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Design and Development of Biosurfactant induced Nanosponges using QBD approach. Part-I

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ABSTRACT

The objective of this research work was to formulate and develop a nanosponge delivery system comprised of the polymers viz. β- Cyclodextrin, Chitosan and Hyaluronic acid using Biosurfactant (Rhamnolipid). The prepared nanosponges were optimized by Plackett Burman design and Box Behnken Design. The optimized batch of the Blank Carboxymethyl β –Cyclodextrins Chitosan rhamnolipids coated with Hyaluronic acid Nanosponges (CCHBS/NS) had the particle size of 510±10 nm, PDI 0.22 ± 0.01 and Zeta Potential of - 30.8 ± 0.4mV. The optimized formulation was found to be stable for 6 months at real time stability conditions of 4°C±2°C and 25°C±2°C /60%±5% RH respectively..

Keywords: Nanosponges, Plackett Burman design, Box Behnken Design, Biosurfactant, carboxymethyl β-Cyclodextrin, Chitosan, QBD

INTRODUCTION

A recent study showed that bacterial biofilms are responsible for resistance to infection rather than planktonic bacteria [1]. Biofilm refers to any group of microorganisms in which cells bind to one another on the surface. These adherent cells are often enclosed inside an extracellular polymeric substance (EPS) self-produced matrix. In addition to the quorum sensing effect, bacterial biofilms show several distinct properties than plankton, such as morphology and phenotype [2]. Such properties play a significant role in the tolerance and resistance of bacteria to antimicrobial agents [3]. First, EPS, consisting of polysaccharides, proteins, lipids, and DNA, acts as a protective barrier to delay the dissemination of antimicrobial agents [4]; second, the nutritional limitation is confronted by bacteria in a biofilm and thus exists in a slow-growing or starving state that is not very responsive to antibiotics attacking the proliferation process [5]; Third, biofilms prevent being cleared by the host immune system [6,7]; finally, biofilms express unique defensive factors, such as multi-drug efflux pumps and stress response regulators [7], each of which is lethal to antimicrobial agents in the uncommon resistance of biofilms. Compared to their planktonic counterparts, antibacterial resistance to biofilm-growing bacteria can be up to 1000-fold [8]. Biosurfactants emerge as one potential alternative to antibiotics due to their environmentally friendly properties and mild production within some classes [9]. One of the most known biosurfactant classes comprising microorganism-secondary metabolites rhamnolipids (RL) is certainly glycolipid, consisting of a carbohydrate moiety attached

to a fatty acid. Rhamnolipids are glycolipid surfactants, primarily produced as mono-and di-rhamnolipids by various Pseudomonas aeruginosa or similar species [10]. Making membrane degradation and subsequent bacterial death, RL has antimicrobial properties against bacteria [10,11]. Such a biosurfactant may be a beneficial solution to enhancing the current strategies against bacterial-mediated biomaterial infections in order to demonstrate low toxicity, be environmentally safe, biodegradable, large-scale production, and existing antimicrobial properties [12]. In this study, we hypothesized that if this biosurfactant is encapsulated in a chitosan particulate system, RL antimicrobial activity against pathogenic bacteria could be enhanced. Biosurfactants incorporated in chitosan-based nanoparticles have therefore been prepared and their physicochemical properties measured.

EXPERIMENTAL WORK

Preparation of Nanosponges

Carboxymethyl β –Cyclodextrins, Chitosan, Hyaluronic acid were purchased from Sigma® Aldrich, Banglore. Rhamnolipid biosurfactant was isolated in house from actinolycetes. Accurately weigh Carboxymethyl β –Cyclodextrins, isolated Bio surfactant and Hyaluronic acid and dissolved them all in distilled water in separately. Weigh chitosan and dissolved in 1% acetic acid solution. Biosurfactant solution was added in Cyclodextrins solution and mix thoroughly. Add Cyclodextrin solution drop wise in Chitosan solution kept on magnetic stirrer for 4 hrs. CCBS nanoparticles were collected using centrifuge and resuspend in nanopure water. CCBS nanposponges suspension was added drop wise in Hyaluronic acid solution kept on magnetic stirrer. Mixture was kept on magnetic stirrer for 4 hrs followed by circulation of mixture for 2-4 cycles at 800 psi pressure through a high pressure homogenizer. After homogenization sample was further stirred for 24 hrs at room temperature for hardening.

Collection of CCHBS Nanosponges

The nanosponges were separated by two step centrifugation process using a refrigerated centrifuge. Nano sized particles were separated initially at a low spin of 500 rpm for 5 min, followed by centrifugation of obtained supernatant containing nanoparticles at 20,000 rpm for 30 min. The supernatant was discarded and pellet was freeze dried.

Particle size and Particle size index analysis.

The average particle size and size distribution of CCHBS nanosponges was measured by dynamic light scattering technique using a Malvern particle size analyzer by photon cross-correlation spectrometry. The measurement was done by using laser light scattering which was monitored at a scattering angle of 90^0 at wavelength of 635 nm [13]. The measurements were repeated three times and average was taken. The nanoparticle sample was diluted in distilled water and sonicated gently for about 3-5 minutes in a bath-type sonicator and the dispersion thus obtained was analyzed for particle size by loading into 1 cm^2 cuvettes in a thermostatic chamber at 25^0C. The size distribution obtained is by plotting the relative intensity of light scattered by particles in various size classes and is therefore known as an intensity size distribution. The particle size distribution is exhibited in terms of span value, which is obtained by using formula,

$$\textit{poly dispersity}\ \textbf{index} = \frac{\mathbf{D\ 0.9 - D\ (0.1)}}{\mathbf{D\ (0.5)}}$$

Where,

D (0.9) corresponds to particle size immediately above 90% of the sample

D (0.5) corresponds to particle size immediately above 50% of the sample.

D (0.1) corresponds to particle size immediately above 10 % of the sample.

Zeta potential

Zeta potential is an indirect measurement of the thickness of the diffusion layer and is used to predict long-term stability. The particle electrophoresis movement was measured using the laser Doppler electrophoresis technique. Zeta potential was measured using Malvern Zetasizer [13]. All measurement were carried out at 25^0C using disposable plain folded capillary zeta cells. The measurements were conducted in triplicate after appropriate dilution of freshly prepared particles in double-distilled water to obtain the optimum kilo counts per second of 50–200 for measurement.

Design Space

For construction of process design space (a key element of Quality by design (QbD)), it is imperative to identify the Critical process parameters (CPPs) causing

variabilities in the Critical Quality Attributes (CQAs) and this is possible through thorough understanding of the process so that the variations can be minimized by controlling the CPPs. This is possible by prior knowledge and risk assessment of all the CPPs and critical material attributes (CMAs) which have the potential of hampering the quality of the product. This characterization step is fundamental for the understanding of the impact of the most important variables on overall process performance, because typically not all variables can be analyzed in great detail. Finally, these studies can be used to define the acceptable variability in material attributes and process parameters. Operating within these multivariate ranges (Process Design Space) provides the assurance of quality. Based on the design space and process understanding a control strategy is developed. The CQA are categorized into high, medium and low risk parameters based on knowledge space. Usually high-risk parameters are considered important for Design of Experiments as they are having more effect than others and need to be in accepting multivariate ranges [14].

Preliminary investigation of critical variables

Preliminary optimization and screening for various process and formulation variables was carried out using Plackett- Burman Design. An 8-factor Plackett-Burman design (PBD) at 2 levels was used for initial screening of the main effects of independent variables (Table 1) on particle size, zeta potential and PDI. The lower and upper levels of the selected variables were selected on the basis references [15].

Table 1 a: Independent factors level for screening experiments.

Factors	Code	Levels	
		Low (-1)	High (+1)
β –Cyclodextrins concentration	A	2	6
Chitosan Concentration	B	2	6
BioSurfactant Concentration	C	2	4
Hyaluronic acid concentration	D	2.5	6.5
Homogenization pressure	E	100 mTorr	750 mTorr
pH	F	5.5	7.4
Temperature	G	25 ^{0}C	40 ^{0}C
Hardening Time	K	6 hr	24 hr

Table 1 b: Plackett-Burman screening design output generated using Minitab 17.

Batch code	Carboxymethyl β –Cyclodextrin conc.	Chitosan Conc.	Biosurfactant Conc.	Hyaluronic acid conc.	Homogenization pressure	pH	Temp.	Hardening Time
PB1	6	6	2	6.5	100	5.5	25	24
PB2	6	2	2	2.5	750	7.4	40	6
PB3	6	2	4	6.5	100	7.4	25	6
PB4	2	6	2	2.5	100	7.4	40	24
PB5	2	2	4	6.5	750	5.5	40	24
PB6	2	2	2	2.5	100	5.5	25	6
PB7	2	2	2	6.5	750	7.4	25	24
PB8	6	2	4	2.5	100	5.5	40	24
PB9	2	6	4	6.5	100	7.4	40	6
PB10	2	6	4	2.5	750	5.5	25	6
PB11	6	6	2	6.5	750	5.5	40	6
PB12	6	6	4	2.5	750	7.4	25	24

Optimisation of process using Box-Behnken (BB) design method

Conventional pharmaceutical formulation development is a time consuming task. DoE, however is a cost-effective and efficient method of accessing the critical factors in the development process. For this screening design, the variables were chosen based on the RA [16]. Box Behnken takes into consideration the various variables simultaneously and is one of the common methods to study the relationship between the parameters and their effect on the desired dependent attributes. BB gives the minimum number of experimental trials with a high level of accuracy. The factors identified after RA were evaluated for effect on response variables. Independent factors were the concentration of HA (X1, lower limit: 2.5 mg/ml, upper limit 6.5 mg/ml), concentration ratio of CS: β-CD (X2, lower limit: 1:2, upper limit 1:3 mg /ml) and Concentration of Biosurfactant (X3, lower limit: 2 mg/ml, upper limit 4 mg/ml). A total of 17 runs involving 3 dependent variables (Y1= Particle Size, Y2= Polydispersity Index (PDI), Y3= Zeta Potential) were obtained using Design Expert® 11 software and ANOVA was applied to analyze the statistical significance of each variable ($p < 0.05$).

Table 2 a.: High and low value of independent factors used for the optimization experiment

Factors		-1	+1	Responses	
X1	concentration of HA	2.5 mg/ml	6.5 mg/ml	Y1	Particle Size
X2	concentration ratio of CS: β-CD	6 (2:4) mg/ml	10 (2:6) mg/ml	Y2	Polydispersity Index (PDI)
X3	Concentration of BS	2 mg/ml	4 mg/ml	Y3	Zeta Potential

Table 2b: Box –Bhenken design output generated using Design Expert vs. 11.

Batch code	Factor 1 A:HA mg/ml	Factor 2 B:CD:CS mg/ml	Factor 3 C:BS mg/ml
BB01	4.5	8	4.5
BB02	2.5	8	6
BB03	4.5	6	6
BB04	4.5	8	4.5
BB05	4.5	10	3
BB06	4.5	10	6
BB07	2.5	6	4.5
BB08	6.5	8	6
BB09	6.5	8	3
BB10	2.5	10	4.5
BB11	4.5	8	4.5
BB12	6.5	6	4.5
BB13	4.5	6	3
BB14	6.5	10	4.5
BB15	4.5	8	4.5
BB16	4.5	8	4.5
BB17	2.5	8	3

Stability Studies

The stability Studies of blank Chitosan Gellan Gum Nanoparticles were carried out by storing the optimized batch in a stability chamber at 25±2^{0}C/ 60 ±5% RH for a period of 6 months**.**

RESULT AND DISCUSSION

Preparation and physiochemical properties of CCHBS Nanosponges

CCHBS nanoparticles were formed on the basis of electrostatic interactions. As per the procedure described in this chapter, the addition of the polymers is important as, Carboxymethyl β –Cyclodextrins, biosurfactant (RLs) and hyaluronic acid are negatively charged polymers whereas chitosan is positively charged one. Biosurfactant when mixed with, Carboxymethyl β –Cyclodextrins they mixed but no interaction was taking place as both contains free carboxyl group. When mixture of Biosurfactant and Carboxymethyl β –Cyclodextrins was added in chitosan solution, biosurfactant and Carboxymethyl β –Cyclodextrins made polyelectrolyte complex (PEC) with chitosan as PEC is strong enough we didn't add any crosslinker. It is possible that biosurfactant also worked as to decrease surface tension which help in reducing the size of the nanoparticles. Chitosan and biosurfactant itself capable of forming nanoparticles but still Carboxymethyl β –Cyclodextrins was added intentionally as cyclodextrin structure is bucket like and can carry number of drug molecules. This property of cyclodextrin is used to entrapment number of different drug molecules together in a system. Once the internal structure of nanosponges formed it was coated with Hyaluronic acid. Hyaluronic acid used in this procedure because it contains CD44 like structure. Because of the structural properties of Hyaluronic acid nanosponges can easily be attached with CD44 receptor, which make nanosponges more site specific. Only limitation of the present method is that, it initially formed large nanosponges. To overcome this, nanosponges passed from the high pressure homogenizer to reduce size as well as it also increase the PDI.

Screening design

PB design was applied as a screening method for identifying the most influencing significant factors. Prediction of the main effect of formulation and process parameters on the responses is a crucial requirement in the development of nanosponges formulation. Eight factors that may affect the experimental responses were selected as independent variables at two levels for the study as shown in Table 3 shows the outline and observed responses of PB formulation (PBF) on two levels. Polynomial equations for individual responses reflect the relationship between dependent and independent factors. The effect of independent factors on nanoparticle characteristic are shown below using Pareto charts and Normal Charts.

Table 3: Response of Plackett-Burman screening design.

Batch code	Particle size	PDI	Zeta potential
PB1	558	0.19	-28.2
PB2	430	0.22	-22.3
PB3	484	0.19	-32.8
PB4	520	0.17	-21.2
PB5	474	0.25	-30.0
PB6	452	0.17	-21.5
PB7	484	0.24	-29.7
PB8	434	0.18	-26.9
PB9	556	0.19	-32.1
PB10	524	0.23	-27.5
PB11	548	0.25	-31.6
PB12	521	0.23	-26.9

Particle size

Particle size can play an important role as it can instantly influence the physical stability, cellular uptake, biodistribution and the drug release. Depending on the desired administration route, the size of the particles should be optimized. The mean particle size was in the range of 430- 558 nm and it was strongly affected by the some of the selected variables.

The fitted model describing the influence of variables on the mean of particle size is,

Particle size = 406.48 - 1.458 β –Cyclodextrins + 19.542 Chitosan Concentration
+ 0.083 BioSurfactant Concentration
+ 9.292 Hyaluronic acid concentration
- 0.00590 Homogenization pressure + 0.439 pH
- 0.6778 Temperature - 0.0278 Hardening Time.

The obtained F-value of the model had a significant effect. The significance of F-value and R2 (0.9960) indicated that there was a good linearity between the predicted and the observed values. The predicted R2 (0.9973) and adjusted R2 (0.9882) values implied a good correlation between the obtained and predicted value and those of the fitted models. Pareto chart helps to prioritize main affecting variables amongst all selected variables. It indicates that any effects that extend beyond the reference line are considered as significant. Here the pareto chart shows that polymer concentrations and temperature were found to significantly affect the particle size. Normal plot indicates that chitosan and hyaluronic acid concentration has

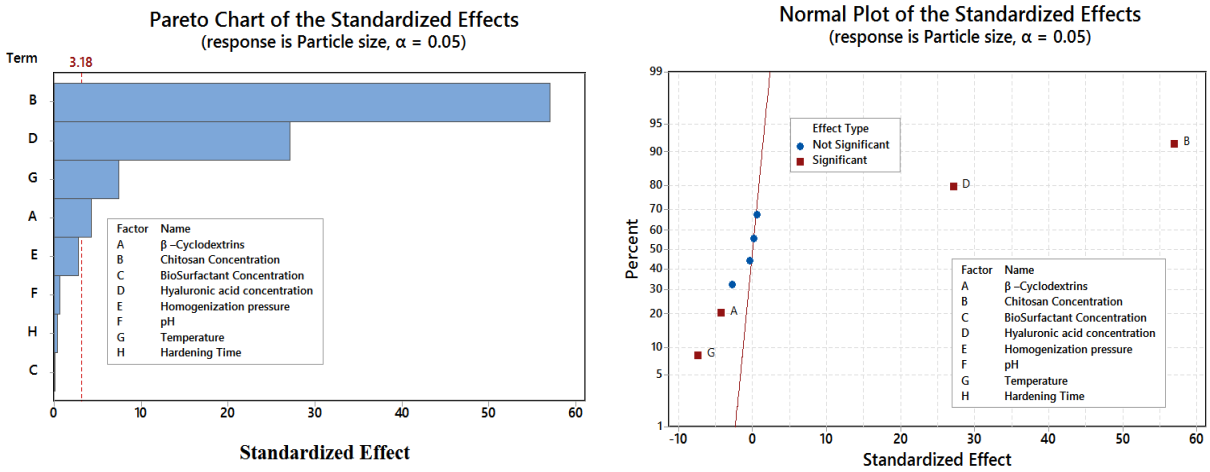


Fig 1: Pareto chart and Normal plot of independent variables for the response of particle size.

positive whereas temperature and Carboxymethyl β –Cyclodextrins has negative effect on particle size. From the result it was evident that chitosan concentration has more effect on particle size then temperature were found to significantly affect the particle size. Normal plot indicates that chitosan and hyaluronic acid concentration. This may be attributed to molecular weight of the chitosan i.e. 50-2000kDa leading to increase in size of nanoparticles. Interesting part of the study is that, Carboxymethyl β –Cyclodextrins decreased size of the nanoparticles. This may be due to molecular weight of cyclodextrin ie. 6000-8000 Da. When concentration of cyclodextrin was increased it replaced chitosan and hence, particle size decreased comparatively to nanosponge containing low concentration of cyclodextrin. Theoretically, surfactant must have pronounced effect on the particle size but here in the present study biosurfactant shows no significant effect on the particle size.

Zeta potential

The fitted model describing the influence of variables on the mean of Zeta potential is,

Zeta potential = -13.93 - 0.208 β –Cyclodextrins - 0.208 Chitosan Concentration
- 1.750 BioSurfactant Concentration- 1.625 Hyaluronic a cid concentration- 0.00128 Homogenization pressure+ 0. 088 pH+ 0.0111 Temperature + 0.0463 Hardening Time.

The obtained F-value of the model had a significant effect. The significance of F-value and R2 (0.9880) indicated that there was a good linearity between the predicted and the observed values. The predicted R2 (0.9554) and adjusted R2 (0.9756) values implied a good correlation between the obtained and predicted value and those of the fitted models.

Result of PB study suggested that zeta potential is influenced by concentration of hyaluronic acid and biosurfactant, both shows positive effect on the zeta potential.

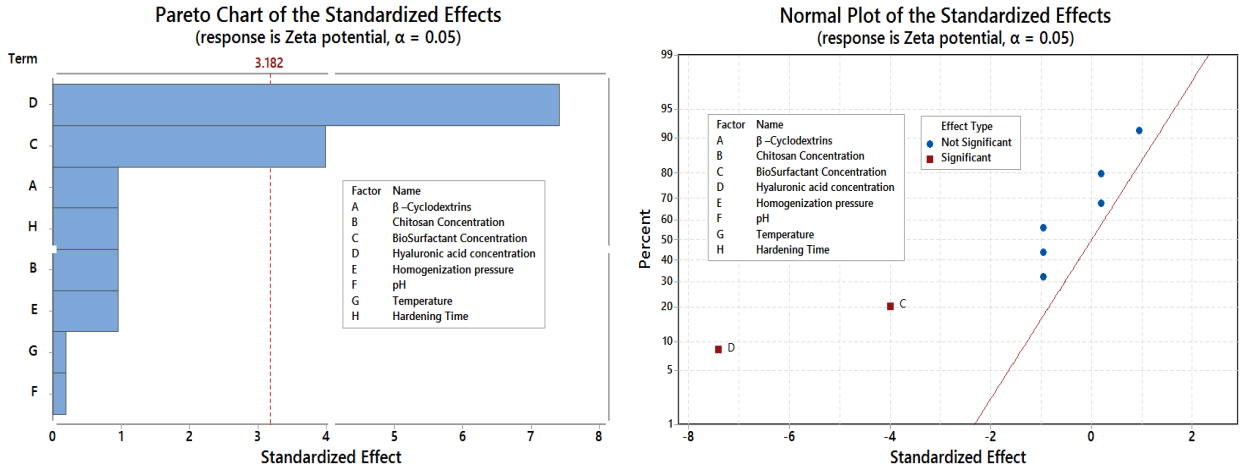


Fig 2: Pareto chart and Normal plot of independent variables for the response of Zetapotential.

Poly dispersibility index (PDI)

The fabricated nanoparticles, size population commonly follows a multimodal distribution. The polydispersity is a significant parameter, which can provide the information about the homogeneity size distribution and it suggest the particles are monodispersity. The effect of variables on the PDI of the particle size and it should be below 0.3. The below 0.3 of polydispersity index shows narrow could be explained by following linear model equation,

PDI = 0.15372 + 0.000417 β –Cyclodextrins
+ 0.000417 Chitosan Concentration
+ 0.002500 BioSurfactant Concentration
+ 0.004583 Hyaluronic acid concentration
+ 0.000085 Homogenization pressure - 0.002632 pH
+ 0.000111 Temperature + 0.000093 Hardening Time

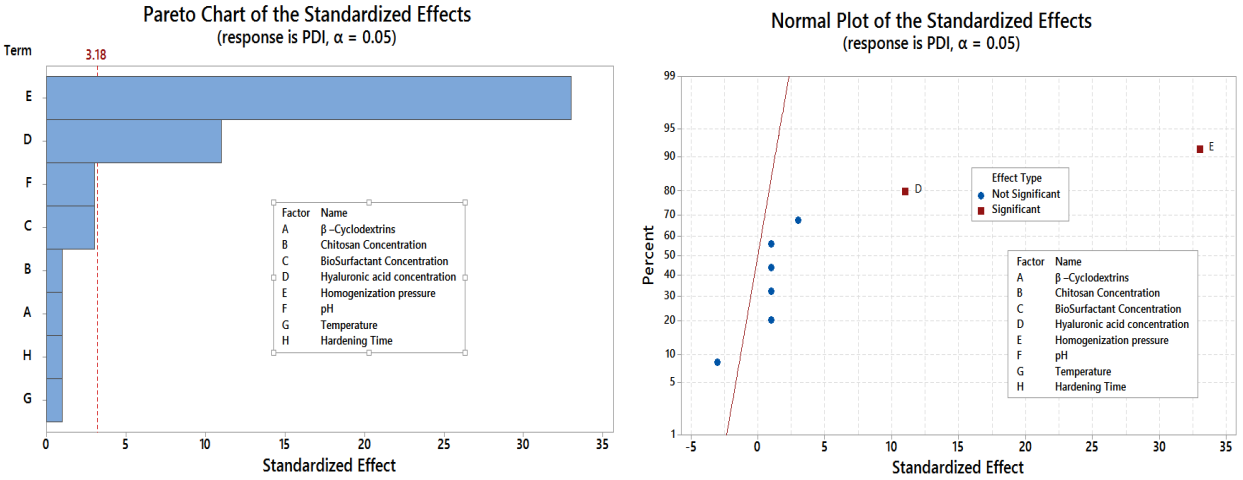


Fig 3: Pareto chart and Normal plot of independent variables for the response of PDI.

Optimization of Nanoparticles

In the current study, particle size, PDI, Zeta Potential were the considered as quality attributes which is the first step of QbD *ie*. determination of CQAs. Several other parameters like polymer concentration, temperature, homogenizer pressure and duration considerably affect the response variables. When these variables were linked with the CQAs, we obtained a design space for the effective formulation design. Hence, the optimization of these variables will be done by Box- Behnken design.

Box- Behnken Design

The preliminary studies revealed that polymers concentration and biosurfactant concentration were the significant factors. Hence, Box- Behnken Design was applied to understand their effect on particle size, PDI and

Zeta Potential. A statistical design was utilized in order to derive the relationship between the response variables and independent variables. Table 4 shows the independent factors and response values of respective batches. The statistical evaluation of the results was carried out by design expert software.

Table 4: Response obtained for coded units of Box Behnken design

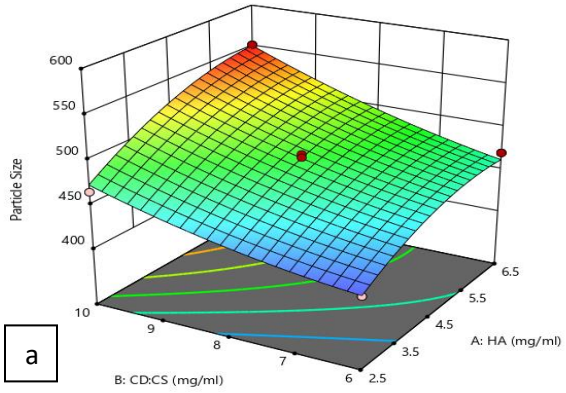
Formulation Code	Particle size	PDI	Zeta potential
B01	488	0.24	-27.6
B02	430	0.16	-26.5
B03	465	0.18	-28.6
B04	482	0.24	-26.2
B05	540	0.22	-26.8
B06	504	0.21	-28.7
B07	415	0.17	-24.6
B08	478	0.24	-31.2
B09	492	0.24	-30.9
B10	465	0.18	-24.5
B11	480	0.22	-26.9
B12	470	0.19	-31.7
B13	418	0.17	-26.4
B14	552	0.23	-31.9
B15	484	0.24	-27.8
B16	483	0.24	-26.4
B17	434	0.18	-24.3

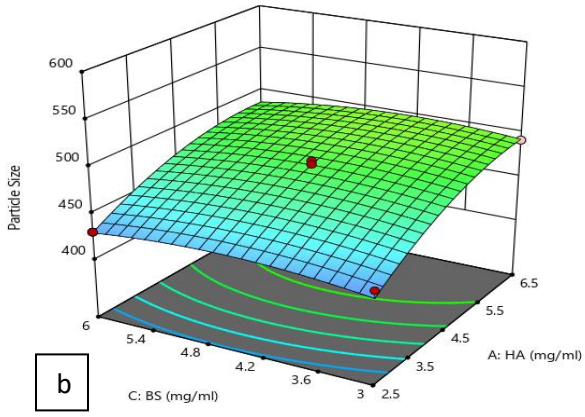
Particle Size

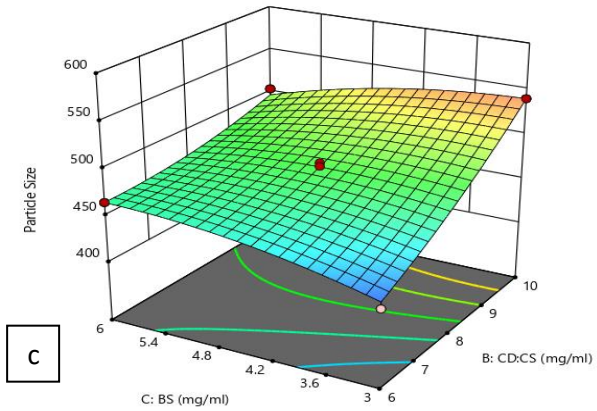
Mathematical Model for Particle Size

Particle Size = 35.92656 +38.29375HA +9.73750CD:CS +95.80000BS +2.00000 HA * CD:CS -0.833333 HA * BS -6.91667 CD:CS * BS -3.89375 HA^2 +1.91875 $CD:CS^2$ -4.14444 BS^2

From the charts it is evident that Carboxymethyl β – Cyclodextrins and chitosan ratio was positively affect the particle size, when ratio increased the particle size also increased and vice versa. Same result was seen with Hyaluronic acid.







Hyaluronic acid and β-Cyclodextrin-Chitosan, Biosurfactant kept fixed at 4.5 (b) hyaluronic acid and Biosurfactant, β-Cyclodextrin-Chitosan kept fixed at 8 (c) β-Cyclodextrin-Chitosan and Biosurfactant, hyaluronic acid kept fixed at 4.5.

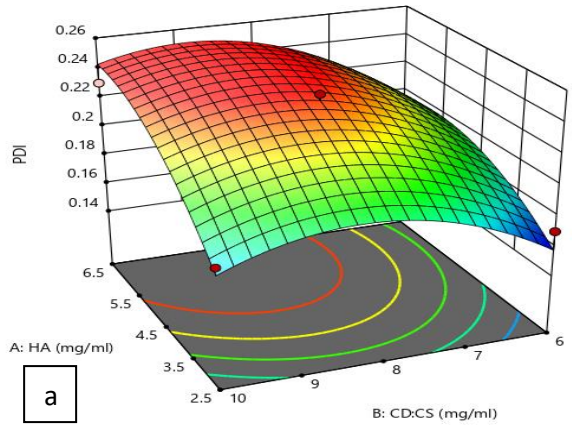
Fig 4: 3D Surface Contour Interaction plot and its effect on particle size.

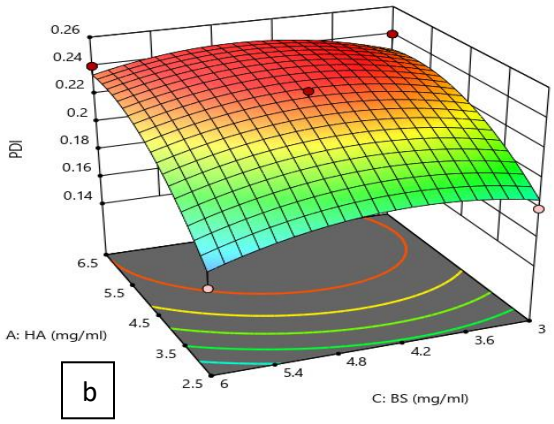
PDI

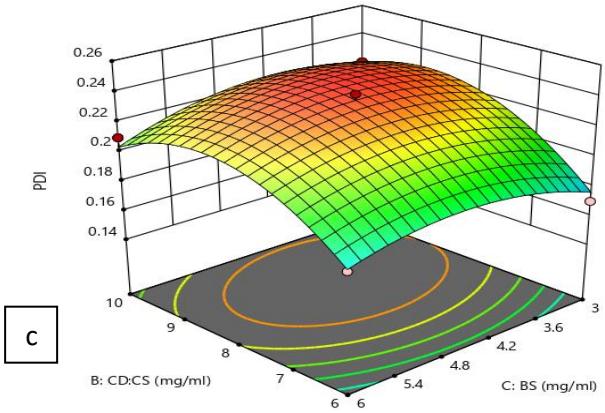
Mathematical Model for PDI

PDI= -0.480359 +0.028313HA +0.114187CD:CS +0.061167BS +0.001875HA * CD:CS +0.001667HA * BS -0.001667CD:CS * BS -0.004187HA² -0.006687CD:CS² -0.006333BS²

Results reveals that PDI was in range and no factors shows any significant effect on PDI. But still Hyaluronic acid pronounces more effect on PDI in comparison to other factors. PDI effect was seem to be silent may be because of homogenization step and as nanosponges were coat separately with hyaluronic acid, hyaluronic acid has greater effect than any other factors.







(a) Hyaluronic acid and β-Cyclodextrin-Chitosan, Biosurfactant kept fixed at 4.5 (b) hyaluronic acid and Biosurfactant, β-Cyclodextrin-Chitosan kept fixed at 8 (c) β-Cyclodextrin-Chitosan and Biosurfactant, hyaluronic acid kept fixed at 4.5.

Fig 5: 3D Surface Contour Interaction plot and its effect on PDI.

Zeta Potential

Mathematical Model for Zeta Potential

Zetapotential = -25.94859 -0.155625 HA +1.12437 CD:CS -0.072500 BS -0.018750 HA * CD:CS +0.158333 HA * BS +0.025000 CD:CS * BS -0.224375 HA^2 -0.074375 $CD:CS^2$ -0.154444BS^2

It can be seen that the Zeta Potential increases with an increase in Hyaluronic acid concentration. While biosurfacant and Cyclodextrin-Chitosan has no effect on the zeta potential. Results clearly indicated that our nanosponges were fully coated with the hyaluronic acid. And biosurfactant, cyclodextrin and chitosan was present in the core.

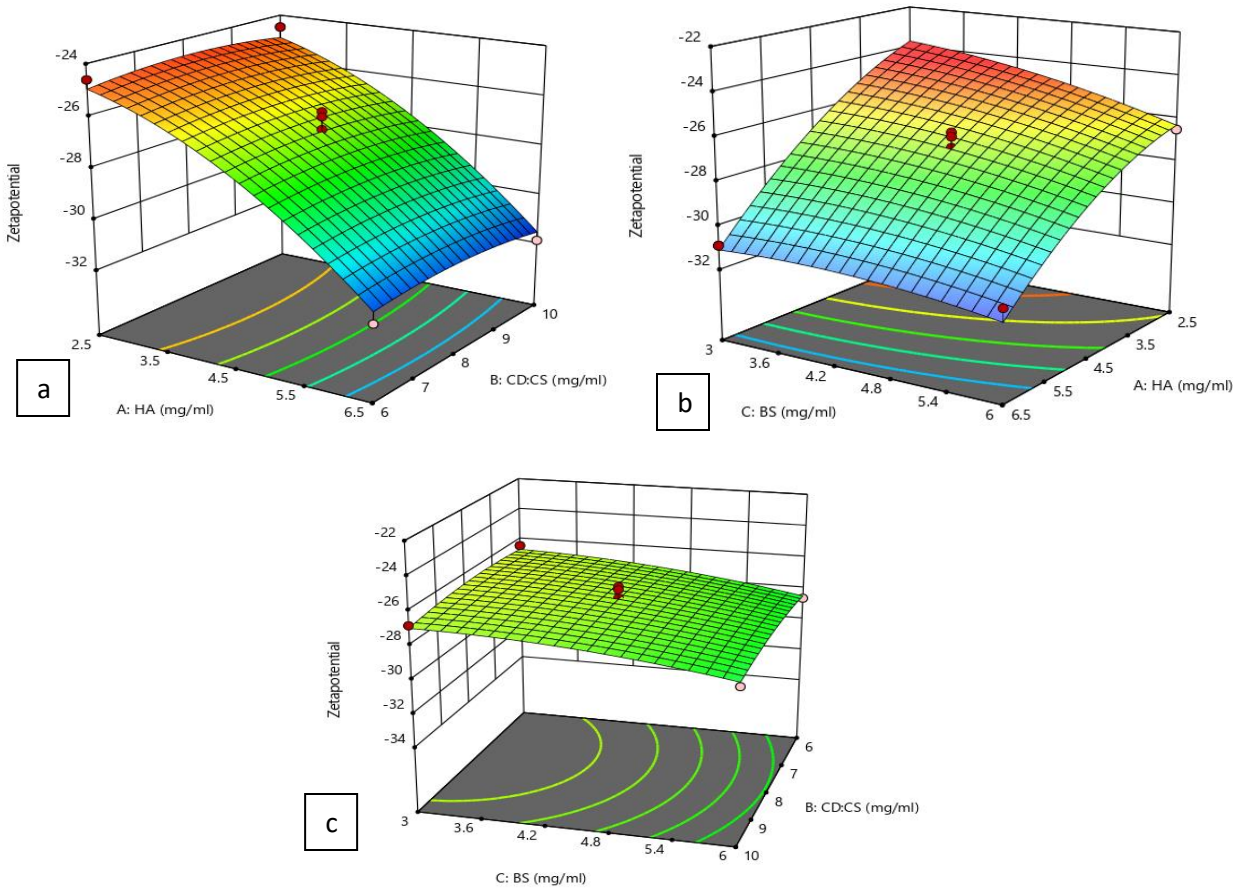
Validation by Check Point Batch Analysis

On the basis of reality, a check point batch was selected and evaluated for average Particle size, PDI and zetapotential.

Check point batch analysis was carried out to validate the optimization process. It provides the predicted values of the response with the optimized values of the variables. These check point batches were prepared and compared with the predicted values of the responses. From the table, it was clear that the obtained values were similar to the predicted values. Therefore, it can be predicted that there was no significant difference between the predicted and observed values (p value >0.05). Hence, the applied Box-Behnken design is validated.

Table 5: Check Point Batch Composition

Check Point Batch	Composition
Hyaluronic acid	5 µg/ml
CD:CS Concentration	9 µg/ml
Biosurfactant	4.5 µg/ml



(a) Hyaluronic acid and β-Cyclodextrin-Chitosan, Biosurfactant kept fixed at 4.5 (b) hyaluronic acid and Biosurfactant, β-Cyclodextrin-Chitosan kept fixed at 8 (c) β-Cyclodextrin-Chitosan and Biosurfactant, hyaluronic acid kept fixed at 4.5.

Fig 6: 3D Surface Contour Interaction plot and its effect on zeta potential

Table 6: Actual and Predicted Values for Check point Batches

Check Point Batch Code	Average Particle size (nm)		PDI		Zeta Potential (mV)	
	Actual Value	Predicted Value	Actual Value	Predicted Value	Actual Value	Predicted Value
CP1	504	489.68	0.22	0.24	32	28
CP2	512		0.22		31	
CP3	506		0.23		31	

Validation by Numerical Optimization – Desirability Plot

Desirability is an objective function that ranges from zero outside of the limits to one at the goal. The numerical optimization finds a point that maximizes the desirability function. The characteristics of a goal may be altered by adjusting the weight or importance. For several responses and factors, all goals get combined into one desirability function.

The value is completely dependent on how closely the lower and upper limits are set relative to the actual optimum. The goal of optimization is to find a good set of conditions that will meet all the goals, not to get to a desirability value of 1.0. Desirability is simply a mathematical method to find the optimum. Here we obtain the desirability of 0.893 which indicates that our optimized batch is closer to 1.

Overlay Plot is obtained by superimposing the contour plots of responses of independent variables. It displays the

areas of feasible response values of the variables in the design space. Regions that don't fit into the optimization criteria look grey in colour while design space is coloured yellow. Overlay plot shows that our check point batch is in the design space as well as actual observation is in the range of the predicted value.

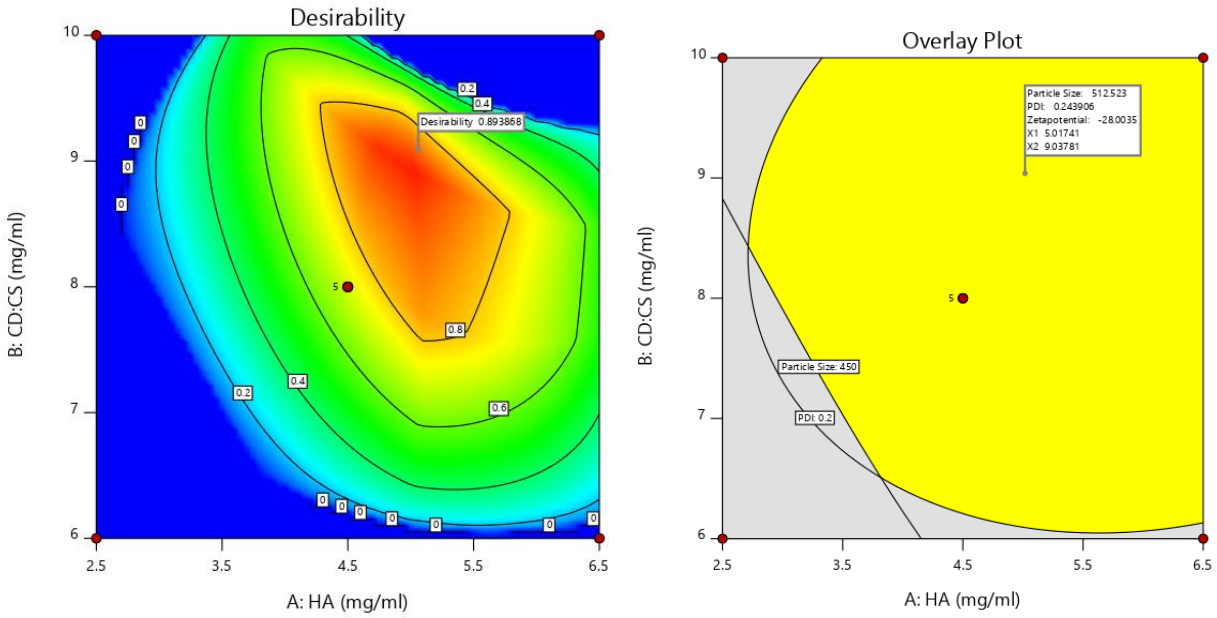


Fig 7 : Desirability and Overlay plot showing the design space for optimized batches

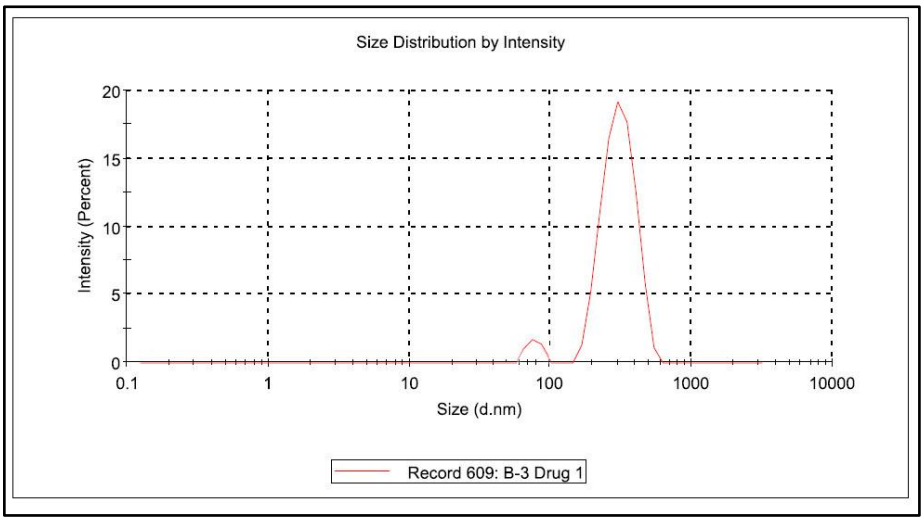


Fig 8 : Optimized Batch Particle size

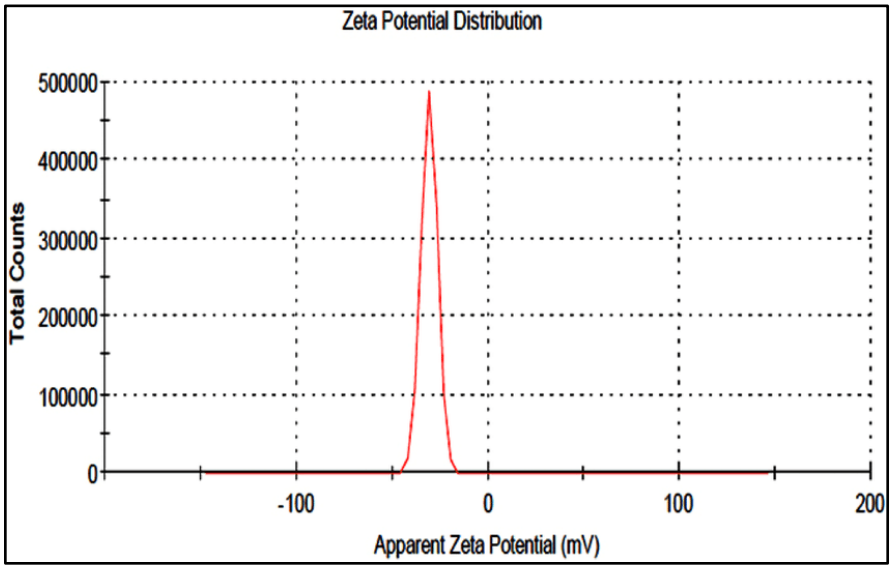


Fig 9: Optimized Batch- Zeta Potential

Stability study

The optimized formulation was evaluated for the change in particles size, dispersibility and physical appearance at 0,1,3,6 months. The particle size was found to slighty increase may be due to the aggregation of nanoparticles. But they were easily redispersed after reconstitution. No significant difference was observed in the appearance of the nanosponges.

Table 7: stability study data for optimized nanosponge batch.

Sr no.	Parameters	0 month	1 month	3 month	6 month
1	Physical Appearance	White/ off white	White/ off white	White/ off white	White/ off white
2	Avg Particle Size	508	521	530	554
3	Dispersibility	+	+	+	+

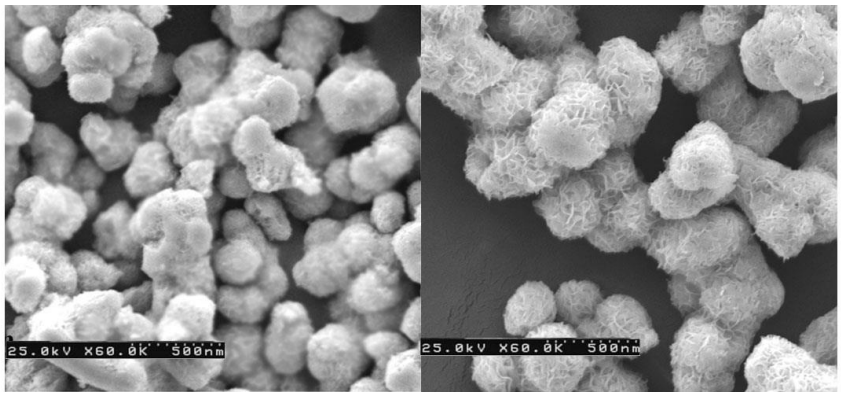


Fig 10: SEM of optimized Batch nanosponges.

CONCLUSION

In the present study, various process and formulation variables were successfully evaluated and screened using Placket-Burman design. The polymer concentration and biosurfactant were the most critical factors which affects particle size and zetapotential. Box- Behnken design gave the idea for the design space, which can be used to produce optimized batch. The subsequent experimentation can be performed using these critical parameters and design space for the formulation design of the drug loaded nanosponges.

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