Cumin and Residronate loaded chitosan microparticles for treatment of osteoporosis

Mohammad Rashid
R.V. Northland Institute, Chithera, Dadri India
Email: mrashid1379@gmail.com
Phone no: +919540324534

*Dr. Shaheen Sultana
Taif University, Saudi Arabia
Email: shaheen634@yahoo.co.uk
Phone no: +966544122485

Mr. Ramji Gupta
R.V. Northland Institute, Chithera, Dadri India
Email: ramjicops@rediffmail.com
Phone no: 097173010303

Dr. Sokindra Kumar
R.V. Northland Institute, Chithera, Dadri India
Email: sokindra_kumar@yahoo.co.in
Phone no: +9968099082

Mohammad Yusuf
Taif University, Saudi Arabia
Email id: yusuf4682@gmail.com
Phone no: +966540464942

Maria Khan
Taif University, Saudi Arabia
Email id: 25mariak@gmail.com
Phone no: +966508761084

* Corresponding author

Abstract

Introduction: Residronate is widely used drug for treatment of osteoporosis. Due to large size, hydrophilicity and negative charge, orally administered bisphosphonates showed poor absorption typically <1%.
Objective: To prepare cumin and residronate loaded chitosan microparticles as penetration and bioavailability enhancer.
Method: Chitosan microparticles were prepared by ionotropic gelation in which TPP was used as a cross-linking agent.
Results: Preliminary evaluation of microparticles was done on the basis of shape and surface characteristics analyzed by scanning electron microscopy. Results of preliminary trials indicated that the chitosan concentration, TPP concentration and cross-linking time had a noticeable effect on shape and surface morphology. A Box-Behnken design was employed to study the effect of independent variables, chitosan concentration (X_1), TPP concentration (X_2) and cross-linking time (X_3) on dependent variables, particle size, drug entrapment efficiency and percentage drug release respectively. Particle size and entrapment efficiency was found to increase with increase in polymer concentration. While an inverse relationship was observed between polymer and TPP concentration and % drug release. 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 chitosan (2.0%), TPP (10.8%) & cross linking time (34.05 minutes) were obtained. These values predict 306.40 µm particle sizes, 92.499 % Entrapment & 271.023 minute's drug release time. Cumin loaded microspheres showed particle size ranged from 258 to 356 µm, % entrapment efficiency from 68.41±2.81% to 91.96±3.01% and 100% release from 120±5.22 min to 240±6.72 min depending on chitosan and TPP concentration.
Conclusion: The microparticles were developed successfully and can be progress further for conducting in-vivo study. The same method can be adopted for bioavailability enhancement of other drugs.

Keywords: *Chitosan, cumin, microparticles, bisphosphonate, particle size, entrapment efficiency, percent drug release*

1. INTRODUCTION

Bisphosphonates are a class of bioactive agents widely used in variety of diseases associated with bone such as Paget's disease, tumour-associated osteolysis and hypercalcaemia, osteoporosis and primary hyper parathyrosis **[1]**. Among the Bisphosphonates approved for the treatment of osteoporosis, Risedronate is one of the strongest inhibitors of farnesyl pyrophosphate synthase (FPPS), an enzyme responsible for the synthesis of cholesterol and bone resorption [**2**].

Due to large size, hydrophilicity and negative charge, orally administered bisphosphonates are unable to cross gastrointestinal epithelium and thus showed poor absorption typically <1% [**3, 4**]. To maintain therapeutic concentration, high dose is required which resulted in severe gastro-intestinal side effects and high cost of treatment. To improve the bioavailability of bisphosphonates, prodrug approach [**1**], use of absorption enhancers [**5**] and design of drug delivery systems have been attempted [**6**].

Natural bioavailability enhancers are agents of natural origin that do not have their own inherent pharmacological activity but have ability to enhance bioavailability and bio efficacy of any pharmaceutical or nutraceuticals agent [**7**]. Several herbal compounds including piperine, quercetin, genistein, naringin, sinomenine, curcumin, cumin, glycyrrhizin have demonstrated potential bioenhancer properties [**8**]. *Cuminum cyminum* Linn. is a small and thin annual herb, which is used as gastric stimulant, beneficial in abdominal lump and flatulence. Various volatile oils, luteolin and other flavonoids present in Cuminum cyminum has been contributed in its bioavailability enhancement activity. Luteolin has already been demonstrated to be a potent P-glycoprotein inhibitor in prior arts [**9**].

The aim of this research is to prepare cumin and residronate loaded chitosan microparticles as penetration and bioavailability enhancer for bisphosphonates. The system developed will show reduced side effects and comparatively less expensive as 28 days therapy at standard daily doses for treatment of osteoporosis management and Paget's diseases costs £21.83.

2. MATERIALS

Risedronate sodium (average M.W. 283.11) was purchased from Jubilant Organosys Ltd (India). Chitosan high molecular weight was obtained as a gift sample from Ranbaxy Laboratory Ltd, India. TPP (sodium tri poly phosphate, average M.W. 367.86) was purchased from Finar Chemicals Ltd. Ahmedabad, India. All other chemicals and reagents were of analytical grade.

3. METHODS

3.1. Aqueous extraction of Cuminum cyminum

Cumin was purchased from local market, identified and then authenticated at Deptt. of phytochemistry, RV Northland Institute, India. Cumin seeds were dried at 60^{O}C in an oven till constant weight was attained. The seeds were powdered in a grinder and boiled in distilled water for about 72 hr on a water bath at 70^{O}C. Collected extract was filtered through filter paper (542, pore size 2.7μm) and then freeze dried.

3.2. Preformulation studies

3.2.1. Ultraviolet spectroscopic analysis of Residronate

Stock solution of residronate (50 μg/ml) containing 2.5 mL of acetate buffer and 2 mL of 2.5 mM copper (II) sulfate solution were scanned for absorbance in the region of 800-200 nm [**10**].

3.2.2. Ultraviolet spectroscopic analysis of Cuminum Cyaminum

UV absorption spectra of stock solution of cumin prepared by dissolving 10 mg of dried extract in 100 mL of distilled water and were scanned for absorbance in the region of 800-200 nm

3.2.3. Interactions study by Infrared spectroscopic analysis

0.3 gm of KBr previously dried at 250^{0}C for 1 hr and cooled, was weighed and powdered. 1.2 mg of test sample was added, mixed perfectly and ground to a uniform mixture. A small quantity of the powder was taken and compressed into the transparent pellets by applying pressure. The IR spectrum of the pellets was reported from 3500 to 500 cm^{-1}.

3.3. Formulation development

3.3.1. Chitosan Microspheres

Chitosan microcapsules were prepared by ionotropic gelation method described by **Sultana and coworker [11]** with slight modification, using sodium Tripoly Phosphate (TPP) as cross linking agent. Different concentration of chitosan is added into acetic acid solution with continuous stirring on magnetic stirrer for 20 minutes to prepare homogenous solution of drug and polymer. Polymeric solution was then sprayed through a spray gun having a pressure of 30 kg/cm^2 with a nozzle size of 1 mm. into aqueous solutions of magnetically stirred sodium TPP solution (w/v). The microspheres were removed from the TPP solution after fixed intervals from the counter ion solution by filtration using What-man filter paper no. 542, pore size 2.7µm, and washed several times with distilled water and dried.

3.3.2. Cumin Loaded microspheres

The dried aqueous extract of cumin was dissolved in water- chitosan solution in 1% acetic acid (1:10) by stirring using a magnetic stirrer for 20 min. The ratio of oil and aqueous phase was 1:10. O/W emulsion was dropped into TPP solution gently agitated using a magnetic stirrer by spray gun. After the cross linking time, the chitosan microspheres were washed with distilled water repeatedly, filtered and dried at room temperature. Formulae for preparation of microspheres are shown in **Table 1.**

Table 1. Formulae for preparation of cumin microspheres.

Formulation code	**Cumin extract**	**Chitosan**	**TPP**	**Cross linking Time**
C-1	100 mg	0.50 **%**	5 **%**	60
C-2	100 mg	1.25**%**	10**%**	60
C-3	100 mg	2.00**%**	15**%**	60

Designing of formulations with optimized three independent variables

An experimental design of 17 runs (**Table 2**) was made according to the software Design-Expert for three selected parameters (Concentration of polymer coded as X_1, Concentration of Cross linking agent-TPP coded as X_2 and Cross linking time coded as X_3). Experimental study was done for the individual and interactive effects of these independent variables at different levels.

Table 2. Levels of process parameters used in Experiments

CODES	INDEPENDENT VARIABLES	LEVELS		
		-1	0	+1
X_1	Chitosan-concentration (w/v)	0.50	1.25	2.00
X_2	TPP- concentration (w/v)	5.00	10.00	15.00
X_3	Cross linking Time (minutes)	20.00	40.00	60.00

3.4. Characterization of microspheres

3.4.1. Particles size

About 300 microspheres were selected randomly and size was determined by optical microscopy fitted with a stage and an ocular micrometer and their mean diameter was determined using optical microscope fitted with a stage and an ocular micrometer.

3.4.2. Percentage drug entrapment efficiency

10 mg of microspheres were taken in mortar-pastel and added to 10 ml of distilled water. The suspended preparation was sonicated for 40 minutes. It was filtered through 0.22µm Millipore filter paper and the filtrate was analyzed for drug content. The percentage drug entrapment efficiency of particles was calculated as per following formula:

% Drug Entrapment = [Wo/Wt] x 100

where, *Wo- observed drug concentration in microparticles*

Wt- theoretical drug concentration in microparticles

The theoretical drug loading determination: It was assumed that the entire drug present in the polymer-TPP solution gets entrapped in microspheres and drug loss at preparation steps is approaches to zero.

3.4.3. Scanning electron microscopy

The shape and surface characteristics of chitosan microparticles were determined by a scanning electron microscope (JSM 5610 LV SEM, JEOL, Datum Ltd., Japan) chamber. Samples of microspheres were dusted onto a double-sided tape on an aluminium stub. Afterwards, the stub containing the sample was coated with gold using a cool sputter coater (Polaran E 5100) to a thickness of 400 A˚. Photomicrographs were taken at an accelerated voltage of 20 KV and chamber pressure of 0.6 mm Hg.

3.4.4. In vitro release studies

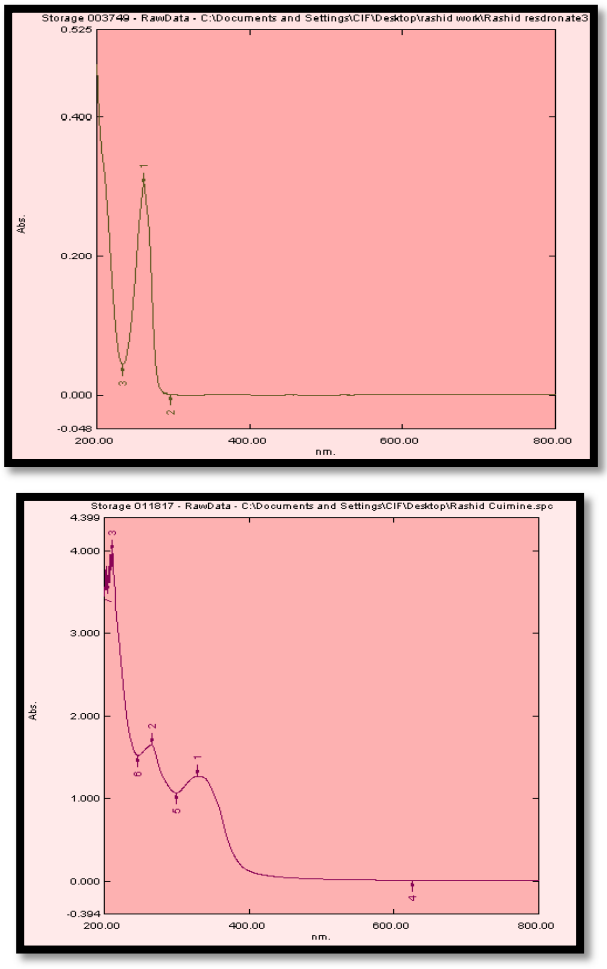
USP dissolution apparatus types 2 (Paddle type) was used to perform in vitro release study. The basket wasfilled with 500 ml of 0.1N HCl maintained at 37^{O}C. The paddle was adjusted at 50 rpm speed. 5ml of sample was removed and fresh medium was added to maintain the removed volume. To each sample 2.5 mL of acetate buffer and 2 mL of 2.5 mM copper (II) sulfate solution were added. The flasks were shaken well and completed to volume with distilled water. The absorbance of the formed copper (II) complex is measured at 264 nm against a reagent blank prepared in the same way without the addition of risedronate [**10**].

4. RESULT AND DISCUSSION

4.1. Preformulation studies

4.1.1. UV Spectra analysis

Risedronate sodium was scanned from 200-800 nm and maximum absorbance (λmax) was found to be at 262 nm (**Fig. 1.a**). λmax of Cuminum Cyaminum was found to be at 340 nm (**Fig. 1.b**).



a **b**

Fig. 1. UV Scan of (a) residronate (b) cumin Extract in distilled water

4.1.2. Interaction studies by Infrared spectroscopy

Drug and Chitosan interaction

IR spectrum of Chitosan + Risedronate : ν_{max} (KBr): 3633, 3523, 3371, 3277, 3099, 29529, 1660, 1556, 1381, 1313, 1213, 1155, 1083, 867, 713 cm^{-1} (**Fig. 2.a).** IR spectrum showed absorption bands for hydroxyl

groups (3622, 3523, 3371, 3099 cm^{-1}) and aromatic ring (1660, 1546, 1083 cm^{-1}). There is no interaction of chitosan with the pyridine ring and hydroxyl groups of Risedronate.

Chitosan and Cumin Extract Interaction

IR spectrum of cumin + chitosan: ν_{max} (KBr) 3568, 3225, 3103, 2953, 2897, 2347, 1639, 1568, 1436, 1323, 1211, 1095, 1014, 935, 887, 802, 744 cm^{-1} (**Fig. 2.b).** IR spectrum showed absorption bands for hydroxyl groups (3568, 3225, 3103 cm^{-1}) and aromatic ring (1639, 1568, 1014 cm^{-1}). Some of the hydroxyl groups are interacted with each other.

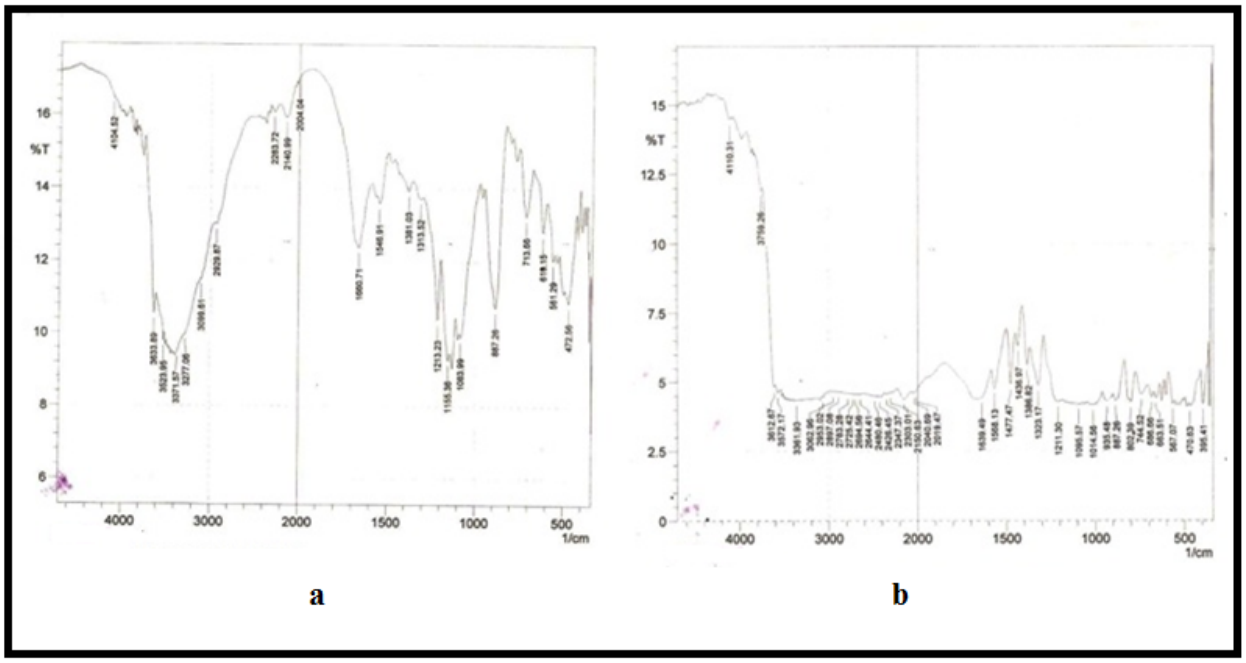


Fig. 2. IR spectrum of (a) Residronate + chitosan (b) Cumin extract +Chitosan

4.2. Formulation development

4.2.1. Chitosan microspheres

Preliminary evaluation was carried out to optimized independent factors (Concentration Of Polymer-X_1, Concentration of cross linking agent-X_2 and cross linking time-X_3). Placebo (without drug) microspheres were prepared by using different concentration of one independent factor with keeping two other independent factors at constant and maximum level. Scanning electron microscope (SEM) is used for optimization of shape and surface characteristics of microparticles.

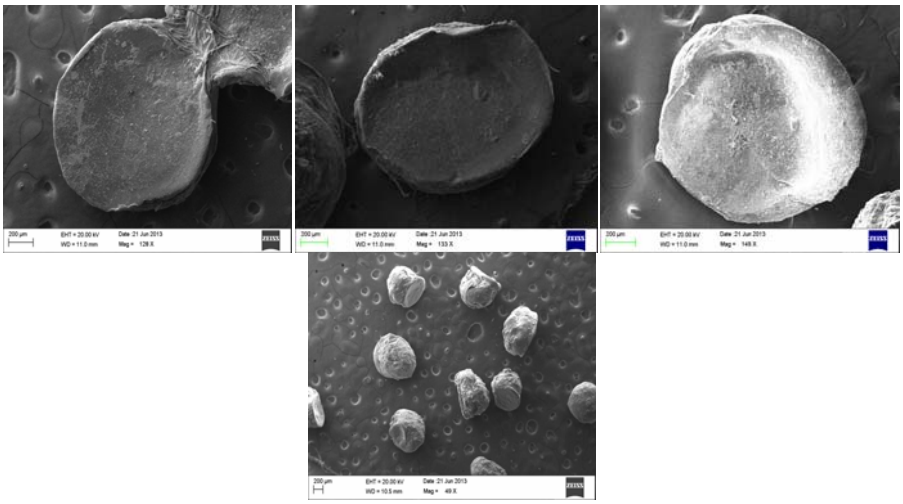
Effect of chitosan concentration on characteristic of placebo microparticles

Six formulations were prepared with different concentrations of Chitosan (0.100%-2.00%) along with constant 15% TPP concentration and maximum cross linking time (60 minutes). The results are shown in **Table 3**. As shown in **Fig. 3**, Formulation A_1 & A_2 was unsuccessful as polymer concentration is too low to form microparticles and only lumps were obtained. At 0.25% chitosan concentration, microspheres obtained were very soft in nature, irregular due to inadequate polymer chains to provide compactness and strength to the molecules. At 0.5% chitosan concentration, microparticles formed were soft and discrete. At high concentration (1.25%-2.00%) full strength discrete microparticles were obtained due to sufficient cross-linking with TPP. At concentrations (> 2.00%), spherical droplets cannot formed due to very viscosity of polymer solution which results in formation of irregular, distorted shaped and rough particles. In view of these results, chitosan concentration0.5%-2.0% was selected for further studies.

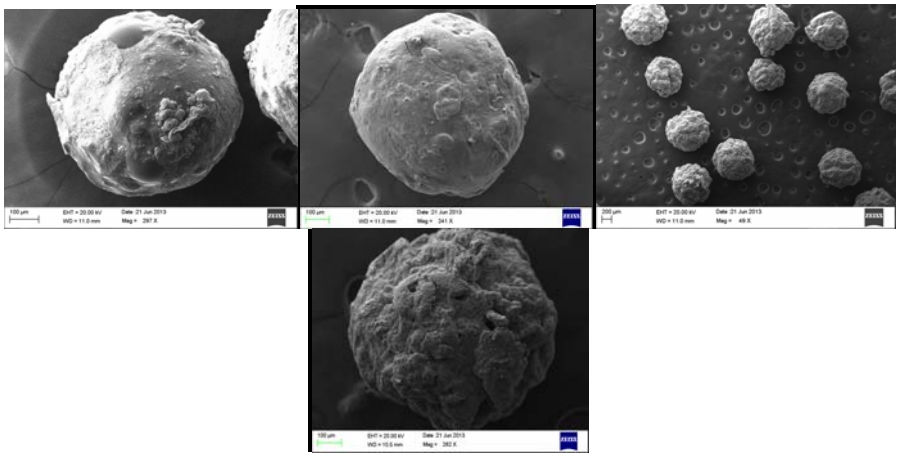
Table 3.Effect of chitosan concentration on characteristic of placebo microparticles

Formulation code	Chitosan (%)	Shape of particles
A_1	0.10	Lumps, no particles
A_2	0.25	Very Soft , irregular shaped lumps
A_3	0.50	Soft, semi-spherical shaped, discrete
A_4	1.25	strengthen, spherical shaped, discrete particles
A_5	2.00	strengthen, spherical shaped, discrete particles
A_6	2.5	Distorted shaped hard particles

***Microparticles prepared using 15% TPP as a crosslinking agent and 60 minutes crosslinking time**



(a) **(b)** **(c)** **(d)**



(e) **(f)** **(g)** **(h)**

Fig. 3. SEM photographs showing (a) lumps and irregular microparticles prepared with 0.1% chitosan (b) & (c) 0.25% chitosan concentration (d) soft, semi-spherical shaped, discrete microparticles prepared using 0.5% chitosan concentration (e) strengthen, spherical shaped, discrete microparticles prepared with 1.25% chitosan concentration (f) well hard, spherical shaped microparticles prepared with 2.00 % chitosan concentration(g) distorted shaped, hard particles (h) zoomed out individual microparticles prepared with 2.5% chitosan concentration.

Effect of cross linking agent (sodium Tripolyphosphate-TPP) on characteristic of placebo microparticles

Six formulations were prepared with different concentrations of TPP (1.00%-20.00%) along with constant 2.00% chitosan concentration and maximum time of cross linking 60 minutes. The results obtained are in the **Table 4**. As shown in **Fig. 4**, Formulation T_5 prepared with 15% concentration produced well rigid microspheres. This is due to sufficient concentration of TPP which can cross-link with polymer chains and form rigid structure. Low TPP concentration (<5%) gives soft and irregular microbarticles because of insufficient cross-linking which is unable to form compact structure. Formulation T_6 prepared with high concentration (20%) results in high cross linking and compaction, thus decreases the size of microparticles as well renders the spheres very hard and rough. Based on the above observation, range of 5- 15% TPP concentration was selected for gelation and microencapsulation process.

Table 4. Effect of TPP concentration on characteristic of placebo microparticles

Formula code	TPP (%)	Shape of Particles
T_1	1.0	Lumps, no particles
T_2	2.5	Soft & irregular shape
T_3	5.0	Soft, Hemi-spherical shaped , discrete
T_4	10	Strengthen, spherical shaped, discrete
T_5	15	Strengthen, spherical shaped, discrete
T_6	20	Rough, spherical shaped, hard particles

*** All microparticles prepared with 2% chitosan concentration and 60 minutes crosslinking time**

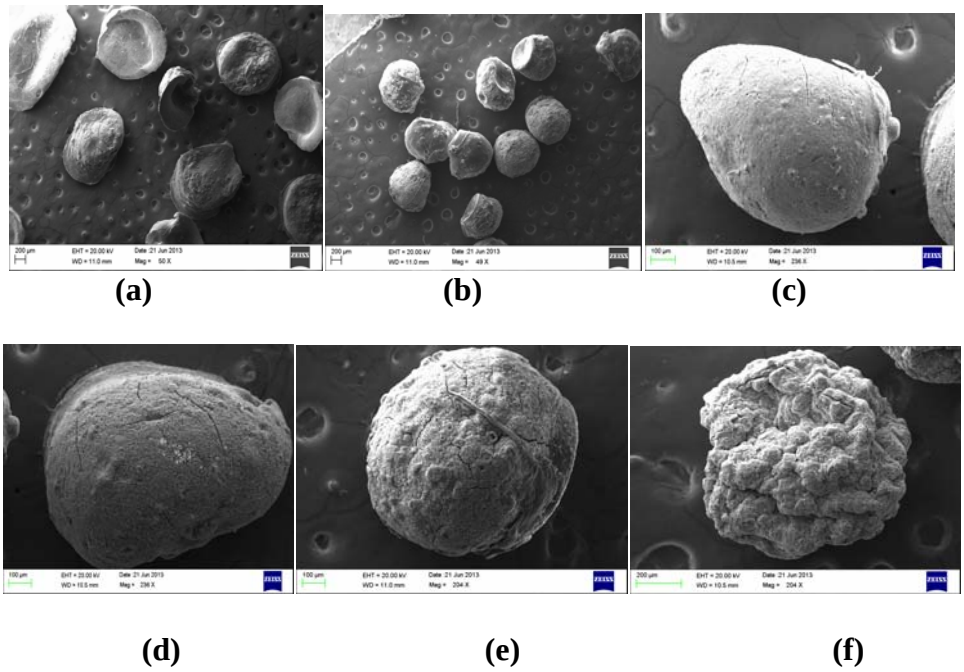


Fig. 4. SEM photomicrographs showing (a) irregular shaped microparticles prepared with 1.00% TPP **(b)**soft & irregular shape microparticles prepared with 2.5% TPP **(c)** soft, discrete strengthen, irregular shaped, discrete particles prepared with 5.00% TPP **(d)** strengthen, smooth, spherical shaped microparticles prepared with 10% TPP **(e)** strengthen, spherical shaped, hard, discrete microparticles having cracks on the surface prepared with15% TPP **(f)** rough, spherical shaped microparticles with irregular surface particles prepared with 20.00% TPP

Effect of cross-linking time on characteristics of placebo microparticles

Different formulations were prepared with different cross linking time (5 minutes to 120 minutes) keeping TPP concentration (20.00%) and chitosan concentration (2.00%) constant. The results are shown in **Table 5**.

Table 5. Effect of crosslinking time on characteristics of placebo microparticles

Formula	Time(minutes)	shape of particles
C_1	5	Lumps no particles
C_2	10	Gel form & irregular particles
C_3	20	Soft, hemispherical discrete particles
C_4	40	Granular, spherical, hard & discrete particles
C_5	60	Granular, spherical, hard & discrete particles
C_6	120	very hard, irregular, rough, discrete, spherical particles

*** All microparticles prepared with 2% chitosan and 20% TPP concentration**

As shown in **Table 5**, Formulation C_1 to C_3 produced very irregular shaped microparticles due to insufficient crosslinking process. After 20 minutes of cross-linking process a homogenous, granular Soft, hemispherical discrete particles were obtained. The hardness, regularity of the particles increase as cross linking time increases because of proper interaction between the polymer and cross-linking agentwhich ultimately result in formation of suitable compact molecular structure. As a result formulation C_4 and C_5 produced rigid, spherical and smooth microspheres (**Fig. 5**). On the other hand, formulation C_6 produced microparticles which were very hard, irregular, rough, discrete, spherical particles due to shrinking of the particles because of extent crosslinking time. Therefore 20 minutes to 60 minutes cross-linking time was selected for further studies.

To identify the optimum levels of different process parameters influencing particle size, entrapment efficiency and time for 100% drug release, an experimental design of 17 runs was made according to the Box-Behnken statistical design for three selected parameters (**Table 6**). The individual and interactive effects of these process variables were studied by conducting the process at different levels of all factors. The results of experimental data and simulated values are listed in **Table 7**.

Table 6. Experimental design for Box-Behnken statistical design using DESIGN EXPERT.

Formula No.	chitosan conc.	TPP Conc.	Cross Linking Time
F-1	0.5	5	40 min
F-2	0.5	10	20 min
F-3	0.5	10	60 min
F-4	0.5	15	40 min
F-5	1.25	5	20 min
F-6	1.25	5	60 min
F-7	1.25	10	40 min
F-8	1.25	10	40 min
F-9	1.25	10	40 min
F-10	1.25	10	40 min
F-11	1.25	10	40 min
F-12	1.25	15	20 min
F-13	1.25	15	60 min
F-14	2	5	40 min
F-15	2	10	20 min
F-16	2	10	60 min
F-17	2	15	40 min

Table 7. Effect of independent variables factors on size, entrapment efficiency and time % release of microparticles

Formulations	X_1	X_2	X_3	Particles Size (μm)	%Entrapment	Time for 100% drug release (minutes)
F-1	-1	-1	0	221.4	84.9	45
F-2	-1	0	-1	240.0	86.19	90
F-3	-1	0	+1	221	74.34	60
F-4	-1	+1	0	194.24	72	45
F-5	0	-1	-1	262	90	150
F-6	0	-1	+1	254.2	88.19	120
F-7	0	0	0	280	92.67	215
F-8	0	0	0	282.7	91.32	210
F-9	0	0	0	282.9	90.2	205
F-10	0	0	0	284.3	89.8	205
F-11	0	0	0	281.7	89.6	205
F-12	0	+1	-1	250.1	89.6	165
F-13	0	+1	+1	246	78	135
F-14	+1	-1	0	278.8	89.44	180
F-15	+1	0	-1	304	91	275
F-16	+1	0	+1	312.6	90	225
F-17	+1	+1	0	279.6	90	240

There are many statistical out comes after data processing by the DESIGN EXPERT software using **Box-Behnken** design through quadratic model. These are in the form of graphs, values and results from. The analysis of variance (ANOVA) of the model for different responses has been analyzed for significance of different combinations of factors and lack of fit was analyzed with its no significance. These are signs of good results of the experiment.

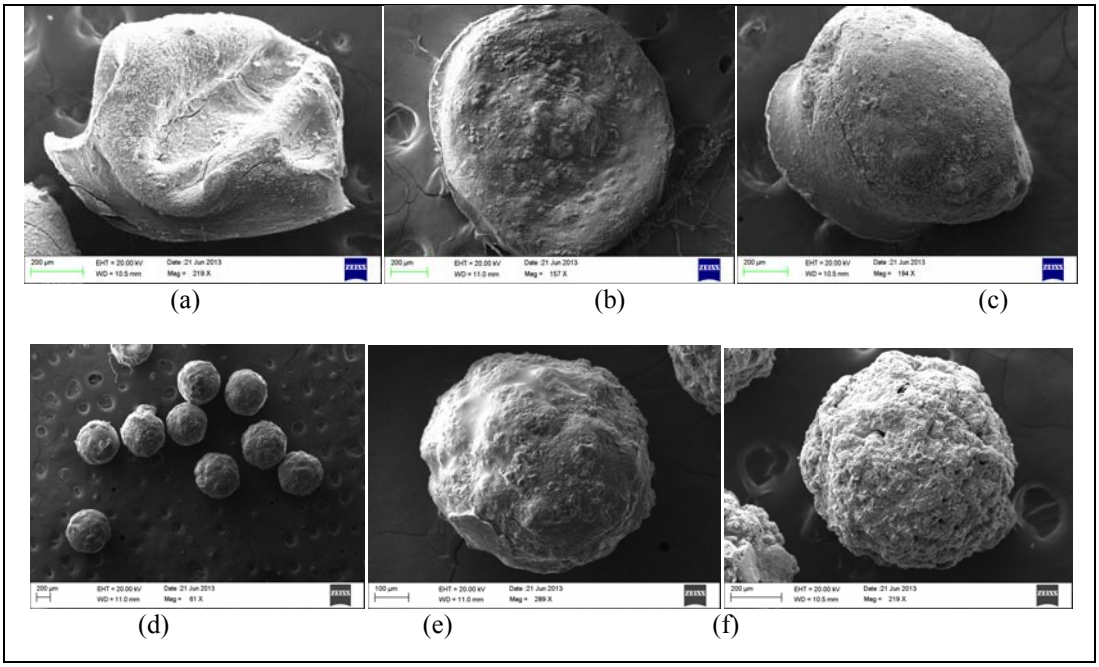


Fig. 5. SEM Photomicrographs showing (a) irregular microparticles prepared with 5 minutes cross linking time (b) Soft & irregular shape microparticles prepared with 10 minutes cross linking time (c) Soft, hemispherical discrete particles prepared with 20 minutes cross linking time (d) Granular, spherical, hard & discrete particles prepared with 40 minutes cross linking time (e) Granular, spherical, hard & discrete prepared with 60 minutes cross linking time (f) very hard, irregular, rough, discrete, spherical particlesprepared with 120 minutes cross linking time

Evaluation of Design Matrix for Response Surface Quadratic Model by applying ANOVA

The ANOVA results are summerised in **Table 8**. No aliases found for Quadratic Model-Degrees of Freedom applied for Evaluation of data. A recommendation is a minimum of 3 lack of fit and 4 df for pure error.This ensures a valid lack of fit test. Fewer df will lead to a test that may not detect lack of fit. According to desing expert software, standard errors should be similar within type of coefficient. Smaller is better. Ideal VIF is 1.0.VIFs above 10 are cause for alarm,indicating coefficients are poorly estimated due to multicollinearity. Ri-squared is nearly 0. High Ri-squared means terms are correlated with each other, possibly leading to poor models.If the design has multilinear constraints multicollinearitywill exist to a greater degree,thus increasing the VIFs and the Ri-squareds, rendering these statistic useless. After applying the statistical design it is found that the actual value and predicted values for the size of the particles (X_1), entrapment efficiency (X_2), and 100% drug release time (X_3), are very nearby with non significant residuals and leverages. Maximum residual for particle size (X_1), entrapment efficiency (X_2) and maximum release time (X_3) was ± 2.387, ± 1.952 and ± 7.00, respectively, with maximum leverage 0.75 which is under limit of consideration. So we can proceed for the next level of analysis of data.

As shown in **Table 8**, the Model F-value of 341.5824 for particle size, 61.926 for entrapment & 210.3601for time for maximum release implies the model is significant. Values of "Prob > F" less than 0.0500 indicate model terms are significant. The "Lack of Fit F-value" of 3.381576, 0.195423 & 3.541667 for particle size, entrapment efficiency & time for maximum release respectivly implies that thelack of Fit is not significant. "Predicted R-Squared" values are in reasonable agreement with the "Adjusted R-Squared" values for the three factors and responses (**Fig. 6**). The "Adeq Precision" ratio of 67.534, 67.534 & 23.759 for all three independent factors indicates an adequate signal and hence this model can be used to navigate the design space. Now we can conlude that the the factors and there purturbation are sinificantly affective for the changes in size of particles, entrapment efficiency and time for maximum release and thus model terms can be used to find an optimum formulation.

Table 8. ANOVA results of Box-Behnken design

Results of analysis of variance	**Particle size**	**Drug Entrapment**	**$t_{100\ \%}$ drug release**
Regression			
Sum of squares	15791	596.45	79110.4
Df	9	9	9
Mean squares	1754.6	66.27	8790
F-value	341.58	61.92	210.36
p	<0.0001	<0.0001	<0.0001
Residual			
Sum of squares	35.96	7.49	292.5
Df	9	7	7
Mean square	5.14	1.07	41.78
Lack of fit test			
Sum of squares	25.78	0.96	212.5
Df	3	3	3
Mean squares	8.59	0.31	70.83
F-value	3.38	0.19	3.5
Correlation coeffecient (R_2)	0.9977	0.9878	0.9876
Correlation of variation (% CV)	0.86	1.19	1.19
SD	2.7	1.03	1.03

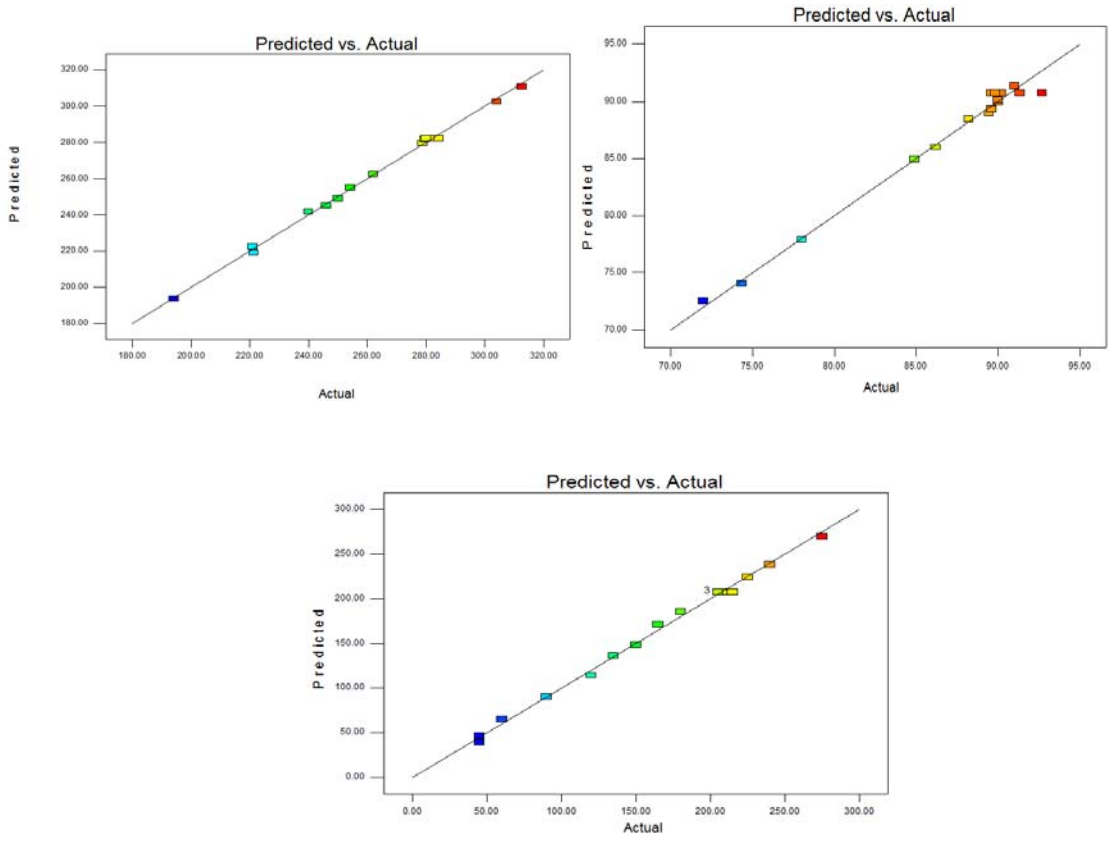


Fig. 6. Graph of predicted & actual values for (a) size of microparticles (b) entrapment efficiency (c) Maximum drug release

The model proposes the following polynomial equation for microparticles for the different responses

$$\mathbf{Y_1 = 282.32+37.30X_1 -5.81\ X_2-2.79\ X_3+6.99\ X_1X_2+6.90\ X_1\ X_3+0.9\ X_2\ X_3-11.24\ X_1^2- 27.57\ X_2^2 -1.68\ X_3^2} \quad \mathbf{(1)}$$

$$\mathbf{Y2 = 90.72 + 5.38\ X_1 - 2.87\ X_2- 3.28\ X_3+ 3.36X\ _1X_2 + 2.71X_1\ X_3-2.4\ X_2\ X_3\ -3.85X_1^2 - 2.78\ X_2^2- 1.49\ X_3^2} \quad \mathbf{(2)}$$

$$\mathbf{Y2 = 90.72 + 5.38\ X_1 - 2.87\ X_2- 3.28\ X_3+ 3.36X\ _1X_2 + 2.71X_1\ X_3-2.4\ X_2\ X_3\ -3.85X_1^2 - 2.78\ X_2^2- 1.49\ X_3^2} \quad \mathbf{(3)}$$

All the three equation of response and factotrs are quadratic equations which means the independent variables X have two values for desired value of dependent variable Y
{ $aX^2+bX+c=0$}

Discussion of cosiquences of pertubations in factors
Size of particles (Y_1)
As shown inequation (1),effects of X_1, X_2, X_3, X_1X_2, X_1X_3, X_1^2, X_2^2 are significant. A positive value of X_1 represents a direct relationship of chitosan concentration on particle size. Particle size increases with increase in concentration of chitosan polymer.This is due to increase viscosity of polymeric solution with increase in concentration which lead to large size particles. At 0.5%, 1.25% and 2% chitosan concentration, mean average size was found to be 219.16 ± 18.83008 µm, 269.3222 ± 16.01014 µm and 293.75 ± 17.16693 µm, respectively.
At low concentration of TPP, insufficient molecules of TPP for cross linking process are available, thus remaining molecule get separated unbound in solution, rendering the size of particles smaller. Gradually, as the TPP concentration increases from 5 to 10%, micropartices size also increases254.1 ± 24.09 to $276.5778 \pm 28.8\ \mu m$. Further increase in TPP concentration, resulted in decrease in the size of microparticles ($242 \pm 35.47509 \mu m$). This is due toavailability of multiple crosslinking sites (NH_2) of chitosan which are being bonded with increased number of TPP, this results in more compaction of molecules and contraction of the particles.

Long Cross linking time (CLT) decreases the size due to maximum interaction of polymer and crosslinking agent and complete bonding of polymeric chains which resulted into compact structure. According to the results, crosslinked time for 20, 40 and 60 minutes produced microparticles having average size $264.025 \pm 28.12595\mu m$, $265.0711 \pm 33.20687\mu m$ and $258.45 \pm 38.76334\ \mu m$, respectively (**Fig. 7**). But the effect of cross linking time is much less significant as the cross linking is an ionic interaction between $(-NH_2)^{+\delta}$ of chitosan and negative $[(PO_4)_3]^{-5}$ of Tri-Poly-Phosphates which is very fast and happens abruptly approaching to maximum as soon as the drops of chitosan is poured into TPP solution. Interactive effect of independent variables is further shown in contour plot (**Fig. 7.A**) and response surface graphs (**Fig. 7.B**).

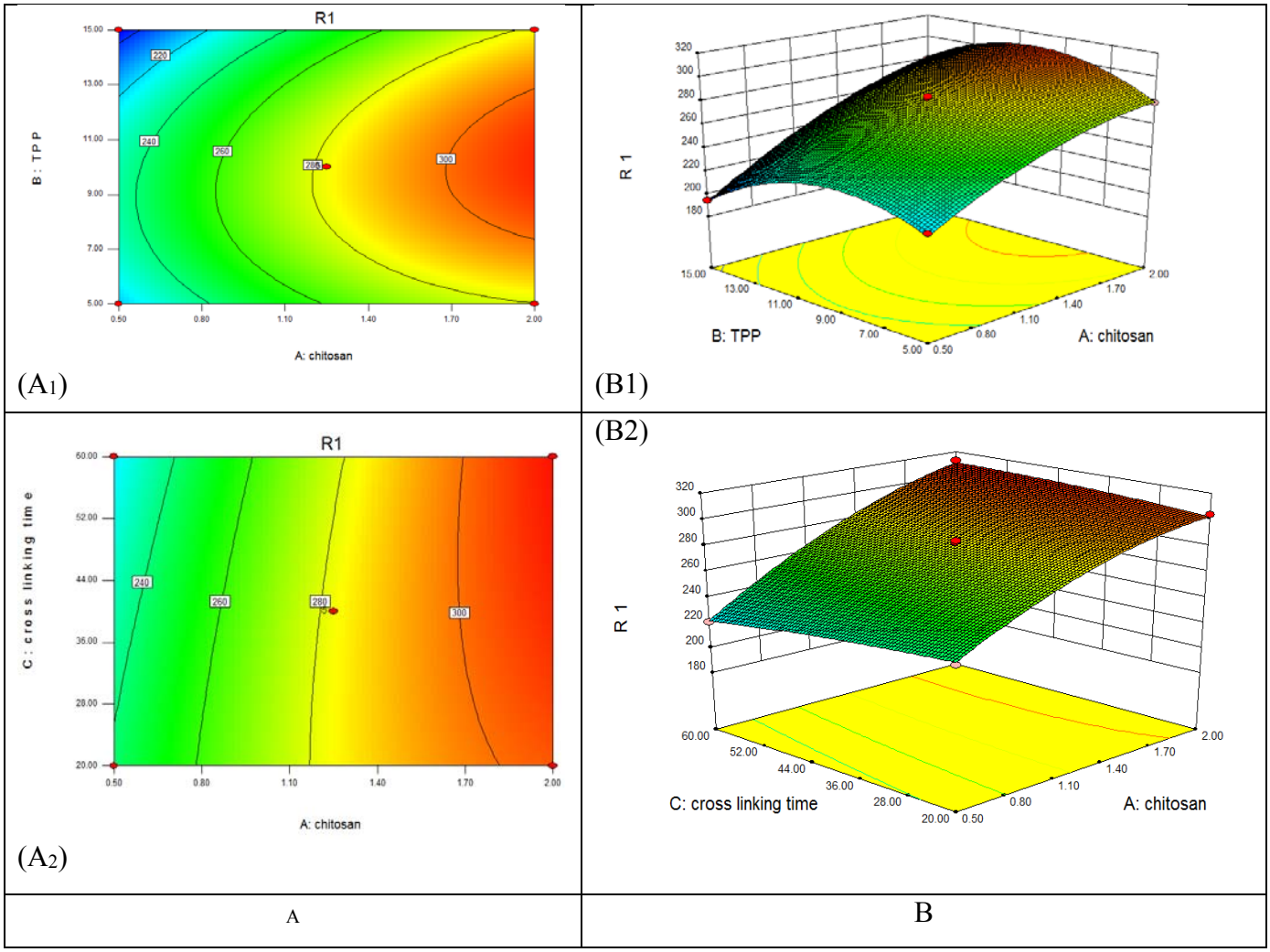


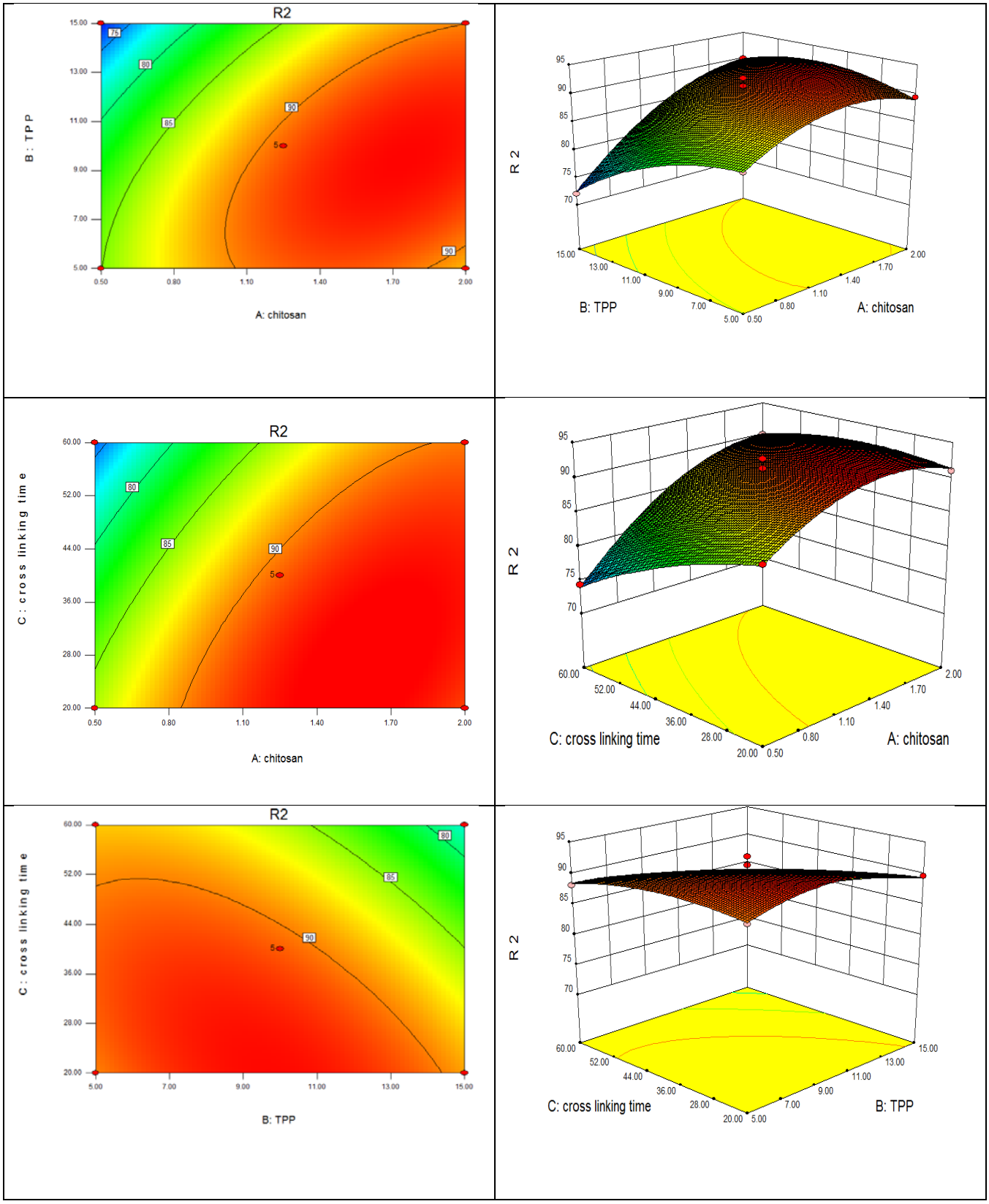
Figure 7.A. Contour plots and B. Response surface graphs showing interactive effect of independent variables on particle size

Entrapment effeciency (Y_2)

Polynomial equation (2) showing X_1, X_2, X_3, X_1X_2, X_2X_3, X_1X_3, X_1^2, X_2^2, X_3^2, are significant model terms which means individually as well as in interaction, the three factors affect the entrapment significantly. A positive value of X_1 represents a favorable optimization process while negative value of X_2 and X_3indicates an inverse relationship. Entrapment of drug increases with increase of concentration of chitosan polymer.As more will be the chitosan concnetration, larger will be the particlessize and hence higher drug will be the entrapment. Moreover, probability of the loss of drug from the denser matrix is quite less during cross-linking and washing process. At chitosan concentration 0.5%, 1.25% & 2.00% w/v, entrapment effeciency of Risedronate was found to be 79.3575 ± 7.227505 %, 88.82 ± 4.24873%, &90.11 ± 0.64941%, respectively.

With increase in concentration of TPP, the micoparticles size also decreases which resultedin squeezing out of drug and ultimately decrease in entrapment efficiency. Formulations prepared with 5.0%, 10.0% & 15.0% w/v concentration of TPP, entrapments effeciencies was found to be 88.1325 ± 2.283964 %, 88.34667 ± 5.536937%, &82.4 ± 8.890444%, respectively.Long Cross liinking time decreases the entrapment by dual effects which is size reduction and diffusion of drug from particles rendering the less drug entrapment.

Formulations prepared with crosslinking time 20, 40 and 60 minutes showed entrapment $89.11975 \pm 2.09\%$, $87.77 \pm 6.27\%$ and $82.6325 \pm 7.64\%$ respectively. Response surface graphs and contour plots (**Fig. 8)** showing quadratic effect of independent variables on entrapment.



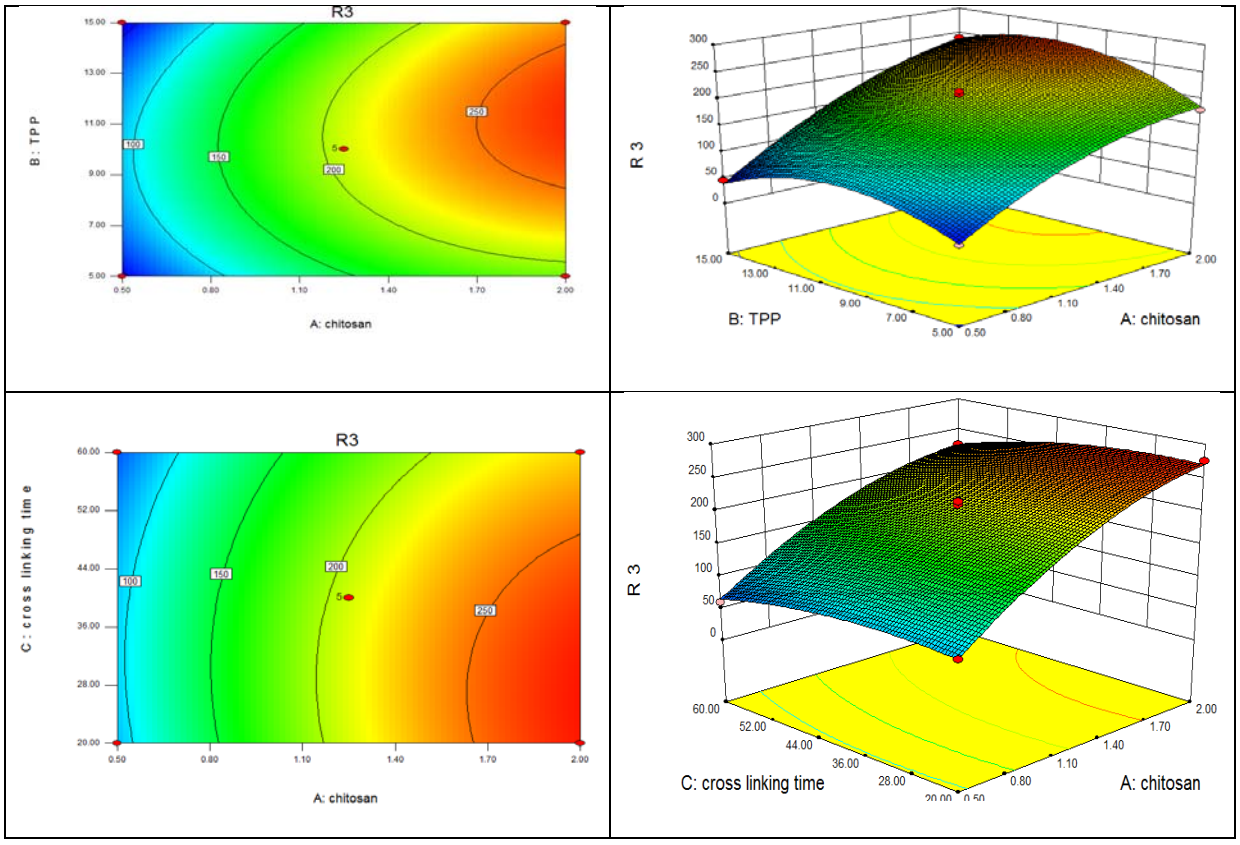
A **B**

Fig. 8.(A) Contour plots (B) response surface graphs showing effect of independent variables on entrapment efficiency.

Time for 100% drug release

As shown in polynomial eqution (3),the effects of X_1, X_2, X_3, X_1X_2, X_{12}, X_2^2, X_3^2 are significant model terms. A positive value of X_1 &X_2represent a favorable optimization process while negative value X_3indicates an inverse relationship. Drug release increases with increase in concentration of chitosan due to increase in polymer chains density which result in slow diffusion of drug particles. Also increased in particle size and high entrapment due to high concentration of chitosan will take more time to release the drug. At chitosan concentration 0.5%, 1.25% and 2%, $t_{100\%}$ was found to be is 60 ± 21.2 minutes, 178.8 ± 36.6 minutes and 230.00 ± 39.37 minutes, respectively.

With increase in concentration of TPP, crosslinking process also increases which results in denser and compact polymer matrix and ultimately slower rate of drug diffusion [**12**]. Formulations prepared using 5%, 10% and 15% TPP concentration releases 100% drug in123.7 ± 57minutes, 187.7 ± 68.01 minutes and 146.25 ± 80.7 minutes, respectively. Similarly, long Cross linking time decreases the release time due to more interactions of crosslinker with polymer chains which form compact structure results to slow squeezing out of drug from patricles. Fomulations prepared with 20 minuts, 40 minutes and 60 minuts of CLT results releases 100% drug in170.00 ± 77.1, 172.2 ± 73.7 and 135.00 ± 68.19 minutes. Interactive effect of independent variables on release is shown by contour plots and response surface graphs (**Fig. 9**). Interactive effect chitosan and TPP as well as chitosan and crosslinking time slower the release rate of residronate. Whereas non-significant quadratic effect was observed at different concentration of TPP and cross-linking time.



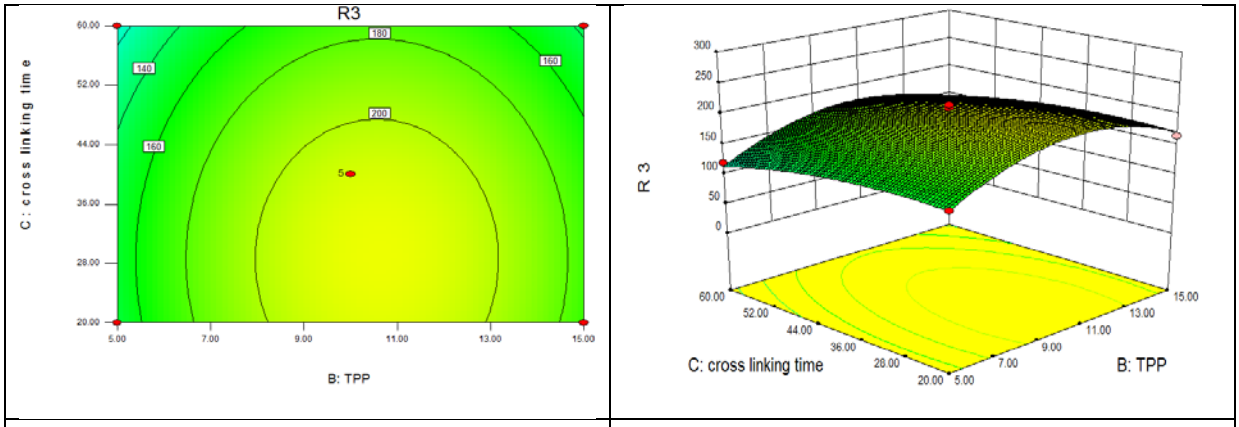


Fig. 9.A. Contour plots and (B) response surface graphs showing effect of independent variables on $t_{100\%}$ release.

Selection of optimized formulation & result validation

Point prediction of the design expert software was used to determine the optimum values of the factors for maximum entrapment. The optimum formulation was selected based on the criteria of attaining the maximum entrapment for microparticles. Total 6 checkpoint formulations were chosen and their predicted and experimental values are shown in **Table 9**. Finally, the optimum values of chitosan (1.90%), TPP (10.73%) & cross linking time (34.10 minutes) were obtained. These formulations predicts values 305.84±2.659 µm & experimental value 305.56±2.0440 of particle sizes, with correlation coefficient between predicted value (PV) and experimented value (EP) (r) = .935621 indicates 6.5% prediction error. Similarly 91.72917±2.024 PV & 91.31±1.886 EV with r = 0.958652 indicates 4.135 % prediction error for evaluation of % entrapment & 271.044±2.350298 PV & 270.8793±2.458076 EV with r = 0.993249 indicates 0.675% prediction error for MRT evaluation.

Upon comparison of the observed responses with that of the anticipated responses, the positive correlation values are +0.935621,+ 0.958652, +0.993249 which are near to +1.0 confirming the excellence of validation of results of optimized values of variables and their effects. Thus, the low magnitudes of error and the significant values of correlation coefficient in the current study indicated a high prognostic ability.

Table 9. Composition of the checkpoint formulations, the predicted and experimental values of response variables

Code	Compositions $X_1 : X_1 : X_1$	Particle size		%EE*		$T_{100\%}$ release	
		E.V	P.V	E.V**	P.V***	E.V	P.V
OF_1	1.80:10.8:34.05	302.22	304.424	90.092	90.589	271.009	271.023
OF_2	1.80:10.8:34.15	303.82	304.230	91.642	92.499	269.899	270.025
OF_3	2.0:10.2:34.05	306.85	306.424	92.405	92.999	272.225	272.520
OF_4	2.0:11.2:34.05	306.66	306.880	91.955	92.000	272.000	272.053
OF_5	2.0:10.2:34.15	307.25	306.980	91.544	91.799	269.022	269.420
OF_6	1.80:11.2:34.15	306.55	306.120	90.222	90.489	271.121	271.223

*%EE: Percent Entrapment Efficiency
**E.V: Experimental values
***P.V: Predicted values

4.2.2. Cumin loaded chitosan microspheres

Cumin loaded microspheres were prepared according to formulae shown in **Table 10**. Formulations showed particle size vary from 258 to 356 µm depending on chitosan and TPP concentration. Similarly Formulation C-3 showed maximum entrapment and slowest reason as compared to Formulation C-2 and Formulation C-3, respectively. SEM photomicrograph shown in **Fig. 10** showed aggregated and less spherical particles when compared with chitosan alone.

Table 10. Formulae for preparation of Cumin Loaded microspheres

Experiment No.	Weight of micro particles	Particles Size (µm)	% Entrapment Efficiency	$T_{100\%}$ Release (min)
C-1	**180 mg**	258.4±3.45	68.41±2.81	120±5.22
C-2	**212mg**	356.2±5.66	89.04±1.22	180±4.98
C-3	**242mg**	321.8±4.98	91.96±3.01	240±6.72

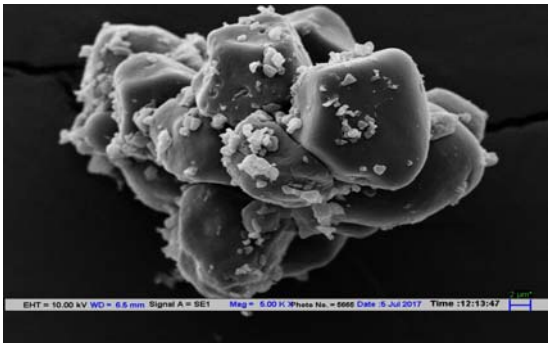


Fig. 10. Cumin loaded chitosan microsphere

5. CONCLUSION

The results of Box‑Behnken design revealed that chitosan concentration, TPP concentration and time of cross linking had a significant effect on dependent variables such as size, percent drug entrapment and maximum release time. Polymers concentration had a positive impact while cross-linker concentration had a negative impact on drug entrapment. The interaction of TPP and Cross linking time had a more pronounced effect on dug entrapment than chitosan concentration. Microcapsules of best batch based on point prediction tool of design software exhibited 91.0 % drug entrapment and 270 minutes for maximum drug release 96% experimental validity. The SEM analysis showed smooth, stable, round and strengthen microparticles which can be used for further studies.

Conflict of interest: Authors declares no conflict of interest

6. REFERENCES

[1] A. Ezra, Golomb G. Administration routes and delivery systems of bisphosphonates for the treatment of bone resorption. Adv Drug Del Rev., 2000, 42: 175–195.
[2] N.B. Watts, D.L. Diab. Long-term use of bisphosphonates in osteoporosis. J Clin Endocrinol Metab., 2010, 95: 1555–1565.
[3] E.M. Lewiecki. Bisphosphonates for the treatment of osteoporosis: insights for clinicians. Ther Adv Chronic Dis., 2010, 1(3): 115-28.
[4] Drake MT, Clarke BL, Khosla S. Bisphosphonates: Mechanism of Action and Role in Clinical Practice. Mayo Clin Proc 2008; 83(9): 1032–1045.
[5] A.J. Bruce. Absorption Enhancers: Applications and Advances. AAPS J., 2012, 14(1): 10–18.
[6] B. Aderibigbe, I. Aderibigbe, P. Popoola. Design and Biological Evaluation of Delivery Systems Containing Bisphosphonates. Pharmaceutics., 2017, 9(2): 1-24.
[7] G.B. Dudhatra, S.K. Mody, M.M. Awale, H.B. Patel *et al*. A comprehensive review on pharmacotherapeutics of herbal bioenhancers. Scientific World J., 2012, 637953.
[8] B. Jhanwar, S. Gupta. Biopotentiation using Herbs: Novel Technique for Poor Bioavailable Drugs. Int J PharmTech Res., 2014, 6(2): 443-454.
[9] K. Kesarwani, R. Gupta. Bioavailability enhancers of herbal origin: An overview. Asian Pacific J Tropical Biomedicine., 2013, 3(4): 253-266 ·
[10] M.I. Walash, M.E. Metwally, M. Eid, R.N. El-Shaheny. Spectrophotometric Determination of Risedronate in Pharmaceutical Formulations via Complex Formation with Cu (II) Ions: Application to Content Uniformity Testing. Int J Biomed Sci., 2008, 4(4): 303-9.
[11] S. Sultana, S. Talegaonkar, D. Singh, R. Ahmad *et al*. An approach for lacidipine loaded gastroretentive formulation prepared by different methods for gastroparesis in diabetic patients. Saudi Pharm J., 2013, 21(3): 293–304.
[12] A. Srinatha, J.K. Pandit, S. Singh. Ionic Cross-linked Chitosan Beads for Extended Release of Ciprofloxacin: *In vitro* Characterization. Indian J Pharm Sci., 2008,70(1).