linking barrier to attach two alginate strand together to form core. (c) Polyelectrolyte complex formation between chitosan and aginate to form coat or outer polymeric membrane. (d) "Egg box model" in which alginate cross-linked with $CaCl_2$ to form inner core

Morphological characterization

The shape and surface characteristics of microparticles were followed by a scanning electron microscope (JSM 5610 LV SEM, JEOL, Datum Ltd., Japan) chamber. Samples of microcapsules were dusted onto a double-sided tape on an aluminium stub. Afterwords, the stub containing the sample were coated with gold using a cool sputter coater (Polaran E 5100) to a thickness of 400 A. Photomicrogarphs was taken at the accelerated voltage of 15 KV and chamber pressure of 0.6 mm Hg. The size was determined by optical microscopy fitted with a stage and an ocular micrometer. About 300 microspheres were selected randomly and their mean diameter was determined using optical microscope fitted with a stage and an ocular micrometer.

Drug content of microparticles

A weighed amount of microparticles (50 mg) were crushed in glass mortar-pestle and the powdered microparticles were suspended in 10 ml of methanol. Then the solution was filtered through 0.22 µm Millipore filter and the filtrate was analyzed for drug content. The drug entrapment efficiency was calculated as per the following formulae:

$$\% \text{ Drug entrapment} = [Wa/Wt] \times 100 \quad (1)$$

Where Wa is the actual nifedipine content and Wt is theoretical nifedipine content.

The theoretical drug loading was determined by calculation assuming that the entire drug present in the polymer gets entrapped in microparticles and no loss occurs at any stage of preparation of microcapsules.[20]

Table 1:Effect of different process variables on characteristics of nifedipine loaded microcapsules (*n*=3)

Alginate (w/v)	$CaCl_2$ (w/v)	Chitosan (w/v)	pH (min)	Sirring time (µm±S.D)	Mean diameter	Surface morphology
2*	10	1	3.55	60	-	
3*	10	1	3.55	60	-	Coagulated, irregular
4	10	1	3.55	60	80.77±0.91	Slightly irregular
5	10	1	3.55	60	86.17±0.21	Spherical, regular
6	10	1	3.55	60	92.49±0.37	Spherical, discrete
6	5	1	3.55	60	96.13±0.56	Spherical, discrete
6	15	1	3.55	60	85.33±0.45	Spherical, discrete
6	10	0.5	3.55	60	78.14±0.31	Spherical, discrete
6	10	2	3.55	60	91.79±0.45	Spherical, discrete
6	10	1	2.95	60	87.45±0.12	Spherical, discrete
6	10	1	4.10	60	88.76±0.66	Spherical, discrete
6	10	2	3.55	15	106.41±0.3	Spherical and soft
6	10	2	3.55	30	95.32±0.74	Spherical, discrete
6	10	2	3.55	90	96.18±0.93	Spherical, discrete

*No microcapsules were obtained

In vitro release studies

The prepared formulation was evaluated for *in vitro* release by USP dissolution apparatus 2 (paddle apparatus), using 900 ml of the dissolution medium (0.1 N HCl, pH: 1.2). The paddle speed was adjusted at 50 rpm. The 5ml sample was withdrawn and replaced with 5 mL fresh medium. The samples were filtered using millipore filter (0.45 µm) assembly and the drug content was estimated spectrophotometrically (Shimadzu 1601 UV-VIS Spectrophotometer) at 239 nm.

In-vitro bioadhesion

The mucoadhesive property of the prepared microparticles was evaluated by *in-vitro* adhesion testing method known as wash-off method as reported previously.[21] A rat stomach mucosa was tied onto the glass slide using a thread. A constant number of microparticles (50) were spread onto wet rinsed tissue specimen and the prepared slide was hung onto one of the groves of a USP tablet disintegrating test apparatus. The disintegrating test apparatus was operated where by the tissue specimen was given up and down movements for one hour in the beaker of the disintegration test apparatus, which contained the gastric fluid (pH 1.2). At the end, the numbers of microparticles still adhering to the tissue were counted. Percent bioadhesion was given by the following formula:

$$\text{Percent bioadhesion} = \frac{\text{No of adhered microparticles}}{\text{No of applied microparticles}} \times 100 \qquad (2)$$

Experimental design

Box-Behnken statistical design was used to optimize the formulation parameters and systemically investigate the effect of wide range of independent and dependent variables. Alginate concentration (X_1), calcium chloride (X_2), chitosan concentration (X_3) and pH of encapsulation medium (X_4) were four independent variables (factors) considered in the preparation of alginate microcapsules, while the drug entrapment and percent drug release were dependent variables (response). The details of design are shown in Table 2. For each factor, the experimental range based on the result of preliminary experiment was selected and process parameters were studied by conducting the runs at different levels of all factors. Data collected for responses in each run were analyzed using the software DESIGN EXPERT 7.1 (Statease, USA) and fitted into a multiple linear regression model.

RESULTS AND DISCUSSION

Morphological studies

Nifedipine encapsulated alginate-chitosan microcapsules were prepared by ionotropic gelation mechanism using calcium chloride as a cross-linker. Being an anionic polysaccharide, alginate can be cross-linked with cations such as calcium ions.[22] Gelled cores were formed instantaneously by ionotropic gelation in which intermolecular cross-links were formed between the divalent calcium ions and negative charged carboxyl groups of the alginate molecules,[23,24] whereas chitosan constituted the majority of outer layer. Both the formation of outer chitosan-alginate complex membrane and cured inner alginate core leads to the fixing of the sphericity of the microcapsules. SEM photomicrographs of one of the batch showed spherical particles with smooth surface and relatively rough inner core suggesting that the microcapsules consist of inner core and outer skin coat [Figure 2]. It must be pointed out that the mechanical stability of the microcapsules is influenced by sodium alginate concentration. Alginate concentration was varied between 2 to 6%. Microcapsules were not obtained when alginate was used in low concentration (1 to 2% w/v).

Table 2: Levels of process parameters used in experiments

Code	Process parameters	Levels		
		−1	0	+1
X_1	Alginate concentration (w/v)	4	5	6
X_2	Calcium chloride concentration (w/v)	5	10	15
X_3	Chitosan cocentration (w/v)	0.5	1.25	2.0

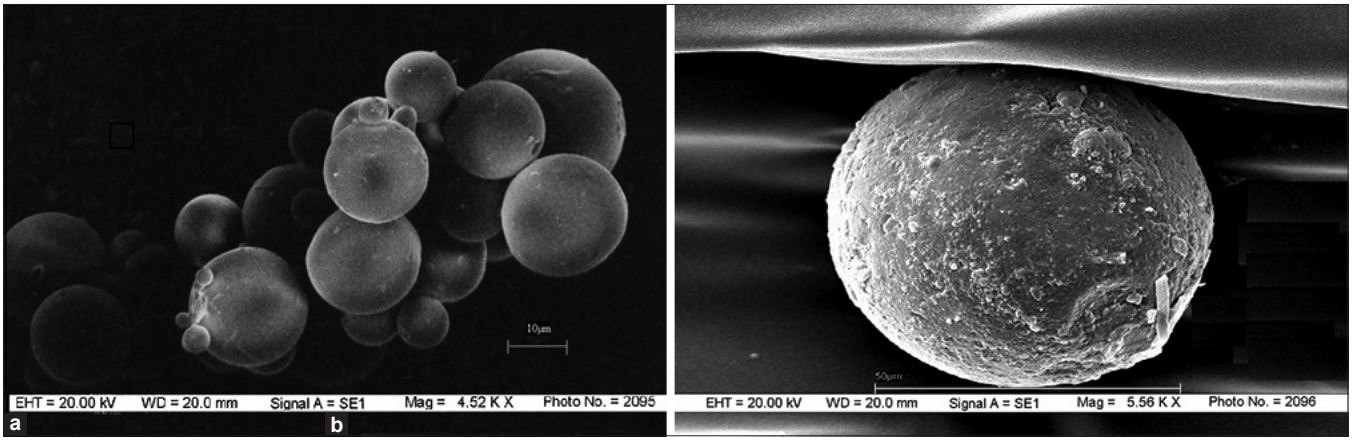


Figure 2: A photomicrograph of Alginate-chitosan microcapsule prepared by ionotropic gelation (a) Optimized formulation. (b) Overall

While soft, weak and non-spherical microparticles were obtained at 3% alginate concentration due to poor molecular packing and cross-linking. However, well discrete and spherical microcapsules were obtained at high alginate concentration (4 to 6%). On the other hand, at a concentration of above 6%, it was difficult to prepare the microcapsules, due to enhanced viscosity of polymer solution.[25] In view of these results, the rest of experiments were carried out using 4 to 6% alginate concentration.

It was observed that the microcapsules prepared from more concentrated and more viscous alginate solution (6%) were large in size (92.49 ± 0.37 µm) in comparison to microcapsules prepared from low (4%) and medium (5%) polymeric concentrations [Table 1]. This effect is presumably due to the fact that with increasing number of biopolymer molecules, the viscosity of solution also increases that result in spraying of large droplets. A decreased in particle size from 96.13 ± 0.56 to 85.33 ± 0.45 µm was observed when calcium chloride concentration was increased from 5% to 15%. This trend of decreased in size with increased cross-linker was due to variation in availability of reacting sites for crosslinking process.[26] Similarly microcapsules prepared with high amount of $CaCl_2$ were well cross linked and hence, they were small, smooth, discrete and more spherical in shape. So, 5 to 15% was found to be sufficient to initiate a cross-linking reaction and was optimized in further experimental design on the basis of entrapment.

Polyionic complexation in microcapsule formation involves interaction between the carboxylate group (COO^-) of alginate with protonated amino group ($-NH_2$) of chitosan. Therefore, the thickness of the complexed membrane is directly related with the amount of its interactive moieties.[27] As expected, increased in chitosan concentration from 0.5 to 2% increased average diameter due to formation of high viscous barrier upon interaction between polyionic components [Table 1]. Above 2%, highly viscous encapsulation medium was obtained that was difficult to stirred and filtered. So, 0.5 to 2% concentration was selected for further experimental trials.

The different pH of encapsulation medium (2.95 to 4.10) was maintained by glacial acetic acid. It was found that the selected range of pH had no significant effect on morphological characteristics of microcapsules.

The droplet size sprayed through spray gun was ultimately determined the size and shape of microcapsules rather than stirring speed. Therefore, high stirring speed was avoided during cross-linking process as it can affect the structural integrity of microcapsules because no further division of droplets is possible once the polymer solution dropped into encapsulation medium. Taking this into consideration, it was decided to carry out stirring at constant and low speed i.e., 500 rpm.

As alginate solution dropped into cross-linking solution, instantaneous cross-linked layer was formed by chitosan comprising inner polymeric core. As cross-linking reaction proceeds, Ca^{2+}slowly diffused from the capsular membrane to inner non cross-linked core. Table 1 shows that after 60 minutes of cross-linking process a homogenous cross-linked microcapsules were formed as no change in the membrane thickness was observed when longer gelation time were employed, indicating complete utilization of chitosan and calcium for the cross-linking of alginate.

The addition of 0.5% w/v of tween 80 to the dispersion medium was found to be essential to produce non-aggregated, discrete and freely flowing microparticles.

Fitting of data to the model

To identify the optimum levels of different process parameters influencing entrapment efficiency and percent drug release, an experimental design of 29 runs containing central points was made according to the box-behnken statistical design for four selected parameters. The individual and interactive effects of these process variables were studied by conducting the process at different levels of all factors. All the responses observed in 29 formulation prepared were simultaneously fitted to first order-, second order- and quadratic models using DESIGN EXPERT. It was observed that the best-fitted model was quadratic model. The results of experimental data and simulated values are listed in Table 3. This quadratic model resulted in several response surface graphs. A few representative response surface plots of the calculated model for percent entrapment and percent drug release are shown in Figures 3 and 4. The analysis of variance of the model for different responses is represented in Table 4.

Effect on entrapment efficiency

The model proposes the following polynomial equation for Percent drug entrapment:

$Y_1 = 32.47 + 21.92X_1 - 0.76X_2 + 10.78X_3 + 0.31X_4 + 1.07X_1X_2 + 1.27X_1X_3 - 0.17X_1X_4 - 7.77X_2X_3 - 1.32X_2X_4 - 0.63X_3X_4 + 11.46X_1^2 - 10.10X_2^2 + 1.45X_3^2 - 0.21X_{42}$. The Model F-value of 292.20 implies the model is significant ($P < 0.0001$). The "Lack of Fit F-value" of 1.59 (P: 0.3470) implies that lack of fit is insignificant. A correlation plot between actual and predicted values are shown in Figure 5. The "Pred R-Squared" of 0.9832 is in reasonable agreement with the "Adj R-Squared" of 0.9932 [Figure 5a]. Adequate Precision measures the signal to noise ratio. A ratio greater than 4 is desirable.Our ratio of 67.60 indicates an adequate signal. In this case $X1$, X_3, X_2X_3, X_1^2, X_2^2, X_3^2 are significant model terms. So, this model can be used to navigate the design space. A positive value of X_1 and X_3 represent a favorable optimization process while negative value of X_2 indicates an inverse relationship. The results showed that an increased in alginate concentration result in an increased in percent entrapment efficiency. Formulation C_{24} prepared with high alginate concentration (6%) showed a maximum

Table 3: Box–Behnken design with results

Runs	Batch	X^1	X^2	X^3	X^4	Drug entrapment (%)		Drug release (%)	
						Actual	Predicted	Actual	Predicted
1	C_1	–1	0	0	–1	19.45	21.32	74.76	75.51
2	C_2	0	+1	+1	0	26.31	26.07	51.35	51.23
3	C_3	+1	0	+1	+1	42.30	43.50	58.28	58.10
4	C_4	0	+1	0	+1	19.67	20.39	52.16	52.45
5	C_5	0	0	–1	–1	24.29	21.99	60.88	61.23
6	C_6	0	–1	0	–1	21.23	21.28	71.25	70.47
7	C_7	0	0	+1	+1	24.24	24.87	60.25	62.54
8	C_8	0	+1	–1	0	19.27	20.05	54.34	53.66
9	C_9	+1	0	+1	0	79.21	79.35	54.35	54.72
10	C_{10}	+1	0	–1	0	53.86	55.24	58.91	57.37
11	C_{11}	–1	–1	0	0	13.87	13.73	80.56	80.65
12	C_{12}	–1	0	–1	0	13.31	13.94	75.97	75.57
13	C_{13}	–1	+1	0	0	11.24	10.07	69.21	69.36
14	C_{14}	+1	–1	0	0	54.67	55.42	68.38	68.40
15	C_{15}	0	0	+1	–1	44.85	44.81	63.25	61.13
16	C_{16}	+1	+1	0	0	56.34	56.06	45.28	45.36
17	C_{17}	0	–1	0	+1	23.95	24.54	70.66	69.87
18	C_{18}	+1	0	0	–1	65.27	65.50	55.17	56.68
19	C_{19}	–1	0	0	+1	22.87	22.28	75.14	73.95
20	C_{20}	0	–1	–1	0	6.14	6.02	70.21	70.66
21	C_{21}	0	–1	+1	0	44.27	43.13	67.56	68.56
22	C_{22}	0	+1	0	–1	22.23	22.41	53.27	53.56
23	C_{23}	–1	0	+1	0	33.58	32.97	72.18	73.23
24	C_{24}	+1	0	0	+1	68.01	65.78	56.95	56.52
25	C_{25}	0	0	0	0	33.21	32.47	60.26	61.77
26	C_{26}	0	0	0	0	33.28	32.47	65.28	61.77
27	C_{27}	0	0	0	0	32.96	32.47	61.66	61.77
28	C_{28}	0	0	0	0	32.64	32.47	61.32	61.77
29	C_{29}	0	0	0	0	30.25	32.47	60.33	61.77

Table 4: Analysis of variance of calculated model for responses

Results of the analysis of variance	Drug entrapment	Drug release (%)
Regression		
Sum of squares	9308.37	2059.91
Df	14	14
Mean squares	664.88	147.14
F ratio	292.20	53.25
P	<0.0001	<0.0001
Residual		
Sum of squares	31.86	38.68
Df	14	15
Mean squares	2.28	2.76
Lack of fit test		
Sum of squares	25.45	21.76
Df	10	10
Mean squares	2.55	2.18
F value	1.59	0.52
Correlation coeffecient (R_2)	0.9966	0.9816
Coeffecient of variation (CV%)	4.50	2.64
Std. Dev.	1.51	1.66

Analysis of variance from DESIGN EXPERT 7.0 df Degree of freedom

entrapment of 68.01% when compared with formulation C_{19} (4%) that showed 22.87% drug entrapment. This trend of high entrapment with increased in polymer concentration is due to increase in viscosity of polymeric solution which leads to difficulty in dispersion and subdivision of droplets, resulting into large microspheres entrapping greater amount of drug.[28] Moreover, the loss of drug from this highly dense matrix is quite difficult during cross-linking and washing process. Increasing $CaCl_2$ from 5 to 10% increased entrapment efficiency from 54.67 to 68.01% for microcapsules prepared with 6% alginate concentration [Table 3]. This may be explained by the increase in the gel strength as the calcium ions increased. Consequently, the cross linking of the polymer and compactness of the formed insoluble matrices also increased. This would result in more drug entrapment in the microcapsules. These results are in agreement with Takka, *et al.*[29] and Mirghani, *et al.*[30] On the other hand, Ostberg, *et al.*[31] reported lesser drug encapsulation at higher calcium concentration. It was also found that further increase in the concentration of calcium chloride (up to 15%) decreased the drug entrapment. The poor retention of nifedipine in microcapsules prepared with

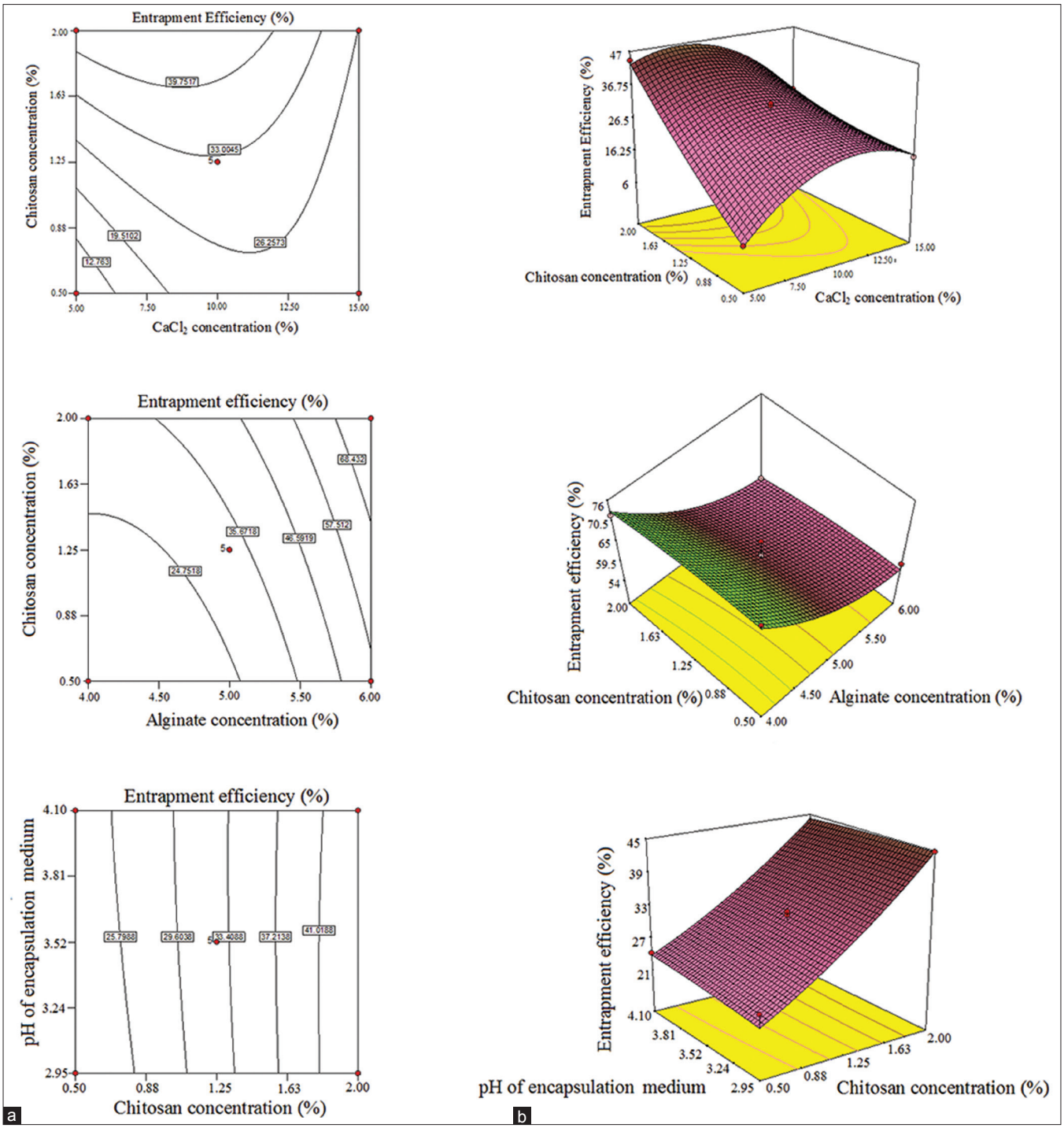


Figure 3: (a) Contour plots. (b) Response surface plot showing the effect of different process parameters on % drug entrapment

high content of $CaCl_2$ is due to high degree of cross-linking, which results in squeezing of drug content in encapsulation medium [Table 3]. The encapsulation efficiency was found to be directly proportional to X_3. Formulation C_9 containing 2% chitosan showed maximum entrapment of 79.21% when compared with C_{10}, which showed 53.86% drug entrapment. This effect was presumably due to the fact that the addition of a chitosan in the gelation medium provided a skin coating upon the alginate capsules due to its polycationic property, as a result less amount of drug leached during gelation process.[16] Moreover, the interactive effect of X_1 and X_3 was found to be more favorable for response Y_1, whereas it was unfavorable for X_2 and X_3. The significant negative impact of X_2X_3 interaction can be explained by the fact that $CaCl_2$ competes with chitosan to interact with carboxylate group of alginate if used in higher concentration (>10%). This interaction resulted in saturation of most of the binding sites by Ca^{2+} that ultimately causes shrinkage of gel network and

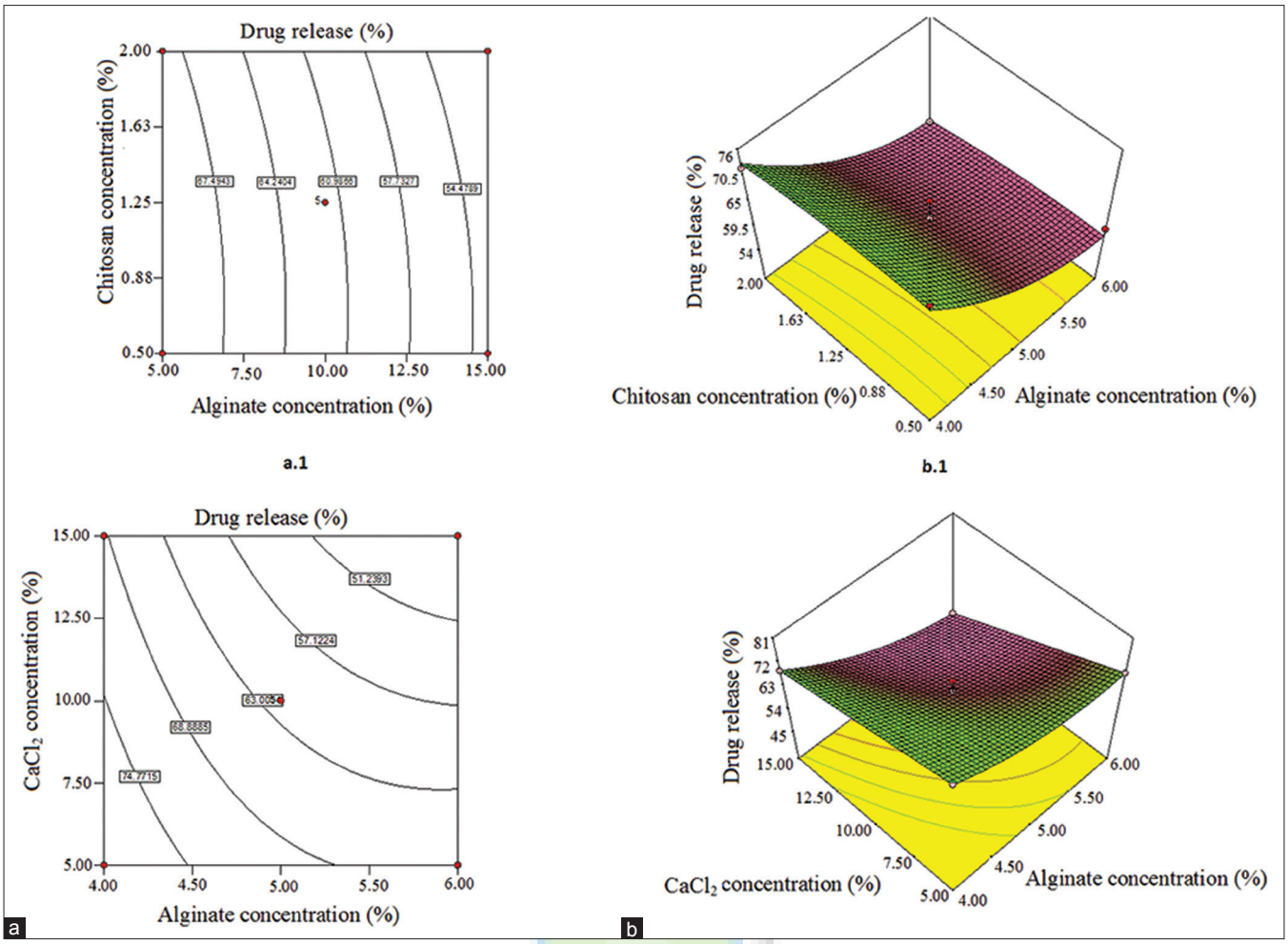


Figure 4: (a) Contour plots. (b) Response surface plot showing the effect of different process parameters on % drug release

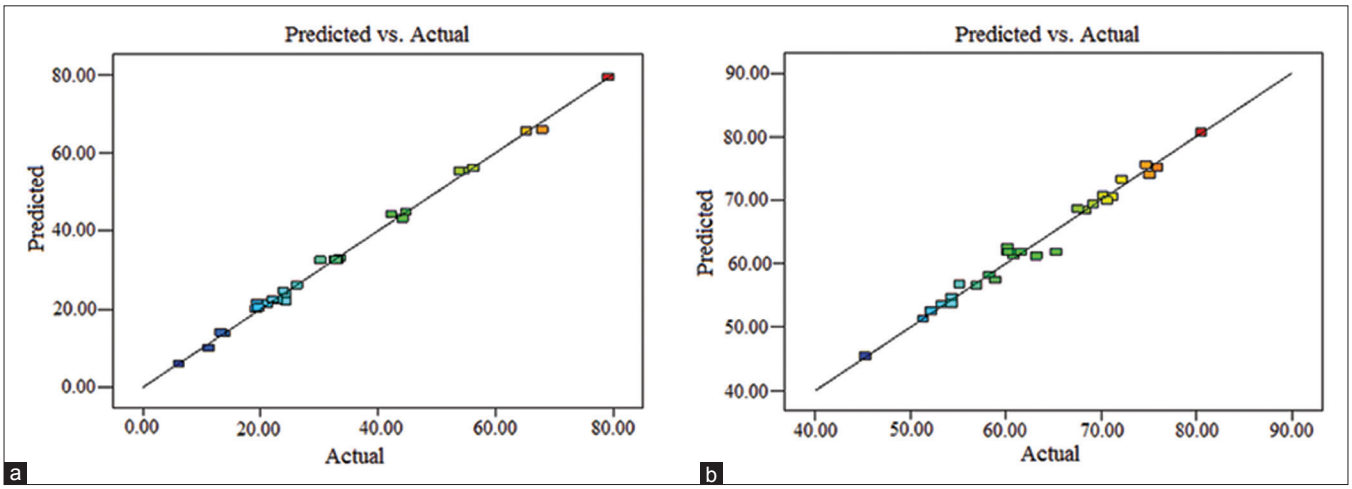


Figure 5: Correlation plot between actual and predicted values for. (a) % drug entrapment. (b) % drug release

squeezing of drug content. Maximum entrapment could be achieved at maximum chitosan concentration [Figure 3a]. As chitosan concentration decreases from 1.83 to 0.3% and $CaCl_2$ concentration increases from 8.5 to 12%, the drug entrapment also decreases from 39.75 to 26.26%, respectively.

Higher positive quadratic effect of X_1 and X_3 further confirmed the interactive effect of two polymers on drug entrapment. Figure 3a represented two-dimensional contour plot that showed interactive effect of X_1 and X_3 on percent drug entrapment at one time, while rest of the factors kept at

constant level. As clearly showed, all the factors were exhibit non-linear relationship at all levels. This interaction even more clearly depicted by response surface plot [Figure 3b] that showed highest entrapment at maximum X_1 and X_3 concentration.

The chitosan solution at 2% concentration in 5% acetic acid had a pH of 3.55. At this acidic pH, chitosan molecules are expected to be mostly protonated and the presence of abundance of positive ions reduced the electrostatic repulsions between the negatively charged alginate molecules in the core[14] that result in better interaction and hence, better entrapment efficiency of drug molecules. As the pH of solution increased or decreased, fewer amino groups were available for cross-linking reaction that result in lower entrapment efficiency.[32] Figure 3a shows that increase in chitosan concentration from 0.8 to 1.8%, increases drug entrapment from 25.80 to 41.02% respectively, at constant pH of 3.55. These results further supported the hypothesis to increase in protonation efficiency of chitosan with increase in concentration at selected pH of 3.55.

Effect on percent drug release

The model proposes the following polynomial equation for Percent drug entrapment:

$Y_1 = 61.77 - 9.07X_1 - 8.58X_2 - 1.13X_3 - 0.43X_4 - 2.94X_1X_2 - 0.19X_1X_3 + 0.35X_1X_4 - 0.085X_2X_3 - 0.13X_2X_4 - 1.08X_3X_4 + 4.13X_1^2 + 0.047X_2^2 - 0.79X_3^2 - 0.23X_4^2$ The Model F-value of 53.25 implies the model is significant ($P < 0.0001$). The "Lack of Fit F-value" of 0.52 implies the Lack of Fit is not significant (P: 0.8193). A correlation plot between actual and predicted values of percent drug release are shown in Figure 5b. The "Pred R-Squared" of 0.9276 is in reasonable agreement with the "Adj R-Squared" of 0.9631. Adeq Precision ratio of 29.53 indicates an adequate signal. Therefore this model can be used to navigate the design space. In this case X_1, X_2, X_3, X_1X_2, X_1^2 are significant model terms.It is evident from inverse relation between polymer and cross-linker that the two independent variables viz. the concentration of alginate (X_1), $CaCl_2$ (X_2) have a negative effect on response Y_2. Formulation C_1 prepared with 4% alginate concentration released 74.76% drug in 7 hours, when compared with C_{25} and $C_{18,}$ which released only 60.26% and 55.17% drug in the same period. This trend of decreased release with increased in polymer concentration was due to increase in density of the polymer matrix and diffusional path length that the drug molecule has to traverse. Similarly, as $CaCl_2$ concentration increased, cross-linking reaction favored, that decreased the drug.[28] A slight decrease in release was observed from 58.91% to 54.35% when X_3 (chitosan concentration) was increased from 0.5% to 2%. This trend of decreased response with increased in X_3 was due to formation of relatively strong walled microcapsules that resulted in less swelling ability and hence, slower diffusion of drug molecules. These results indicate that the suppression of erosion profile of the microparticles is relative to the viscosity of chitosan solution [Figure 4a].

Response surface plot shown in Figure 4b clearly indicates that the nifedipine release decreases with increasing concentrations of polymer and cross-linker. A significant interactive effect of X_1X_2 for response Y_2 confirms that the cross-linking reaction is highly depending upon the viscosity of alginate solution. Minimum drug release was observed at maximum alginate (6%) and calcium chloride concentration (15%). Similarly a negative interactive effect of X_1X_3 supports the hypothesis that the polyionic complexation between chitosan and alginate depends upon their concentration. As concentration of two polymers increases, complexation reaction initiates that slow the diffusion of drug molecules through viscous coacervate barrier. It can be depicted from Response surface methodology (RSM) plots that the interactive effects of X_1X_2 in controlling drug release is 15 folds more significant then the effect of X_1X_3 that confirmed the dominating effect of X_2 as a cross-linker when compared with chitosan. This effect can be explained on the basis of fast diffusivity of small molecules of calcium in solution, as a result of which concentration of chitosan in the inner layer was lower then that of calcium.[33] Moreover, chitosan is highly soluble and cationic in acidic medium due to conversion of amine units into soluble forms NH_3^+. The interaction between amino group and protonated carboxylic group is weak leading to distortion of membrane and fast drug release. Thus the total release behavior is dominating by the calcium alginate matrix. Simultaneously this protonization phenomenon creates repulsive forces that cause increment in swelling of the chitosan membrane with fast diffusion of drug molecules. Contour plots are presented in Figure 4 that exhibited nearly non-linear relationship at all levels of the two variables.

Validation of RSM results

Point prediction of the design expert software was used to determine the optimum values of the factors for maximum entrapment. For all the 6 checkpoint formulations, the result of drug entrapment was found to be >60%. Table 4 lists the composition of the checkpoints, their predicted and experimental values of the response variables and the percentage error in prognosis. Upon comparison of the observed responses with that of the anticipated responses, the prediction error varied between +5.11% and −5.89% for entrapment and +5.07 and −5.11 for % drug release. Figure 6 shows linear correlation plots between observed and predicted response variables. The linear correlation plots drawn between the predicted and observed responses demonstrated high values of R^2 for % drug entrapment (0.842) and % drug release (0.834) indicating excellent goodness of fit ($P < 0.001$). All checkpoints formulation exhibited >90% experimental validity. Thus, the low magnitudes of error and the significant values of R^2 in the current study indicated a high prognostic ability of RSM.

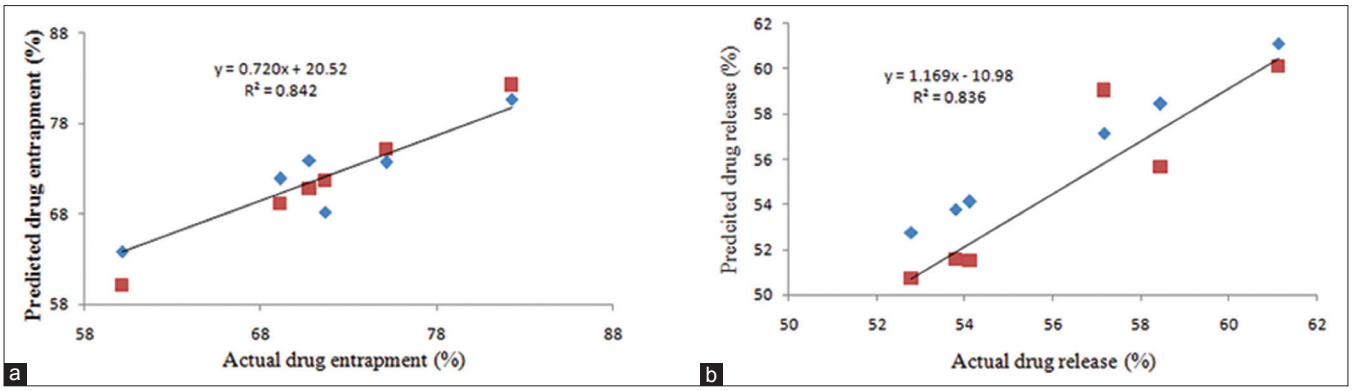


Figure 6: Linear correlation plot between actual versus predicted values for (a) % drug entrapment (b) % drug release

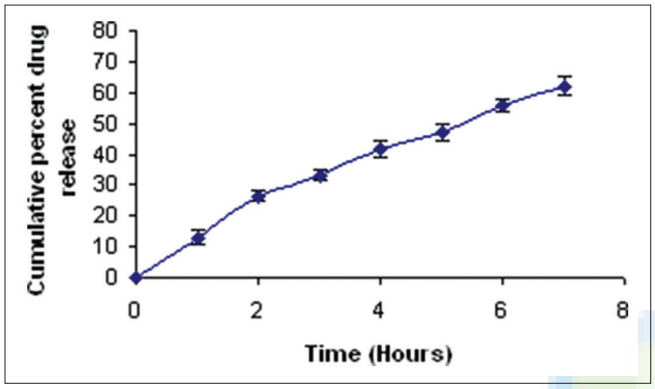
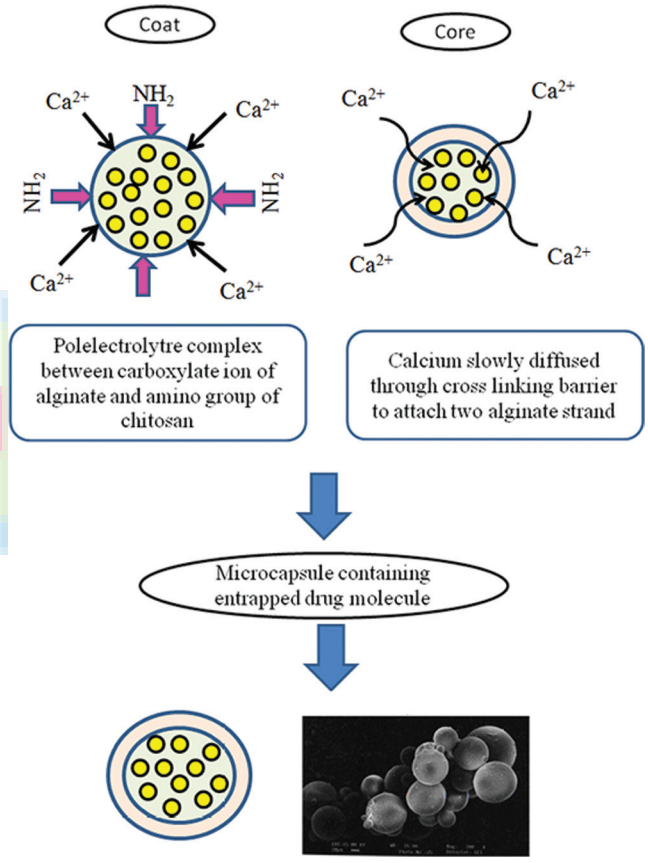


Figure 7: *In vitro* release profile of nifedipine from optimized microcapsules



Graphical abstract

Optimization

The optimum formulation was selected based on the criteria of attaining the maximum entrapment for microcapsules. Point prediction tool of the design expert software was used to determine the optimum values of the factors for maximum entrapment. Finally, the optimum values of alginate (6%), calcium chloride (8.11%), chitosan (2%) and pH of encapsulation medium (3.55) were obtained. These values predict 80.72% drug entrapment and 59.05% drug release in 7 hours. These predicted values of responses were validated by further conducting *in vitro* studies of nifedipine loaded microcapsules prepared with previously optimized process parameters, and an average of 82.26% entrapment and 57.17% release was obtained. This shows 101.91% and 96.82% validity of the predicted model for drug entrapment and percent drug release [Table 5].

The dissolution release profile of optimized batch is shown in Figure 7. While conducting dissolution studies of optimized formulation, it was found that microcapsules settled down at the bottom of dissolution medium within a few seconds, which confirmed its high density potential in gastric fluid. To ascertain the release mechanism, the *in vitro* dissolution data were applied to various kinetics models. The best fit with highest regression coeffecient correlation value (R^2) was predicted by Higuchi model (0.9957) rather than zero (0.9871) or first model (0.8859). This clearly indicates that the release of nifedipine from alginate-chitosan microcapsules is diffusion controlled. To predict the better release pattern, the experimental data were further fitted to Korsemeyer and Peppas equation, which characterized the transport mechanism. The value of release exponent (n) for the proposed model was 0.7805, indicating that the transport of the drug molecules across polymeric matrix takes place through diffusion controlled drug release and swelling controlled drug release (anomalous transport). The actual drug release mechanism includes two apparent

Table 5: Composition of the checkpoint formulations, the predicted and experimental values of response variables and percentage prediction error

S.No	Composition (X_1:X_2:X_3:X_4)	Experimental value		Predicted value		Percent Error		Experimental validity	
		PE	% Release	PE	% Release	PE	% Release	PE	% Release
SS_1	6:12.30:1.25:3.52	60.11	53.78	63.87	51.55	-5.89	+4.32	94.11	104.32
SS_2	6:12.03:1.55:3.51	71.67	54.11	68.18	51.50	+5.11	+5.07	105.12	104.94
SS_3	6:12.03.1.82:3.51	69.12	52.78	71.91	50.71	-3.88	+4.08	96.12	102.11
SS_4	6:8.11:2:3.55	82.26	57.17	80.72	59.05	+1.90	-3.18	101.91	96.82
SS_5	6:5:1.72:3.55	70.76	58.45	73.89	55.66	-4.24	+5.01	95.76	105.01
SS_6	6:8.11:1.70:3.55	75.11	61.12	73.72	60.11	+1.88	+1.68	101.88	101.68

*PE: Percent entrapment

phenomenon: Penetration of the acidic medium inside the microcapsules and hence, swelling of the chitosan network at early stage that moves drug molecules, and the diffusion of the drug molecules out of the alginate coat as solvent hydrates the microparticles.[34]

Bioadhesion studies

Optimized formulation obtained from point prediction tool of design expert software was subjected to mucoadhesion studies. Alginate has strong hydrogen bonding chemical groups (two hydroxy and one carboxylic group for each C6 buiding units) that favors the adhesion with negatively charged mucus glycoprotein through weak chemical bonds formation. Therefore, by counting a number of microparticles still adhering to gastric mucosa after a specified time period, mucoadhesive potential can be determined. *In vitro* wash off test revealed that optimized formulation exhibited good bioadhesive property i.e., 71.65 ± 0.95% ($n = 3$) after 1 hour.

CONCLUSION

The results of Box-Behnken design revealed that alginate, chitosan and $CaCl_2$ concentration had a significant effect on dependent variables, percent drug entrapment and percent drug release. Polymers concentration had a positive impact while cross-linker concentration had a negative impact on drug entrapment. The interaction of alginate and $CaCl_2$ in controlling the drug release is more significant than chitosan. Microcapsules of best batch based on point prediction tool of design software exhibited 82.26% drug entrapment and 57.17% drug release at 7 hours with more than 97% experimental validity. The release pattern followed higuchi model via non-fickian diffusion. The optimized formulation showed 71% bioadhesion in *in vitro* wash-off test after 1 hours.

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