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Evaluation of Protein Microencapsulation Efficiency in Alginate/Hydroxyethyl Cellulose Polymer Composite

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Considering how much protein is loaded into the process, the crosslinking solution concentration and the weight percentage of the alginate/hydroxyethyl cellulose coating material, this study presented the details of optimization of the microencapsulation process. Optimization was done with an objective of maximizing the encapsulation efficiency of the microparticle fabricated using external ionotropic gelation method. Results showed that the process is capable of achieving around 75% encapsulation efficiency when protein loading is around 15 wt%, alginate/hydroxyethyl cellulos is about 2 wt% and the $CaCl_2$ solution should be 3 wt%. This was done using Box-Behnken methodology wherein the predicted model was found to have good predictive capability. Analysis also showed that the process is affected by how much protein drug is loaded into the system and the interactions between crosslinking solution concentration with bovine serum albumin (BSA) loading as well as the strong relationship between alginate/hydroxyethyl cellulos concentration with itself.

Keywords: Microencapsulation, Protein, Alginate, Cellulose, Ionotropic gelation.

INTRODUCTION

In design of oral delivery of peptide or protein drugs, pH-sensitive hydrogels like alginate and other biopolymers have attracted increasing attention. This is because most of the synthetic polymers are immunogenic and adding of therapeutic proteins into these polymers require a harsh environment that causes denaturation and inactivity to the desired protein [1]. Alginate and cellulose, cellulose derivative for that matter, are natural polymers that are biocompatible, biodegradable and produce no systemic toxicity when administered into the human body. Alginate comes from a family of polysaccharides which is composed of α-L-gluronic acid (G) and β-D-mannuronic acid (M) residues, arranged in homopolymeric blocks of each type (MM, GG) and heteropolymeric blocks (MG) [2,3]. Hydroxyethyl cellulose (HEC), a type of cellulose derivative, on the other hand, is a non-ionic polysaccharide cellulose derivative which can be compounded with alginate to form an improved hydrogel blend. Several studies have used hydroxyethyl cellulose (HEC) as a component in drug delivery systems. Alginate has the property of shrinking in low pH and getting dissolved in higher pH. Integration of HEC into its polymer complex, improves the pH-responsive property of alginate hydrogels resulting to more improved microencapsulation properties [4,5]. The HEC component of the composite microparticles contributes as more of an entrapment enhancer rather than a swellable matrix like alginate because of the presence of hydrogen bonds between the two polymers (Fig. 1). Previous swelling study [6] reported that the alginate matrix swells in the presence of neutral media while HEC degrades and dissolves leading to a higher rate of drug release.

In this regard, alginate gelled by the addition of calcium ions will be used to prepare the microparticles. A mild encapsulation method can enhance the protein stability and retain the biological activity of the encapsulated materials [7-10]. In addition, the microparticles can protect the model protein as it passes through the acidic and enzymatic environment of the stomach and can release the protein *via* diffusion and micro-

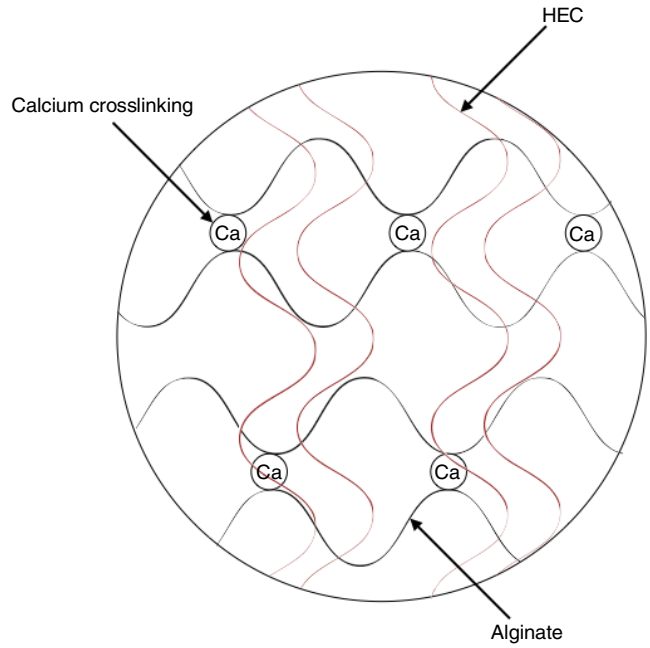

Fig 1. Schematic illustration of the alginate/hydroxyethyl cellulose microparticle. Hydrogen bonding enhances the drug entrapment property of the calcium alginate polymer network

capsule degradation once they reach the absorption region, at which the protein is effectively absorbed into the blood stream [11-13]. From previous studies, the encapsulation efficiency of the microencapsulation process was predominantly affected by how much protein is loaded into the process, the concentration of the crosslinking solution, and the weight percentage of the alginate and HEC as coating material [14].

On the basis of these considerations, this study present the details of optimization of the microencapsulation process of the microencapsulation of bovine serum albumin (BSA) as model protein. Optimization was done with an objective of maximizing the response or the encapsulation efficiency of the microparticle fabricated. A desirability based approach will be followed for analyzing the accuracy of optimization process [15]. Although there are different colloidal devices for protein delivery using alginate and other polymers [16], to date, there have been no attempts that have been made to correlate statistically the formulation variables with the final microparticle properties with respect to alginate and HEC as coating material for the microencapsulation of protein. Therefore, in this work, the optimization of the microparticle′s encapsulation efficiency using response surface design, Box Behnken to be specific is carried out. This design was used to simultaneously study the effect of the three formulation variables of the alginate-HEC delivery system on one response variable.

EXPERIMENTAL

Sodium alginate provided by Sigma-Aldrich Chemical Co., USA was used in this study. This sodium alginate was low in viscosity and has a viscosity of 100-300 cP when in a 2% aqueous solution at room temperature. Calcium chloride ($CaCl_2$) was supplied by Merck KGaA. Furthermore, hydroxyethyl cellulose (HEC, viscosity of 800 to 1500 cP when in a 2% aqueous solution) was provided by Tokyo Chemical Inc. Bovine serum albumin (BSA), a model protein, was purchased from MP Biochemicals New Zealand Ltd. Other reagents and the bicinchoninic acid (BCA) protein assay kit were of analytical grade and used as received.

Preparation of BSA-loaded microparticles: Weight percentages of sodium alginate and HEC (Table-1) were dissolved in distilled water until solution was homogeneous. Known amounts of BSA was then added to the solution until it forms a uniform blend. Microencapsulation process was done using Buchi B-390® microencapsulator (Buchi Labortechnik AG, Switzerland) under constant feed rate (1500 Hz, 1150 V). Beads were formed when droplets from a 200 µm nozzle come in contact with the 5 wt% $CaCl_2$ solution under room temperature with constant stirring (Fig. 2). Curing time for the microspheres were done for 30 min, then BSA loaded microbeads were collected *via* filtration and washed with distilled water twice. Collected samples were then freeze dried using GEA Smart Lyo SL2 for a 24 h freeze drying cycle starting from -4 ºC.

TABLE-1
PROCESS PARAMETERS WITH THEIR VALUES AT THREE LEVELS TO BE USED IN THE BOX-BEHNKEN EXPERIMENTAL DESIGN

Factor	Factor level		
	–1	0	+1
X_1, wt. % BSA loading	5	10	15
X_2, wt. % Alginate/HEC	1	2	3
X_3, wt. % $CaCl_2$	3	5	7

Experimental design: A response surface method, Box-Behnken experimental design, was applied to evaluate the relationship between the independent variables and their responses as well as their interactions in an effective model. The model contains 12 factorial design points and three center points. Three variables and one response were involved in the experimental design. The dependent response factor variable measured was encapsulation efficiency [13]. The independent variables are the percent BSA loading (X_1), concentration of alginate/HEC (X_2) and oncentration of $CaCl_2$ (X_3) (Table-1).

Determination of BSA content: Weighted amount of alginate/HEC composite microparticles were dispersed in 0.1 M phos-phate buffer solution (PBS) of pH 7.4. Samples were incubated in 37 ºC in a shaking water bath (100 rpm, 2 h). Then the samples were centrifuged and the supernatants were collected. The BSA content (protein estimation) in the supernatant was determined using the bicinchoninic acid (BCA) method. The principle of this method is that proteins can reduce Cu^{2+} to Cu^{+} in an alkaline solution and result in a purple color formation by bicinchoninic acid. Supernatants were mixed with 2 mL BCA working reagents provided by the BCA assay kit. Afterwards, solutions were measured within the 562 nm absorbance wavelength with a UV spectrophotometer (UV1800, Shimadzu, Japan). Encapsulation efficiency (EE) was then determined using the following formula:

$$E_e\ (\%) = \left(\frac{\text{Actual loading}}{\text{Theoretical loading}}\right) \times 100 \qquad (1)$$

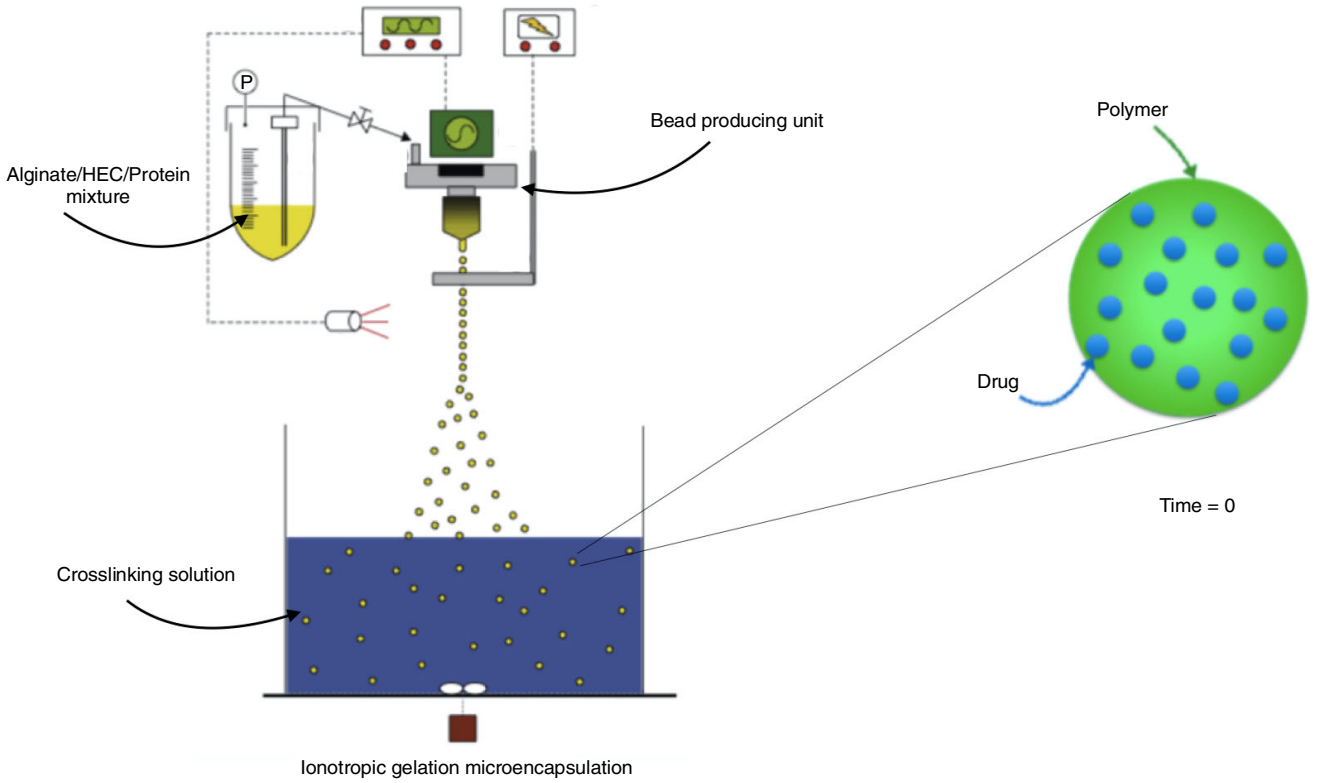

Fig. 2. Schematic diagram of the preparation of the protein loaded alginate/HEC microparticles *via* ionotropic gelation method. Alginate/HEC/protein mixture is extruded out of the nozzle of the bead producing unit with the aid of a vibration unit to create droplets forming beads in the crosslinking solution. Encapsulator diagram was retrieved from Buchi B-390® manual

Statistical analysis: The statistical analysis data through regression model and plotting the response surface graphs were achieved by JMP Version 9.0. The developed models were tested for its significance using analysis of variance (ANOVA). All tests were performed at a 95% level of significance (α = 0.05).

RESULTS AND DISCUSSION

Effect of formulation variables in encapsulation efficiency: Response surfaces for Box-Behnken were generated by JMP (Version 9.0) statistical analysis software. Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA). The levels chosen for the independent variables, % drug loading (X_1), % alginate/HEC (X_2) and % $CaCl_2$ (X_3) are shown in Table-2.

Based on the Box-Behnken design, encapsulation efficiency of each experimental group (total 15 data points) was determined for the protein content (%BSA loading), coating material (% alginate/HEC), and crosslinking solution (% $CaCl_2$). The encapsulation efficiency of BSA in alginate/HEC microparticles are in the range from 15.20 to 65.75% as shown in Table-2.

The regression coefficients calculated for the efficiency of microencapsulation were shown in Table-3. Among the linear, quadratic and cross product forms of independent variables, X_1, X_1X_3 and X_2^2 were significant at the level of $p < 0.05$. Thus, the regression model equation for the microencapsulation could be predicted as follows:

TABLE-2
BOX BEHNKEN DESIGN IN VARIOUS RUNS WITH THEIR CORRESPONDING RESPONSE WITH RESPECT TO ENCAPSULATION EFFICIENCY (n =3)

Formulation code	Coded variables			Encapsulation efficiency (%)
	BSA loading (%) (X_1)	Alginate/HEC (%) (X_2)	$CaCl_2$ (%) (X_3)	
P1	-1	-1	0	16.78
P2	-1	+1	0	15.20
P3	+1	-1	0	65.52
P4	+1	+1	0	45.04
P5	0	-1	-1	41.27
P6	0	-1	+1	34.10
P7	0	+1	-1	34.16
P8	0	+1	+1	34.66
P9	-1	0	-1	17.08
P10	+1	0	-1	65.75
P11	-1	0	+1	54.11
P12	+1	0	+1	56.57
P13	0	0	0	58.98
P14	0	0	0	58.98
P15	0	0	0	58.98

$$Y = 58.98 + 16.21\left(\frac{X_1 - 10}{5}\right) - 3.577(X_2 - 2) + 2.646\left(\frac{X_3 - 5}{2}\right)$$

$$+\left(\frac{X_1 - 10}{5}\right)[(X_2 - 2)(-4.727)] + \left(\frac{X_1 - 10}{5}\right)\left[\left(\frac{X_3 - 5}{2}\right)(-11.55)\right]$$

TABLE-3
SORTED PARAMETER ESTIMATES. TABLE SHOWS THAT THE ENCAPSULATION EFFICIENCY IS SIGNIFICANTLY AFFECTED BY THE PROTEIN LOADING AND INTERACTIONS BETWEEN PROTEIN LOADING AND CROSSLINKING SOLUTION CONCENTRATION, AS WELL AS THE STRONG RELATIONSHIP BETWEEN ALGINATE/HEC CONCENTRATION WITH ITSELF

Term	Estimate	Std Error	t Ratio	t Ratio	Prob>|t|
X_1	16.21	2.614	6.20		0.0016*
X_2*X_2	-17.84	3.847	-4.64		0.0057*
X_1*X_3	-11.55	3.696	-3.12		0.0261*
X_1*X_1	-5.507	3.847	-1.43		0.2117
X_2	-3.577	2.614	-1.37		0.2295
X_3*X_3	-5.096	3.847	-1.32		0.2426
X_1*X_2	-4.727	3.696	-1.28		0.2571
X_3	2.646	2.614	1.01		0.3578
X_2*X_3	1.918	3.696	0.52		0.6260

$$+(X_2-2)\left[\left(\frac{X_3-5}{2}\right)(1.918)\right]+\left(\frac{X_1-10}{5}\right)\left[\left(\frac{X_1-10}{5}\right)(-5.507)\right]$$

According to the model equation, the amount of protein (BSA) incorporated or loaded into the process is the most important factor affecting the encapsulation efficiency of the microencapsulation process. Also, %EE is affected by the interaction between the amount of BSA loaded and the concentration of $CaCl_2$ used during the process as well as the quadratic effect of % alginate/HEC.

Optimization of encapsulation efficiency by Box Behnken methodology: The model terms for Y (encapsulation efficiency) were found to be significant with an F value of 0.0148 as shown in Table-4. High r^2 value of 0.9387 indicates the fitting of regression model.

TABLE-4
ANALYSIS OF VARIANCE. ANOVA SHOWS THAT THE MODEL IS CONSIDERED ADEQUATE WITHIN THE CONFIDENCE LIMIT

Source	DF	Sum of squares	Mean square	F ratio
Model	9	4181	464.6	8.502
Error	5	273.2	54.65	Prob > F
C. total	14	4455		0.0148*
Lack of fit	3	273.2	91.08	
Pure error	2	0.0000	0.0000	
Total error	5	273.2		
				Max RSq
				1.0000

The analysis of variance (ANOVA) technique was used to check the adequacy of the developed models at 95% confidence level. The criteria followed in this technique is that if the calculated value of the F-ratio of the regression model is more than the standard value specified (F-table) for 95% confidence level and then the model is considered adequate within the confidence limit. This is consequently presented in Fig. 3, where it is observed that the model satisfy the adequacy conditions in non-linear form. This also presents a high value of r^2 equivalent to around 94%.

Response surface plots: The purpose application of the response surface method, Box-Behnken for this matter, is to increase efficiency of encapsulation and to understand how this

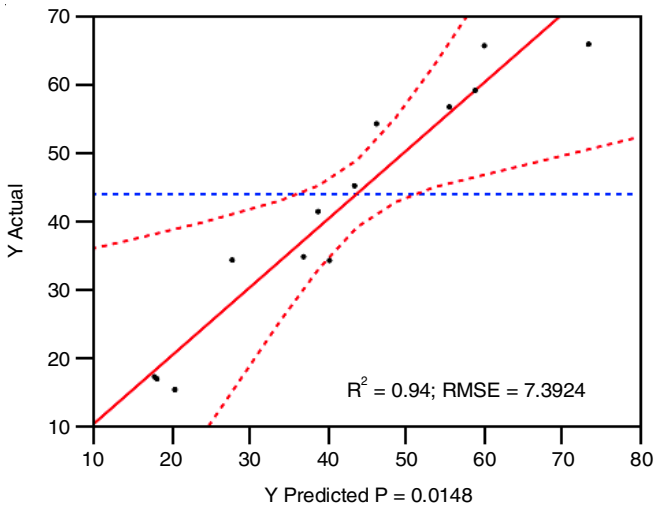

Fig. 3. Actual data by predicted encapsulation efficiency plot. Actual encapsulation efficiency data from the experimental design are plotted against the values of encapsulation efficiency derived from the regression model equation

response changes in a given direction by adjusting the design variables. In general, the response surface can be visualized graphically (Fig. 4). Regions where optimal conditions can be achieved are represented by the red color areas of 3D graphs.

Fig. 4.1a shows that encapsulation efficiency can be increased from 1 wt.% of alginate/HEC to around 2 wt.% only, while increasing the amount of BSA-loaded into the process. Fig. 4.1b shows encapsulation efficiency of greater than 55% can be achieved in this region while Fig. 4.1a predicts that it will likely be about 70%. Fig. 4.2a and 4.2b on the other hand show that the optimal region occurs % BSA loading is increased up to its limits, which corresponds with the decrease in value of $CaCl_2$. Fig. 4.2b shows that the optimal region can offer an encapsulation efficiency of greater than 55% while Fig. 4.2a narrows it down to in between 70-75%.

Finally, increase in encapsulation efficiency according to Fig. 4.3a lies in the middle region of the inverse saddle 3D relationship of %alginate/HEC and %$CaCl_2$. This result is accordance to the strong quadratic interaction of alginate/HEC with itself. Thus increasing its value as well as increasing the concentration of the crosslinking solution will guarantee an increase in this study's response. However, up to a certain point within

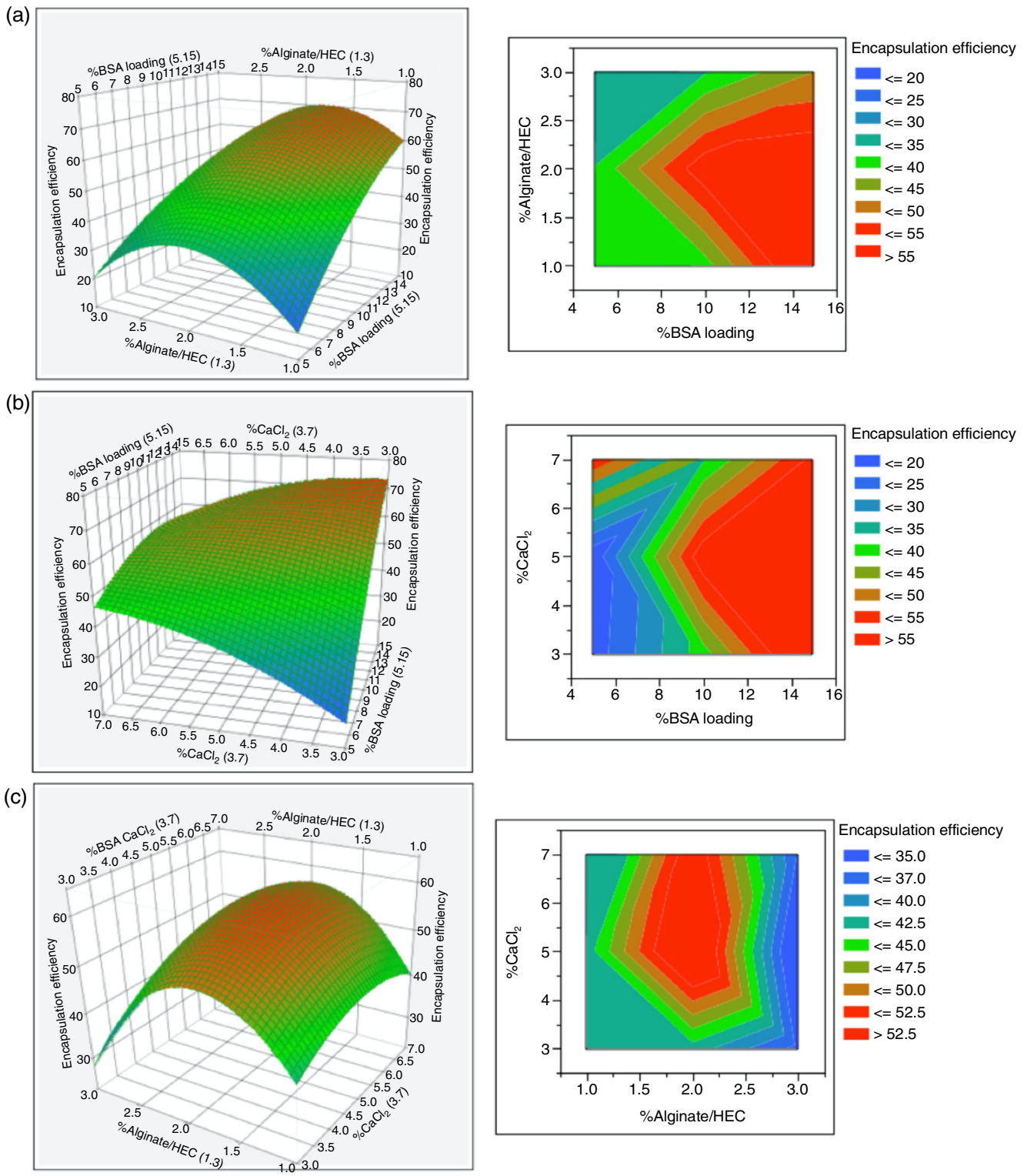

Fig. 4. Response surface plot and contour profile showing the effect of (a) alginate/HEC concentration and % protein loading; (b) crosslinking solution concentration and % protein loading; and (c) polymer/cellulose derivative concentration and crosslinking solution concentration on encapsulation efficiency. Red regions on both profiles represents the optimal areas wherein encapsulation efficiency can be maxed out

their limits, values of %EE will also decrease when %alginate/HEC and %$CaCl_2$ is also decreased. Both Figs. 3.3a and 3.3b suggested that at the optimum conditions with respect to these two factors, %EE would be in between 50% to 60%. It is observed that all regions where optimal values of factors can be achieved are in agreement with each other. Also, each of them corresponds to the interaction profiles presented above.

Optimization using the desirability approach: Desirabilities range from zero to one for any given response. The program combines individual desirabilities into a single number and then searches for the greatest overall desirability. A value of one represents the ideal case. A zero indicates that one or more responses fall outside desirable limits [17,18]. In this optimization study, the goal is to maximize the efficiency of encapsulation of the microencapsulation process and find out the values of the factors noted that will contribute to this goal. JMP (version 9.0) provides a prediction profiler for all factors/variables used in this study and sets it in the normal level (000) values as presented in Fig. 5.

At 10% BSA Loading, 2 wt.% alginate/HEC and 5wt% $CaCl_2$, the prediction profiler gives its %EE of about 58.98%. These values correspond to a desirability value of 0.8027. To find for the optimal conditions to achieve the highest %EE possible using the model equation presented earlier, desirability value should be increased to a value of 1, or more or less close to it. This process is shown in Fig. 6.

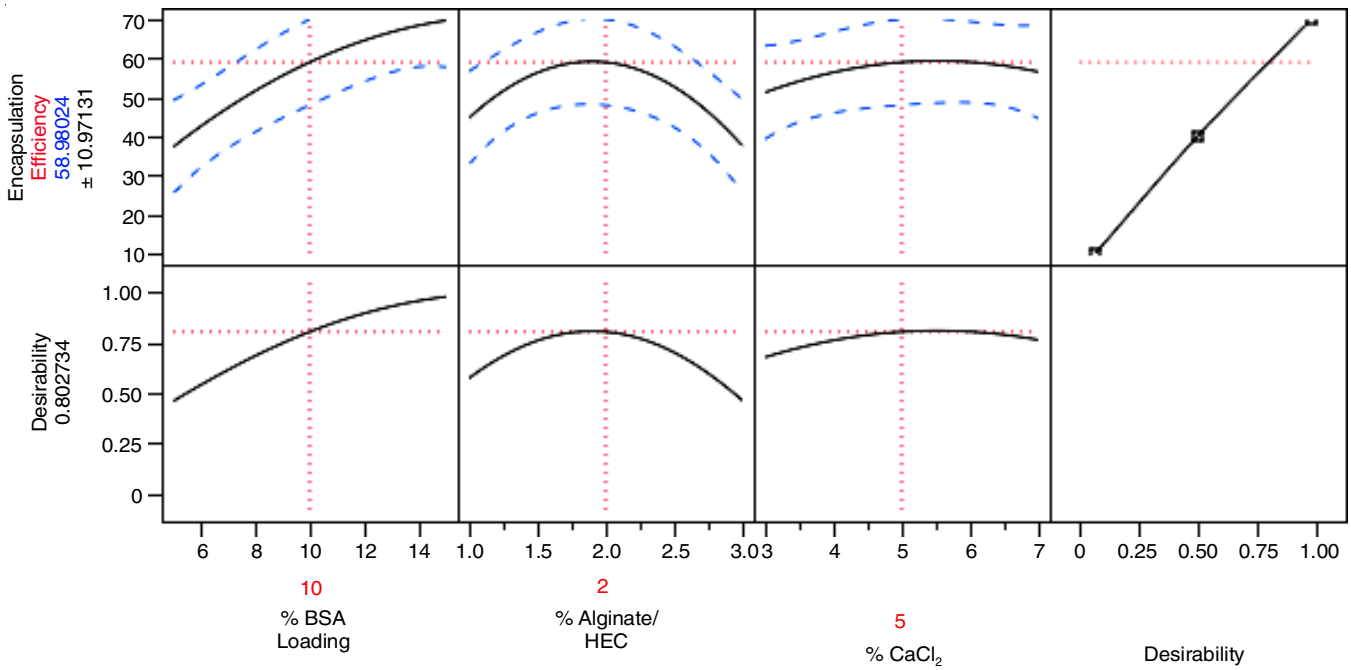

Fig. 5. Prediction profiles at standard conditions. These curves present the current encapsulation efficiency result using standard values for the three different factors used

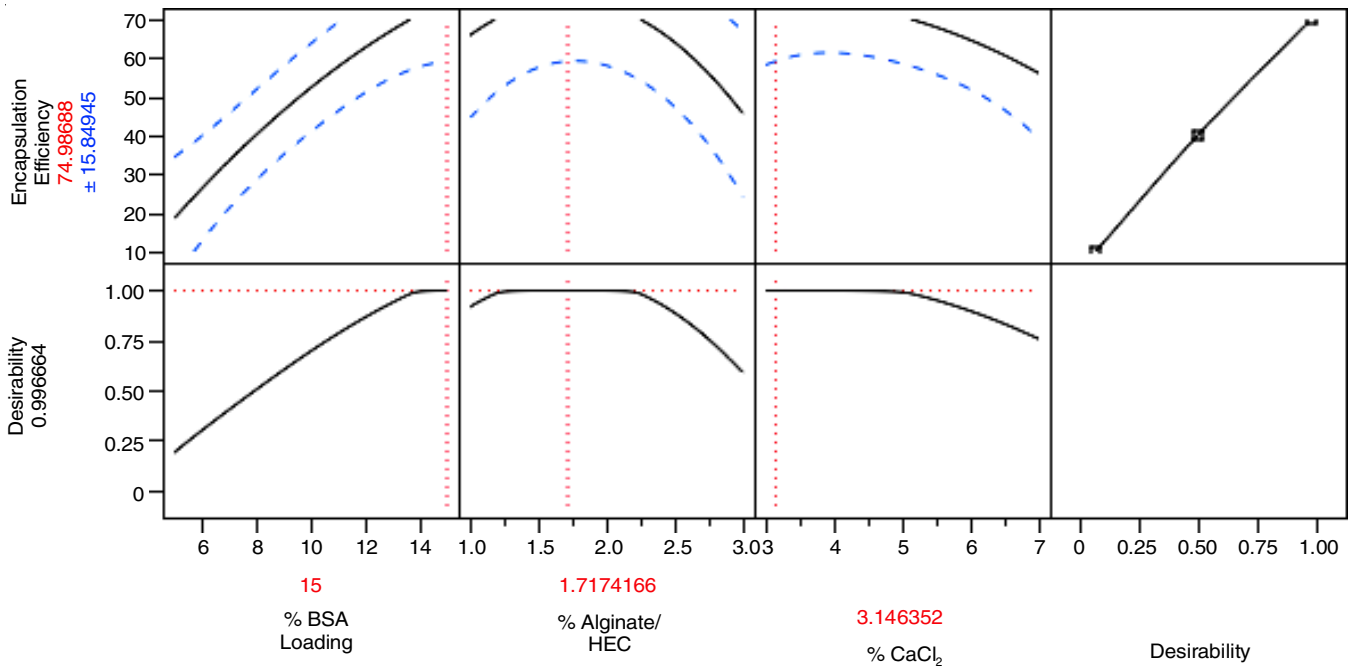

Fig. 6. Prediction profiles when desirability approaches 1.0. These curves present the predicted optimal value of encapsulation efficiency if values for the three different factors are optimized

Maximum desirability function value that can be achieve is about 0.9967. At this point, an encapsulation efficiency of about 75% is predicted to be achieved provided that the optimal conditions for the following factors must be met: % BSA loading is 15%, % alginate/HEC is around 2 wt.% and % $CaCl_2$ concentration is about 3 wt.%. Comparing this maximized theoretical value of encapsulating efficiency with the experimental value obtained from the optimized process conditions gives a percent error of about 12.32%.

Conclusion

Optimization was done with an objective of maximizing the response, which is basically the encapsulation efficiency of the microparticle fabricated using Box Behnken methodology. A desirability based approach was followed for analyzing the accuracy of optimization process. From this optimization calculation, it can be concluded that protein loading, polymer concentration, and crosslinking solution concentration all affects the efficiency of the encapsulation process. As a matter of fact that interaction plots showed that the process is greatly affected by how much protein drug is loaded into the system and the interactions between crosslinking solution concentration with protein loading, as well as the quadratic relationship of the alginate/HEC concentration with itself. Furthermore, the optimal conditions for the factors weighed were the following: protein loading of 15%, alginate/HEC concentration of about 2 wt.% and the crosslinking solution concentration of 3 wt.%.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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