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Research paper

Design for optimization of nanoparticles integrating biomaterials for orally dosed insulin

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ABSTRACT

Design of nanoparticles integrating biomaterials that govern the functional behavior of orally dosed insulin is focused on improving insulin stability and absorption by facilitating its uptake and translocation throughout the intestinal membrane, while providing protection from acidic and enzymatic degradation in the gastrointestinal tract. The purpose of the study was to optimize a nanoparticle formulation by investigating the relationship between design factors and experimental data by response surface methodology. Designed nanoparticles consisting of calcium crosslinked alginate, dextran sulfate, poloxamer 188 and chitosan followed by an outermost coating of albumin are described as multilayer complex retaining insulin within the nanoparticle. A 3-factor 3-level Box–Behnken design was used to optimize nanoparticle formulation. The screened independent variables were the concentration of calcium chloride, chitosan and albumin, and the dependent variables were particle size, polydispersity index, zeta potential, entrapment efficiency and insulin release in enzyme-free simulated digestive fluids. Experimental responses of a total of 15 formulations resulted in mean nanoparticle diameters ranging from 394 to 588 nm, with polydispersity index from 0.77 to 1.10, zeta potential values ranging from −36.6 to −44.5 mV, and entrapment efficiency of insulin was over 85%. Insulin release from nanoparticles in enzyme-free digestive fluids was prevented during 120 min in gastric conditions, and over 80% of insulin was released after 180 min in simulated intestinal fluid. Based on the experimental responses and the criteria of desirability defined by constraints, solutions of 0.20% calcium chloride, 0.04% chitosan and 0.47% albumin constitute the optimum formulation of nanoparticles for orally dosed insulin.

1. Introduction

Nanoparticles are under consideration for oral delivery of proteins like insulin by stabilizing, by ensuring biological activity during transit through the gastrointestinal tract and by facilitating absorption and delivery to the target site [1–3]. Insulin absorption from the gastrointestinal tract into the portal vein would mimic the physiological route of insulin undergoing first hepatic bypass. Compared to parenteral delivery, less insulin would be necessary to obtain a therapeutic effect, and consequently secondary effects due to high insulinaemia in extrahepatic tissues would be minimized [4].

Design of nanoparticles involves interconnected processes toward developing and optimizing nanoparticle formulation based on physicochemical, pharmacological and physiological parameters [5]. Considering that the design of nanoparticles for orally dosed insulin is a promising approach for treatment of diabetes mellitus, attempts include the use of biomaterials that have demonstrated favorable characteristics for protein entrapment, absorption across the intestinal membrane and delivery to the target site [2,6,7]. Nanoparticles formulated by ionotropic pregelation are described as a multilayer complex with insulin protected and retained within the nanoparticle with outermost coat consisting of a protease-protective protein. The particle nucleus consists of alginate, dextran sulfate and insulin following complexation with chitosan and poloxamer 188, and coated with bovine serum albumin.

Alginate consists of anionic chains of (1,4)-linked β-D-mannuronic acid and α-L-guluronic acid with mucoadhesive, biodegradable and biocompatible properties, and forms stable and reversible hydrogel in the presence of multivalent cations due to intramolecular and intermolecular crosslinking of polymer chains [8,9]. Chitosan is an unbranched polyamine of D-glucosamine and *N*-acetyl glucosamine with mucoadhesive, biodegradable and biocompatible properties, which stabilizes the alginate hydrogels for the formation of nanoparticles, reduces transepithelial electrical resistance and transiently opens tight junctions between epithelial cells to enhance insulin absorption via paracellular pathway

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[10,11]. Chitosan is important to reinforce the fragile alginate nucleus [12], and is used for incorporation of other polyanionic polymers to modulate insulin entrapment, stability, retention and release in the gastrointestinal tract [2]. Additional incorporation of a polyanionic polymer such as dextran sulfate can improve insulin entrapment efficiency and modulate insulin release from nanoparticles due to enhancement of electrostatic interactions between reinforced polyanionic-based nucleus and insulin [13,14].

Stabilizers are important to modulate the structural properties of nanoparticles with high tendency to aggregate by providing stability in suspension and influencing interaction between particles and enzymes, cells and membranes [15]. Steric stabilization of nanoparticles as previously described [16] is induced by poloxamer 188 that is a nonionic triblock copolymer composed of a central hydrophobic chain of polypropylene oxide and two hydrophilic chains of polyethylene oxide. Poloxamer is bound to the nanoparticle surface by hydrophobic interaction of the polypropylene oxide chains, while the hydrophilic polyethylene oxide chains protrude into the surrounding medium to create a steric barrier [17]. Poloxamer reduces particle aggregation and enzyme adsorption when in the gastrointestinal tract, which may interfere with the nanoparticle carrier function by degrading insulin [17].

Additionally, improvement of insulin protection against enzymatic degradation represents an essential strategy in the successful oral administration of protein-based drugs. Factors that counteracted the pharmacological effect of orally dosed insulin were calculated as 60% due to insulin degradation, 23% to premature insulin release and 17% to lack of mucoadhesion [18]. By these factors, albumin coating is designed to minimize acid degradation and to prevent proteases from accessing insulin within the nanoparticle, acting as a sacrificial target to enzymatic degradation [3,19,20].

Experimental design has been frequently applied for nanoparticle optimization considering the advantages such as reduction in the number of experiments that need to be performed, development of mathematical models to assess the relevance and statistical significance of the factor effects, and evaluation of interaction effect between studied factors [21]. Box–Behnken designs are based on 3-level incomplete factorial design and are useful tools for optimization following response surface methodology because it permits estimation of the parameters of the quadratic model, building of sequential designs and detection of lack of model fit. Box–Behnken design does not contain combinations for which all factors are simultaneously at highest and lowest levels, avoiding experiments performed under extreme conditions for which unsatisfactory results might occur [21]. In the present study, a design matrix of 15 experimental runs was constructed, for which a quadratic model was generated: $Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2$, where Y is the measured response associated with each factor level combination, b_0 is an intercept, b_1 to b_{33} are regression coefficients computed from experimental runs, and X_1, X_2 and X_3 are the coded levels of independent variables. The terms X_i, X_iX_j and X_i^2 (i = 1, 2 or 3) represent the linear effect, interactions and quadratic effect, respectively [22–24].

The purpose of this study was to optimize nanoparticle formulation for orally dosed insulin, investigating the relationship between design factors and experimental responses by response surface methodology combined with Box–Behnken experimental design. Experiments were designed based on the previously observed important effect of divalent calcium on formation of the pregel state with alginate and dextran sulfate, and of chitosan and albumin on the nanoparticle structure and coating [16]. Therefore, the optimum nanoparticle formulation was determined based on the effects of calcium chloride, chitosan and albumin on minimizing particle size to improve particle uptake [25], minimizing polydispersity index for a narrow distribution, reducing zeta potential to less than −30 mV for potential stability in suspension [26], maximizing insulin entrapment efficiency, minimizing insulin release in gastric simulation for protection against harsh gastric conditions, and maximizing insulin release in intestinal simulation to improve insulin absorption through the gastrointestinal tract.

2. Materials and methods

2.1. Materials

Alginic acid sodium salt from *Brown algae*, low molecular weight chitosan (50 kDa), bovine serum albumin (BSA) and trifluoroacetic acid (TFA) 99% were purchased from Sigma–Aldrich Chemie (France), dextran sulfate sodium salt from *Leuconostoc* ssp., and polyvinylpyrrolidone K 30 (PVP K 30) were purchased from Fluka (Switzerland), poloxamer 188 (Lutrol® F68) was kindly supplied by BASF (Germany), calcium chloride and sodium chloride were purchased from Riedel-de-Haën (Germany), lactic acid 90% was purchased from VWR BDH Prolabo (France), acetonitrile LiChrosolv®, hydrochloric acid 37%, potassium dihydrogen phosphate and sodium hydroxide were purchased from Merck KGaA (Germany), and actrapid® INN-insulin human (rDNA) (Novo nordisk A/S, Denmark) was kindly supplied by *Hospitais da Universidade de Coimbra*. Polyelectrolyte solutions were prepared in ultrapure water and chitosan was dissolved in 0.5% (v/v) of lactic acid solution, and solutions were filtered through Millipore # 2 paper filter under vacuum.

2.2. Methods

2.2.1. Preparation of nanoparticles

Nanoparticles were prepared by ionotropic pregelation [9] of alginate/dextran sulfate solution containing poloxamer 188 and insulin with divalent calcium ions, followed by polyelectrolyte complexation with oppositely charged chitosan and albumin [8,16]. Ionotropic pregelation involves dropwise extrusion of 7.5 mL of a calcium chloride solution into 117.5 mL of pH 4.9 0.06% (w/v) alginic sodium salt, 0.04% (w/v) dextran sulfate, 0.04% (w/v) poloxamer 188 and 0.006% (w/v) of insulin at constant stirring. Complexation then involves dropwise addition of 25 mL of chitosan solution at pH 4.6 for stabilization of pregel nuclei into nanoparticles, followed by dropwise addition of 25 mL bovine serum albumin solution at pH 5.1. Nanoparticles were concentrated by dialysis [27] using regenerated cellulose membrane with tubing nominal dry thickness of 10K MWCO (SnakeSkin Pleated Dialysis Tubing Thermo Fisher Scientific Inc., USA) and dialysis solution of 20% PVP K 30 for 24 h at 4 °C.

2.2.2. Particle size analysis

Particle size measurements were taken by Dynamic Light Scattering using N5 Particle Analyser (Beckman Coulter Inc., USA). Measurements were carried out at 25 °C with detection angle of 90°. The instrument was routinely checked and calibrated using standard latex particle kit (Beckman Coulter Inc, USA). DLS data are presented as the mean size of multiple runs (n = 3) and the polydispersity index of the particle size distribution.

2.2.3. Zeta potential analysis

Zeta potential measurements were taken by Laser Doppler Electrophoresis using Zetasizer Nano ZS (Malvern Instruments Ltd., UK). Measurements were taken in folded capillary electrophoresis cell (Malvern Instruments Ltd, UK) at 25 °C and ultrapure water as diluent to proper concentration. Data analysis of zeta potential is presented as mean of triplicate runs per sample.

2.2.4. Insulin entrapment efficiency analysis

Insulin entrapment efficiency (EE) is the difference between the total amount of insulin used to prepare nanoparticles and the amount of unassociated insulin per total amount of insulin [8,16]. For this purpose, nanoparticles containing insulin were separated from aqueous medium containing unassociated insulin by centrifugation at 20,000*g* for 60 min at 4 °C. The amount of unassociated insulin was determined in triplicate by high performance liquid chromatography (HPLC) described below.

2.2.5. Insulin release studies

For determination of cumulative insulin release in enzyme-free simulated digestive fluids, 10 mg of nanoparticles were incubated in 10 mL of simulated gastric fluid without pepsin (USP31-NF26) at 37 °C for 120 min under magnetic stirring at 100 rpm, followed by incubation in 10 mL of simulated intestinal fluid without pancreatin (USP31-NF26) for 180 min after recovering by centrifugation at 20,000*g* for 15 min. Sample aliquots were collected after 120 min in gastric simulation and 180 min after transferring nanoparticles to intestinal conditions. For determination of cumulative insulin released from nanoparticles, samples were centrifuged at 20,000*g* for 15 min, and 200 μl of supernatant were collected for insulin determination by HPLC. The difference between the initial amount of insulin and the concentration in the supernatant corresponds to the amount of insulin retained within the nanoparticles. Release studies were carried out in enzyme-free fluids with the purpose of determining the pH responsive retention/release properties of nanoparticles, minimizing interference of enzymes that may not reveal changes toward the pH shift from the stomach to the small intestine with the subtle variations in concentration of calcium chloride, chitosan and albumin in each nanoparticle formulation. The insulin release studies were carried out in triplicate.

2.2.6. Insulin determination

Insulin was determined using LC-2010 HT HPLC system (Shimadzu Co., Japan) equipped with a quaternary pump, a HP 1050 programmable multiple wavelength detector set at 214 nm, a reversed-phase X-Terra® RP 18 column, 5 μm, 4.6 mm × 250 mm (Waters Co., USA) and Purospher STAR® RP-18 precolumn 5 μm, 4 mm × 4 mm (Merck KGa, Germany). Mobile phase consisted of acetonitrile (A) and 0.1% TFA aqueous solution (B) operated in gradient mode at flow rate of 1.0 mL/min set to 30:70 (A:B), changed to 40:60 (A:B) in 5 min for elution over 5 min, and changed to 30:70 (A:B) in 1 min for elution over 1 min. Peak area responses of the chromatograms were measured with an automatic integrator. The method was validated and is linear in the range of 1.4–112 μg/mL (R^2 = 0.9998).

Table 1
Variables in Box–Behnken design.

Factor	Levels used		
Independent variables	−1	0	1
X_1 = Calcium chloride (% w/v)	0.20	0.22	0.24
X_2 = Chitosan (% w/v)	0.04	0.07	0.11
X_3 = BSA(% w/v)	0.25	0.50	0.75
Dependent variables			Constraints
Y_1 = Particle size (nm)			Minimize
Y_2 = Polydispersity index			Minimize
Y_3 = Zeta Potential (mV)			<−30
Y_4 = Entrapment Efficiency (%)			Maximize
Y_5 = Release simulated gastric fluid (% at 120 min)			Minimize
Y_6 = Release simulated intestinal fluid (% at 180 min)			Maximize

2.2.7. Experimental design

Box–Behnken design was specifically selected since it requires few runs in case of three or four variables. A 3-factor, 3-level Box–Behnken design was used to optimize nanoparticle formulation with calcium chloride (X_1), chitosan (X_2) and albumin (X_3) concentrations as the independent variables with low, medium and high concentration values described in Table 1. The condition defined by the center point was evaluated in triplicate [28]. Range of concentrations was established according to previous studies for development of nanoparticles containing insulin [16], where calcium concentrations were determined as appropriate to produce a pregel with alginate solution, and chitosan amount should reinforce the alginate nucleus and is used for incorporation of other polyanionic polymers like albumin that should act as a sacrificial target to protect insulin within nanoparticles. The dependent variables were particle size (Y_1), polydispersity index (Y_2), zeta potential (Y_3), entrapment efficiency (Y_4) and insulin release in enzyme-free simulated digestive fluids after 120 min (Y_5) and 180 min (Y_6) with constraints applied as described in Table 1. Design-Expert® software (v.7.1.5 Stat-Ease Inc., Minneapolis, USA) was used for the generation and evaluation of the statistical experimental design. The concentrations of the formulation parameters and the corresponding observations for these dependent variables are presented in Table 2.

Table 2
Composition and observed responses in Box–Behnken design.

Batch	Independent variables			Dependent variables					
	X_1 (%)	X_2 (%)	X_3 (%)	Y_1 (nm) mean + SD	Y_2 mean + SD	Y_3 (mV) mean + SD	Y_4 (%) mean + SD	Y_5 (%) mean + SD	Y_6 (%) mean + SD
1	0.20	0.04	0.50	394 + 14	0.91 + 0.02	−44.5 + 1.1	100	0	92 + 3.27
2	0.24	0.04	0.50	540 + 12	1.10 + 0.05	−44.3 + 0.9	100	0	81 + 1.80
3	0.20	0.11	0.50	472 + 28	0.81 + 0.08	−40.0 + 1.1	89 + 0.68	14 + 1.67	93 + 1.57
4	0.24	0.11	0.50	543 + 34	0.96 + 0.09	−39.5 + 1.4	93 + 0.13	12 + 0.61	90 + 0.38
5	0.20	0.07	0.25	488 + 11	0.77 + 0.05	−40.5 + 1.5	94 + 0.30	0	93 + 1.49
6	0.24	0.07	0.25	588 + 26	0.97 + 0.05	−39.5 + 1.3	100	0	91 + 0.94
7	0.20	0.07	0.75	406 + 11	0.78 + 0.03	−38.8 + 1.5	85 + 0.45	33 + 1.04	98 + 0.21
8	0.24	0.07	0.75	507 + 35	0.98 + 0.06	−36.6 + 0.8	88 + 0.18	28 + 0.66	98 + 1.10
9	0.22	0.04	0.25	516 + 12	1.10 + 0.05	−44.3 + 2.1	100	0	89 + 1.31
10	0.22	0.11	0.25	546 + 12	0.86 + 0.01	−38.8 + 1.3	100	8 + 0.54	86 + 0.84
11	0.22	0.04	0.75	444 + 25	0.93 + 0.03	−40.4 + 1.1	90 + 0.70	19 + 0.64	95 + 0.66
12	0.22	0.11	0.75	495 + 11	0.94 + 0.03	−38.2 + 1.0	90 + 0.56	24 + 0.50	93 + 0.28
13[a]	0.22	0.07	0.50	551 + 19	0.91 + 0.09	−37.1 + 0.9	94 + 0.56	0	94 + 1.41
14[a]	0.22	0.07	0.50	552 + 13	0.93 + 0.05	−37.5 + 0.7	94 + 0.42	0	96 + 0.86
15[a]	0.22	0.07	0.50	561 + 18	0.92 + 0.05	−37.7 + 1.2	95 + 0.36	0	94 + 1.06

[a] Indicates the center point of the design.

2.2.8. Optimization of experimental data analysis and model validation

For determination of the optimized nanoparticle formulation, three-dimensional response surface plots from the experimental data were drawn. All responses observed were fitted to linear, second order and quadratic models, and were evaluated in terms of statistically significant coefficients p-values and R^2 values. Polynomial equations involving the main effect and interaction factors were determined based on estimation of statistical parameters such as multiple correlation coefficient, adjusted multiple correlation coefficient and the predicted residual sum of squares generated by Design-Expert software. The statistical validation of the polynomial equations was established by ANOVA provision available in the software. Therefore, the optimum values of the variables were obtained by graphical and numerical analyses using the Design-Expert software and based on the criterion of desirability [29].

Nanoparticles were prepared using the optimum formulation, and resultant experimental values of the responses were quantitatively compared with the predicted values for calculating the percentage of the predicted error. Predicted error is the difference between the experimental value and the predicted value per predicted value [28,30]. Validation of the optimization procedure was demonstrated for predicted errors lower than 5% [30].

3. Results and discussion

3.1. Preparation and characterization of nanoparticles

Box–Behnken experimental design was performed to determine the relationship between calcium chloride, chitosan and albumin on response variables for the optimization of nanoparticle formulation for orally dosed insulin. The observed responses for mean particle size, polydispersity index, zeta potential, entrapment efficiency and the cumulative insulin release in enzyme-free simulated gastric fluid after 120 min and intestinal conditions after 180 min are presented in Table 2.

It can be observed that nanoparticle mean diameter is dependent upon concentration of calcium chloride and chitosan, where the minimum mean size (394 nm) corresponds to the lowest calcium chloride (0.20%) and chitosan (0.04%) concentrations. Moreover, particle size decreased with increasing albumin concentration. At low calcium concentrations, intramolecular crosslinking of individual alginate chains led to a pregel state and compact coiled structure [9,16]. However, in situations of calcium saturation at higher concentrations, lateral association of a number of alginate chains led to larger structures [9]. Chitosan complexation then contributes to the size of the alginate nucleus in proportion to its initial diameter, since larger nuclei have more carboxylic groups to interact with amino groups of chitosan [8]. Moreover, the effect of albumin concentration on decreasing nanoparticle diameter is possibly related to reduction in the electrical repulsion among biopolymers, where modifications in the electrical state cause the particle to swell or to shrink, depending on whether the electrical repulsions increase or decrease, respectively [31].

Polydispersity index was lower (0.77) when calcium chloride concentration was low (0.20%), but it did not depend on chitosan or albumin concentrations. The calcium effect on nanoparticle formation and size may also be the same on the size distribution, represented by the polydispersity index, by reducing particle aggregation, resulting in a narrower but polydisperse particle size distribution.

Results in Table 2 demonstrated that zeta potential of nanoparticles were strongly negative. Zeta potential was lower than −30 mV for all conditions which shows higher electrostatic stabilization of nanoparticles in suspension, suggesting low aggregation tendency independent of the factors studied at levels selected for Box–Behnken design [26]. It was shown previously [16] that additional steric stabilization of nanoparticles in suspension was obtained by adding poloxamer 188 to the formulation. In addition, negative zeta potential show a prevalence of negatively charged groups, suggesting the presence of albumin on the particle surface that interact with positively charged chitosan at pH above the albumin isoelectric point [32]. Stability of nanoparticles in suspension is an important parameter for insulin physicochemical and biological stability in an oral dosage form [28].

Entrapment efficiency of insulin was over 85% and was influenced by calcium ions and albumin concentration. High insulin entrapment with values above 90% was obtained with higher concentrations of calcium chloride, while maintaining the other factors at constant levels. An exception is seen when comparing batches 1 and 2 which could be influenced by other factors like albumin concentration. Calcium ions interact with guluronic residues of alginate chains and establish ionic bridges with carboxylic residues of insulin amino acids, thereby, enhancing insulin association to the alginate nucleus [8]. Moreover, insulin entrapment was affected by albumin concentration as shown in Table 2, where insulin entrapment was 100% with 0.25 or 0.50% albumin, except for batch 5 which may also be influenced by the low concentration of calcium chloride. The addition of albumin solution at pH 5.1 raised the pH of the nanoparticle suspension to the isoelectric point of insulin, decreasing the strength of electrostatic interaction between insulin and alginate/dextran nuclei, and consequently, reducing the insulin entrapment efficiency [16]. The lowest entrapment efficiency (85%) is seen in batch 7 with lower concentration of calcium chloride (0.20%) and higher concentration of albumin (0.75%).

Insulin release in enzyme-free simulated digestive fluids was carried out to determine if insulin is retained within nanoparticles for protection against acidic degradation, and if insulin is released at neutral pH for absorption through the intestinal mucosa. Insulin was retained within nanoparticles in enzyme-free simulated gastric fluid after 120 min, except for batches 3, 4, 7, 8, 10 and 12 with highest concentration of chitosan (0.11%) or albumin (0.75%), where insulin release was up to 33% depending on these factors at the highest level. Higher concentration of chitosan influenced insulin release due to nanoparticle swelling based on chitosan properties at low pH and then the diffusion of ions present in the simulated gastric fluid inside the nanoparticles may break the ionic interaction between insulin and the alginate/dextran nucleus [33]. High concentration of albumin results in higher insulin release likely due to a weakening of the electrostatic interaction between insulin and the alginate/dextran nuclei reliant on the pH conditions [16]. After gastric simulation, nanoparticles were transferred to enzyme-free simulated intestinal fluid. High levels of cumulative insulin release (greater than 80%) were observed after 180 min in the simulated intestinal fluid. Thus, it appears that pH triggers insulin release, potentially as nanoparticles pass from the acidic gastric medium, into a neutral intestinal medium. This combined with the mucoadhesive properties of alginate and chitosan, which are exposed after degradation of the sacrificial-target albumin in the stomach, would result in adhesion of the nanoparticles to the intestinal mucosa for insulin release directly at the site of absorption. Additionally, liquid chromatography is generally used to indicate the integrity of insulin against deleterious conditions observed in release processes [34]. Neither hydrolysis nor deamination products were observed in the HPLC chromatograms.

3.2. Fitting response data to model statistics

As described above, a direct relationship between particle size and concentration of calcium and chitosan, and an inverse relation-

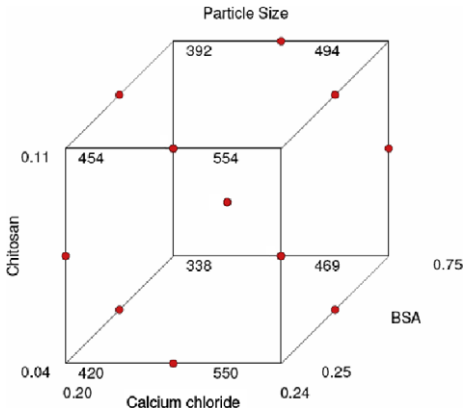

Fig. 1. Model graph showing the effect of independent variables on particle size.

ship depending on albumin concentration were observed in the nanoparticle formulation. Mean particle size varied from 394 to 588 nm depending on calcium ion concentration at constant concentration of albumin and chitosan as observed in Table 2. With increasing chitosan concentration from 0.04 to 0.11% at constant levels of calcium chloride and albumin (batches 1 and 3), particle size increased from 394 to 472 nm. Particle size decreased from 488 to 406 nm by increasing albumin concentration as seen in batches 5 and 7. The particle size effect by changing the levels of the independent factors is represented by Fig. 1.

In Table 2, a direct relationship between polydispersity index and calcium ion concentration was observed, where the polydispersity index increased by increasing calcium chloride concentration from 0.20 to 0.24% and keeping constant chitosan and albumin concentrations as observed in batches 1–8.

Zeta potential of nanoparticles varied in the range of −36.6 to −44.5 mV as shown in Table 2, and higher negative values of zeta potential were dependent upon higher availability of cationic ions and protonated amino groups of chitosan chains. The magnitude of the zeta potential indicates the potential stability of the nanoparticles, with values <−30 mV and >+30 mV representative of stable nanoparticles in suspension [26].

Insulin entrapment efficiency was above 85% and higher entrapment efficiency was dependent on increase in calcium chloride concentration and decrease in chitosan concentration at a constant level of albumin. The direct influence of calcium chloride is seen in batches 3–8 in Table 2 and the inverse effect of chitosan is seen in batches 2 and 4, where insulin entrapment efficiency reduced to 93% by increasing chitosan concentration from 0.04% to 0.11%. Entrapment efficiency was dependent on albumin concentration with inverse relationship between highest insulin entrapment efficiency with low levels of albumin (0.25 or 0.50%) as seen in batches 1, 2, 6, 9 and 10.

Insulin retention in simulated gastric fluid and release in simulated intestinal fluid were influenced by chitosan and albumin concentrations. Insulin was completely retained within nanoparticles formulated with low concentration of chitosan observed when keeping constant calcium chloride and albumin, and also with low concentrations of albumin. Influence of chitosan concentration is seen for insulin release in batches 1–4, where insulin retention decreased to approximately 90% by increasing chitosan concentration from 0.04 to 0.11%. Insulin retention decreased to approximately 70% by increasing albumin concentration from 0.25 to 0.75%. Insulin release in simulated intestinal fluid was higher with high amounts of albumin as seen in Table 2 in batches 7 and 8.

Statistical analysis of the experimental data were used to establish the best fitted models for the independent variables. The quadratic model was established for particle size ($R^2 = 0.9809$), polydispersity index ($R^2 = 0.9834$), zeta potential ($R^2 = 0.9865$) and insulin release in simulated gastric fluid ($R^2 = 0.9451$) and intestinal fluid ($R^2 = 0.8421$). For entrapment efficiency, the best fitted model was linear ($R^2 = 0.7639$) despite poor fit considering the R^2 value, which may be explained by the small variation of entrapment efficiency responses. The regression equations of the fitted models were plotted, and the polynomial equation involving the individual main and interaction effects was selected based on estimation of statistical parameters for optimization of nanoparticle formulation. Table 3 shows the coefficients for independent variables associated with the effect of these factors and their comparative significance on the responses of the dependent variables. A positive value in the regression equation represents an effect that favors the optimization due to synergistic effect, while a negative value indicates an inverse relationship or antagonistic effect between the factor and the response [22].

It is observed in Table 3 that all three independent variables (X_1, X_2 and X_3) presented positive effects on the responses of particle size (Y_1) and zeta potential (Y_3). Polydispersity index (Y_2) and entrapment efficiency (Y_4) were primarily affected by concentrations of calcium chloride. The polydispersity index was lower at low calcium chloride level possibly due to lower tendency in the formation of aggregates. At high concentrations of calcium chloride, the entrapment efficiency was higher by favorable insulin association with alginate/dextran nucleus. Albumin had positive effects on minimizing insulin release in simulated gastric fluid (Y_5) and maximizing insulin release in simulated intestinal fluid (Y_6), whereas calcium ion concentration had a negative effect on

Table 3
Coefficients of regression equation for independent variables and standard error.

Terms	Y_1		Y_2		Y_3		Y_4		Y_5		Y_6	
	C	SE	*C*	SE	*C*	SE	*C*	SE	*C*	SE	*C*	SE
b_0	554.67	8.08	0.92	0.012	−37.43	0.29	94.13	0.71	0.00	2.67	94.67	1.89
b_1	57.87	4.95	0.092	0.007	0.49	0.18	1.62	0.98	−0.87	1.63	−2.75	1.16
b_2	14.67	4.95	−0.059	0.007	2.13	0.18	−2.25	0.98	4.87	1.63	−0.13	1.16
b_3	35.75	4.95	−0.009	0.007	1.14	0.18	−5.13	0.98	12.00	1.63	3.13	1.16
b_1b_2	−7.50	7.00	−0.010	0.010	0.075	0.25	–	–	−0.50	2.31	0.50	1.63
b_1b_3	0.25	7.00	0.00	0.010	0.30	0.25	–	–	−1.25	2.31	0.50	1.63
b_2b_3	5.25	7.00	0.063	0.010	−0.83	0.25	–	–	−0.75	2.31	0.25	1.63
b_1^2	−40.83	7.28	−0.029	0.011	−1.53	0.26	–	–	4.50	2.40	−1.46	1.70
b_2^2	−37.83	7.28	0.054	0.011	−3.11	0.26	–	–	2.00	2.40	−5.71	1.70
b_3^2	−16.58	7.28	−0.016	0.011	0.12	0.26	–	–	10.75	2.40	1.79	1.70

C: coefficient estimate.
SE: standard error.
Regression equation of the fitted model:
$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2$.

responses Y_5 and Y_6, and chitosan concentration had positive effect on response Y_5 but inverse relationship to response Y_6.

Coefficients with more than one factor or higher order terms in the regression equation represent the interaction between terms or the quadratic relationship, respectively, which suggests a non-linear relationship between factors and responses [28]. In this condition, factors can produce different degree of response than predicted by regression equations if they are varied at different levels or more than one factor is changed simultaneously. Except for insulin entrapment efficiency, for which factors presented linear relationship, responses Y_1, Y_2, Y_3, Y_5 and Y_6 were affected by the interactions between factors, demonstrating aquadratic relationship. The interaction effect between X_1 and X_2 was favorable for zeta potential and 60-fold higher for insulin release in simulated intestinal fluid. The interaction effect between X_1 and X_3 was favorable for responses Y_1, Y_3 and Y_6, but unaffected by Y_2 and had an inverse effect for Y_5. Favorable effects were also observed for Y_1, Y_2 and Y_6 by interaction between X_2 and X_3 that were 80- and 20-fold higher for particle size than for polydispersity index and insulin release in simulated intestinal fluid, respectively. The quadratic effects of X_1, X_2 and X_3 were observed for responses Y_1, Y_2, Y_3, Y_5 and Y_6 at different levels and the relationship was determined by statistical analysis. Highest and negative quadratic effects for all factors were observed for Y_1, whereas the highest and positive effects were observed for Y_5.

3.3. Response surface analysis

Response surface analyses were plotted in three-dimensional model graphs for optimization of nanoparticles with physicochemical properties for orally dosed insulin. The three-dimensional response surface plots for particle size, entrapment efficiency and insulin release in gastrointestinal fluids are presented in Figs. 2–5, respectively. The response surface plots are used for interpretation of the interaction effects of two independent variables on the responses or dependent variables when a third factor is kept at constant level. Except for insulin entrapment efficiency where interaction effects of calcium, chitosan and albumin were linear, the relationships among the three variables were non-linear.

It is observed in Fig. 2 that the particle size decreases with decreasing concentration of both calcium chloride and chitosan and increasing albumin concentration. Particles with small size are highly desirable in order to increase contact with the intestinal mucosa due to higher surface area per volume and mucoadhesive strength [35], increasing nanoparticle uptake in the intestinal mucosa [36]. For polydispersity index, lower values are obtained with intermediate concentration of chitosan and decreasing calcium chloride and albumin concentrations. Chitosan primarily influences zeta potential values, in which zeta potential increases with increasing chitosan concentrations, and thus the availability of positively charged amino groups. Intermediate concentration of calcium chloride also increases zeta potential to values lower than −30 mV, affecting the stability of nanoparticles in suspension. Entrapment efficiency is influenced by albumin concentration as seen in Fig. 3, and at constant level of albumin, entrapment efficiency increases by increasing calcium chloride and decreasing chitosan concentrations in a linear mode. Insulin release in enzyme-free simulated digestive fluids is higher by increasing albumin concentration as seen in Figs. 4 and 5. For insulin protection against harsh conditions in gastric simulation, retention within nanoparticles is obtained by intermediate concentration of calcium chloride and low concentration of chitosan at constant level of albumin. Though, at constant level of calcium chloride and chitosan, insulin is retained within nanoparticles in gastric conditions when albumin concentration is lower than 0.50%. This effect is also observed when nanoparticles are transferred to intestinal fluid.

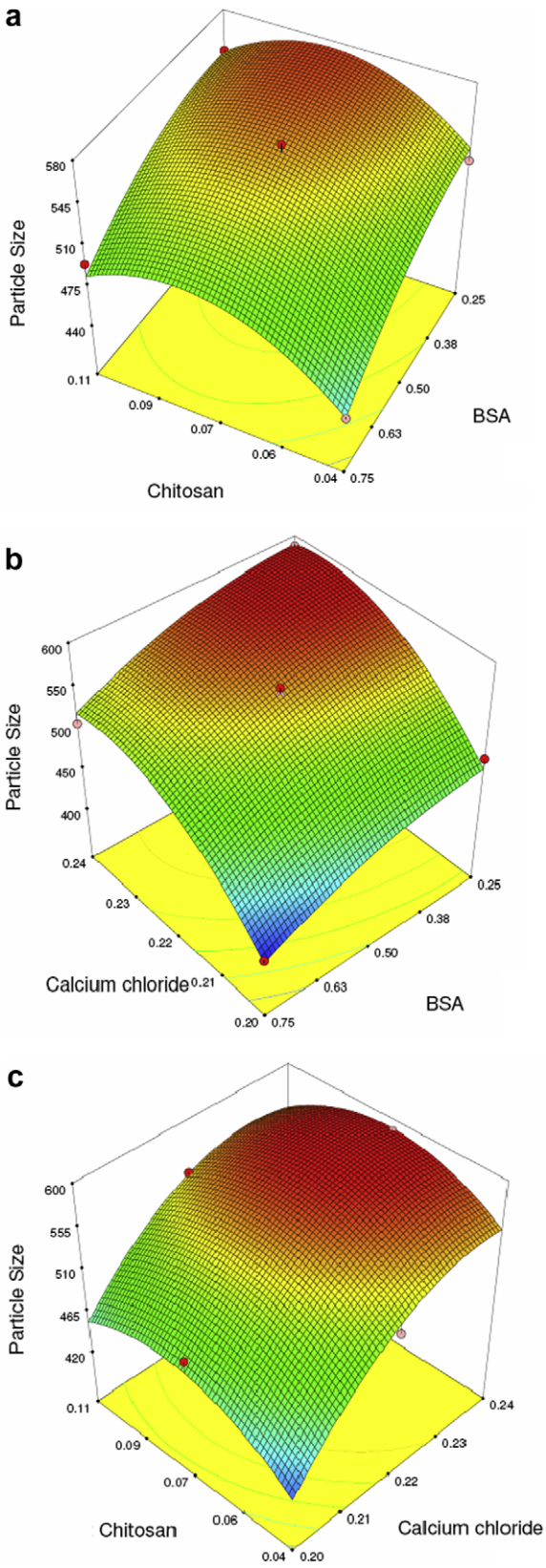

Fig. 2. Response surface plot showing effect of (a) chitosan (X_2) and albumin (X_3), (b) calcium chloride (X_1) and albumin (X_3), and (c) calcium chloride (X_1) and chitosan (X_2) concentrations on particle size (Y_1).

Insulin release from nanoparticles increases when albumin concentration is greater than 0.50%. In addition, at constant level of

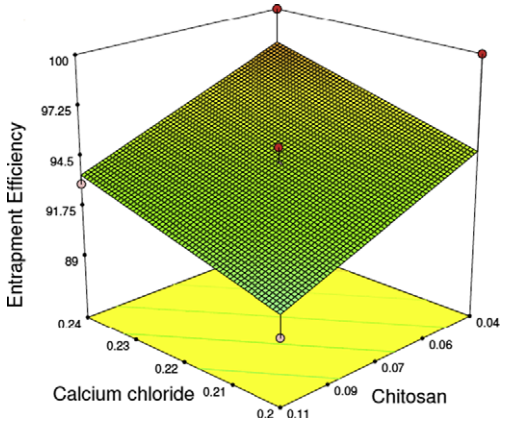

Fig. 3. Response surface plot showing effect of calcium chloride (X_1) and chitosan (X_2) concentrations on entrapment efficiency (Y_1) when albumin is at constant level 0.50% (X_3).

albumin, insulin release from nanoparticles in intestinal conditions increases by decreasing concentration of calcium chloride and at chitosan to intermediate level.

3.4. Optimization and model validation

The optimum nanoparticle formulation integrating biomaterial for orally dosed insulin was selected based on the constraints of the physicochemical parameters established to perform the Box–Behnken design as seen in Table 1. Analyzing various response variables and evaluating the constraints by mathematical approach, the optimum nanoparticle is formulated with solutions of 0.20% calcium chloride, 0.04% chitosan and 0.46% bovine serum albumin, and has the predictive values of particle size of 402 nm, polydispersity index of 0.91, zeta potential of −39.8 mV, 96% of insulin entrapment efficiency, total retention of insulin in gastric conditions and 96% release in intestinal fluid as presented in Table 4.

The formulation of nanoparticle with solution described above showed the validity of the Box–Behnken design for the optimization of nanoparticle formulation demonstrated by experimental values similar to predicted values within 5% of predicted error. Table 4 lists the observed responses and the predicted error compared to the predictive values. The optimized nanoparticle formulation has a mean particle size of 396 nm, polydispersity index of 0.87, zeta potential of −38.2 mV, 100% insulin entrapment efficiency and retention within nanoparticles in enzyme-free simulated gastric fluid during 120 min, and 95% release in enzyme-free intestinal simulation after 180 min.

4. Conclusions

Experimental designs allowed systematic optimization of the nanoparticle formulation by determining physicochemical parameters important for oral dosed insulin, evaluating the most important factors on observed responses, and investigating the relationship between factors by the response surface methodology. Box–Behnken design was successfully used to statistically optimize the formulation parameters and to evaluate the main interaction and quadratic effects of the independent variables on the particle size, polydispersity index, zeta potential, entrapment efficiency and insulin retention and release from nanoparticles in simulated digestive fluids. A 3-factor, 3-level design was used to explore the quadratic and linear response surfaces and for constructing a second order polynomial model. Based on the experimental responses and accordingly to the criteria of desirability, solutions of 0.20% calcium chloride, 0.06% alginic sodium salt, 0.04% dextran sulfate, 0.04% poloxamer 188, 0.006% insulin,

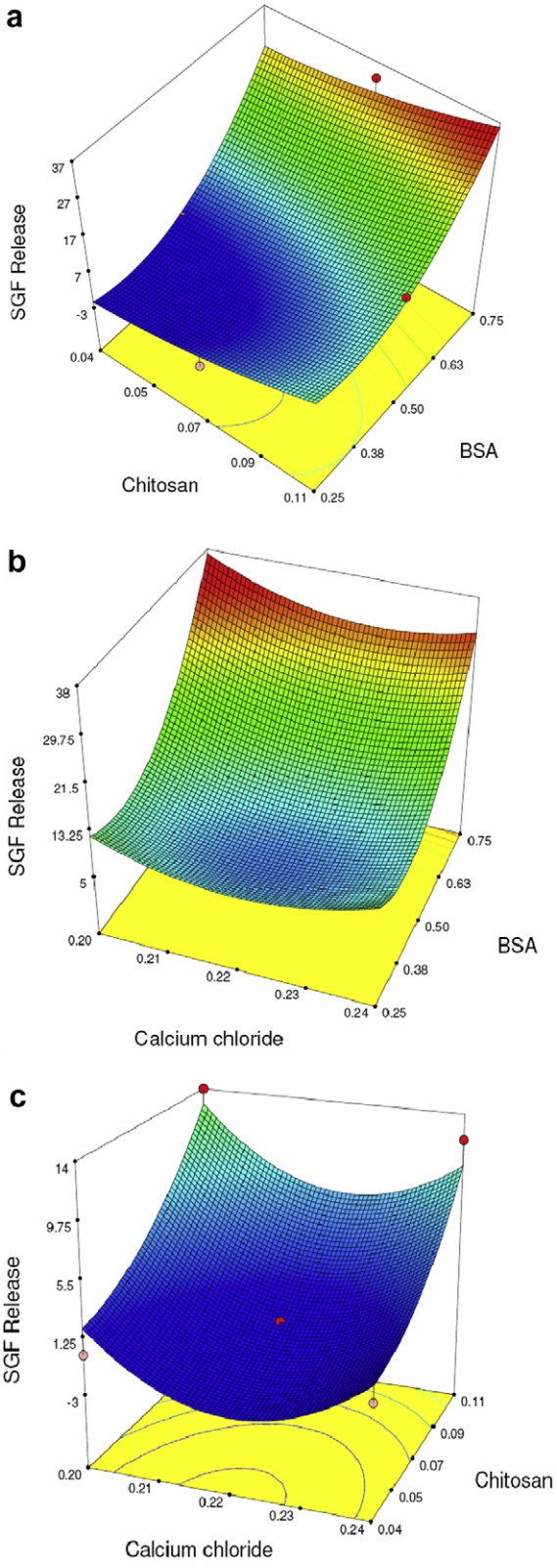

Fig. 4. Response surface plot showing effect of (a) chitosan (X_2) and albumin (X_3), (b) calcium chloride (X_1) and albumin (X_3), and (c) calcium chloride (X_1) and chitosan (X_2) concentrations on insulin release in simulated gastric fluid without pepsin (Y_5).

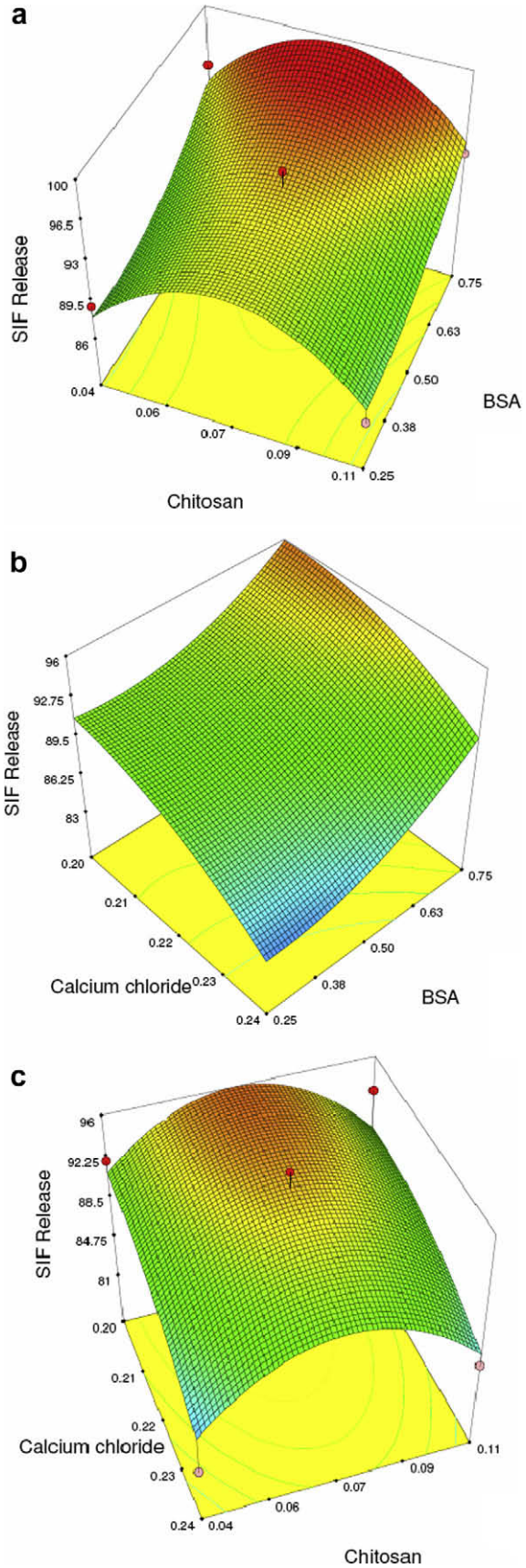

Fig. 5. Response surface plot showing effect of (a) chitosan (X_2) and albumin (X_3), (b) calcium chloride (X_1) and albumin (X_3), and (c) calcium chloride (X_1) and chitosan (X_2) concentrations on insulin release in simulated intestinal fluid without trypsin (Y_6).

Table 4
Comparative values of predicted and experimental responses for optimized nanoparticle formulation.

Dependent variables	Predicted response	Experimental responses	Predicted error (%)
Y_1 (nm)	402	396	−1.49
Y_2	0.91	0.87	−4.40
Y_3 (mV)	−39.8	−38.2	−4.02
Y_4 (%)	96	100	−4.00
Y_5 (%)	0	0	0
Y_6 (%)	96	95	−1.04

0.04% chitosan and 0.46% albumin constitute the optimum formulation of nanoparticles for orally dosed insulin.

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