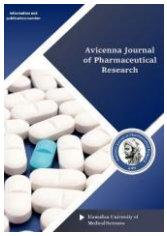
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Original Article

Development, Statistical Optimization and *In Vitro*/ *In Vivo* Characterization of an Intelligent Hydrogel Containing Methylated N-(4-N, N-Dimethylaminobenzyl) Chitosan for Glucose-Responsive Insulin Delivery

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Abstract

Background: The present study aimed to develop an intelligent, glucose-responsive hydrogel composed of methylated N-(4-N,N-dimethylaminobenzyl) chitosan as a quaternized and aromatized derivative of chitosan.

Methods: In this study, a cross-linked hydrogel, incorporating boric acid (BA), was prepared from the synthesized chitosan derivative. The preparation of hydrogel was statistically optimized using box-behnken response surface methodology. The chemical structure and morphology of the optimized hydrogel were examined using FT-IR spectroscopy and scanning electron microscopy, respectively. Furthermore, the glucose-responsive behavior of the prepared hdyrogel was evaluated by *in vitro* release studies. Finally, the efficacy of the prepared hydrogel in glucose-responsive insulin delivery was studied *in vivo* by implementation of the hydrogels in male Wistar rats and their blood glucose levels were evaluated in the pre-determined time intervals.

Results: The optimized hydrogel with proper characteristics was prepared. Following 480 min of incubation, the *in vitro* release study demonstrated 26.1 ± 1.84%, 52.7 ± 3.11% and 78.9 ± 3.85% of cumulative insulin release in glucose-free-, glucose 3%- and glucose 5%-phosphate buffer, respectively. Finally, the obtained *in vivo* results demonstrated 1.5-fold reduction in blood glucose level in diabetic animals compared to non-diabetic ones.

Conclusion: The findings obtained suggests that the hydrogel can efficiently pose glucose-responsive properties for insulin delivery.

Keywords: Boric acid, Glucose-responsive, Hydrogel, Methylated N-(4-N, N-dimethylaminobenzyl) Chitosan, Poly vinyl alcohol (PVA)

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Introduction

Diabetes mellitus, the most prevalent endocrine disease, is characterized primarily by hyperglycemia. Currently, more than 415 million people worldwide are living with diabetes, and this figure is estimated to reach 642 million by 2040 [1].

Insulin, a peptide hormone composed of 51 amino acids with a molecular weight of 5,808 Da, is secreted by the β-cells of the islets of Langerhans in the

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pancreas [2]. Through several mechanisms, this peptide effectively reduces blood glucose levels, and exogenous insulin is mainly used for the treatment of type 1 diabetes mellitus (T_1DM) [3].

Typical and conventional treatments for diabetes using insulin and other glucose-lowering agents do not achieve precise regulation of blood glucose within normal physiological range (i.e., normoglycemia) and are usually associated with varieties of hypoglycemic and hyperglycemic events. To overcome these problems, glucose-triggered drug delivery devices, as a class of intelligent closed-loop systems, have been emerged. In these systems, the level of blood glucose can determine the release rate of the therapeutic compound.

Among several types of glucose-sensitive delivery systems, glucose-responsive hydrogels, have been widely studied in the last decade. Based on the type of sensor, glucose-sensitive hydrogels are divided into three sub-classes, including glucose oxidase (GOX)-bearing hydrogels [4], modified hyrogels containing glucose binding molecules (e.g., concanavalin A or lectin)[4], and cross-linked hydrogels containing boric acid (BA) and its derivatives [5].

Boric acid is a prosthetic compound that poses high constancy and high stability in the physiological environment. This compound and its derivatives are well known for their selective and reversible covalent complexation with poly-ol molecules (e.g., glucose or poly vinyl alcohol (PVA)) through two-diol complexation mechanism [6]. Several studies investigated the development of a glucose-responsive drug delivery system by preparation of a BA-crosslinked PVA hydrogel. In the absence of glucose, the boric acid molecules are attached to PVA, by both covalent and hydrogen bonds, forming BA-PVA complexes. However, in the presence of glucose, the PVA molecules which are incorporated in BA-PVA complexes are replaced by glucose molecules, forming BA-glucose didiol complexes [6]. The BA-glucose complexation can elevate the osmotic pressure inside the hydrogel network which in turn, leads to swelling of the drug delivery system and enhancement of drug release through various diffusion mechanisms [7].

Chitosan is a cationic linear polysaccharide composed of alternating glucosamine and N-acetylglucosamine units. It is produced by the alkaline deacetylation of chitin. Chitosan is a bio-adhesive, muco-adhesive, anti-microbial, bio-degradable, bio-compatible and non-toxic hydrogel and has been widely studied for agricultural, food industrial, cosmetic, gene delivery, tissue engineering, biomedical and drug delivery applications [8]. However, the low and pH-dependent solubility of chitosan in aqueous media restricts its application in development of drug delivery systems. Therefore, soluble quaternary ammonium derivatives of chitosan, such as trimethyl chitosan (TMC), have been synthesized; these derivatives are soluble over a wide pH range owing to the permanent positive charges on their quaternary amines [9].

Moreover, it was reported that due to increase in amine protonation and charge density, quaternized derivatives of chitosan pose more swelling ability that non-modified chitosan [10, 11].

Based on the related literature, aromatic derivatives of quaternized chitosan, such as methylated N-(4-N,N-dimethylaminobenzyl) chitosan (designated as methylated aminobenzyl chitosan), exhibited greater interactions with cell surfaces and, consequently, greater efficiency for drug and gene delivery than chitosan and TMC. This enhanced performance is attributed to the presence of an aromatic ring and, therefore, a proper hydrophilic/lipophilic balance [12, 13]. The chemical structure of methylated aminobenzyl chitosan is presented in Figure 1. Although the introduction of an aromatic ring may decrease the hydrophilic characteristics of methylated aminobenzyl chitosan, the presence of two amino moieties (i.e., aliphatic and aromatic amines) in its monomers, compared with TMC, which possesses only one aliphatic amino-functional group per unit, can increase the swelling ability of the polymer. Moreover, it is hypothesized that the presence of an additional aromatic amine in the subunit structure of methylated aminobenzyl chitosan leads to a higher degree of boric acid cross-linking than that of TMC.

The present study aimed to prepare a hydrogel-based intelligent drug delivery system for glucose-responsive delivery of insulin. The hydrogel was prepared from methlylated aminobenzyl chitosan and PVA and it was cross-linked using BA molecules. The preparation of the hydrogel was optimized using box-behnken response surface methodology. The

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characteristics of the prepared hydrogel, including FT-IR spectroscopy, morphology, glucose-dependent swelling ability (%), and *in vitro* glucose-dependent insulin release profile, were determined. Finally, to investigate the glucose-responsive behavior *in vivo*, the prepared hydrogels were implanted into the abdominal wall of the animals (either diabetic or non-diabetic), and changes in blood glucose levels were monitored.

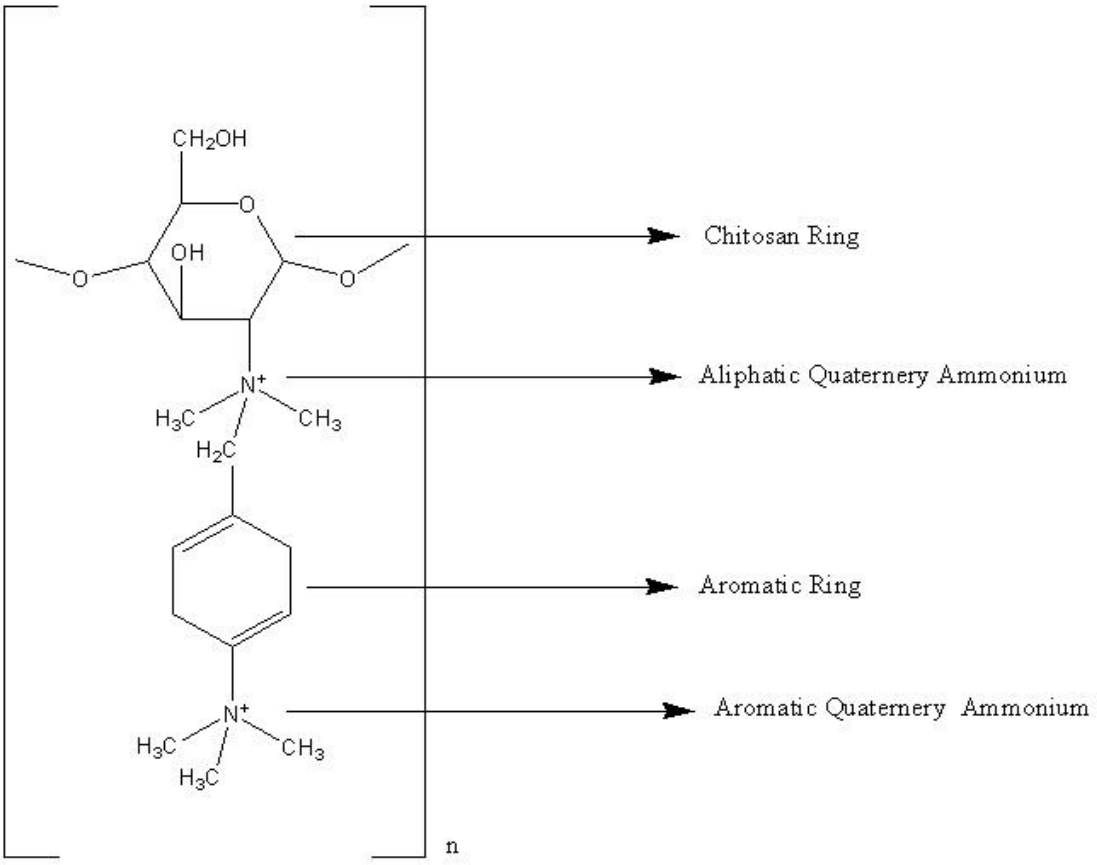


Figure 1. Chemical structure of Methylated N-(4-N, N-dimethylaminobenzyl) chitosan;

Materials and Methods

Materials

Insulin as the active pharmaceutical ingredient was obtained from Novonordisk™ pharmaceutical company (Bagsvaerd, Denmark). Low molecular weight chitosan (viscosity 1% (*w/v*) solution, 20 mPa S) with a high degree of deacetylation (i.e.,>98%) was purchased from Primex™ (Siglufjordur, Iceland). Streptozotocin, glucose, sodium hydroxide (NaOH), sodium chloride (NaCl), potassium chloride (KCl), disodium hydrogen phosphate (Na_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4), methyl iodide (CH_3I), sodium iodide (NaI), phosphoric acid (85%), N-methylpyrrolidone (NMP), polyvinyl alcohol (PVA), boric acid, glacial acetic acid, ketamine, xylazine, and Lichrosolv® analytical grade acetonitrile were all obtained from Merck™ (Darmstadt, Germany). Sodium borohydride and triethanolamine were purchased from Sigma-Aldrich™ (United Kingdom). Doubly distilled deionized water was supplied using Mili-Q® water purification system (Milipore™, United States).

Phosphate-buffered saline (PBS) with adjusted pH of 7.4 was prepared using NaCl (137 mM), KCl (2.7 mM), Na_2HPO_4 (10 mM), and KH_2PO_4 (1.8 mM). All other chemicals were of pharmaceutical grade and were used as received.

Synthesis of methylated N-(4-N,N-dimethylaminobenzyl) chitosan

Methylated N-(4-N, N-dimethylaminobenzyl) chitosan, designated as methylated aminobenzyl chitosan, was synthesized using a Schiff base reaction according to literature, with minor modifications

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[14]. In the first step, chitosan (3 g) was dissolved in acetic acid solution (1% v/v), the solution was kept stirred overnight. The pH of the solution was adjusted to 5.0 by adding NaOH (1N). Then, the excess amount of 4-N,N-dimethylbenzaldehyde (4.5 g) was added to the solution and kept stirred for 24 h at room temperature, followed by filtration through Whatman filter paper 42. Subsequently, sodium borohydride (3 g) was added to the solution to restore the binary bonds of the prepared imine, and the mixture was stirred for 12 h at room temperature. Afterwards, the prepared derivative (i.e., N-(4-N, N-dimethylaminobenzyl) chitosan) was precipitated by elevation of the pH to 10.0 using NaOH (1 N). The sediment (i.e., aminobenzyl chitosan) was collected by filtration. For removal of any impurities, it was dialyzed against distilled water and it was lyophilized to produce the dried powder.

In the next step and to synthesize methylated aminobenzyl chitosan, methylation step was performed on the previously synthesized aminobenzyl chitosan [15]. Briefly, the synthesized aminobenzyl chitosan (2 g) was completely dissolved in NMP at 50 ˚C. Next, NaI (2 g) and NaOH (1N) were added to the solution. Subsequently, under reflux conditions at 60 ºC, methyl iodide (15 mL) was added in five equal portions at 3 h intervals. The solution was dialyzed against distilled water using a dialysis bag (MWCO 10 kDa) and the methylated derivative was precipitated using an excess amount of acetone. Finally, the synthesized derivative (i.e., methylated aminobenzyl chitosan) was washed with methanol and completely dried using a vacuum drier. The chemical structure of the synthesized methylated N-(4-N, N-dimethylaminobenzyl) chitosan, as well as the degree of aromatic substitution and the degree of methylation, were identified using ^{1}H-NMR spectroscopy.

Preparation of BA-crosslinked hydrogel

The boric acid-crosslinked glucose-responsive hydrogel composed of the synthesized methylated aminobenzyl chitosan and PVA was prepared using a frequent melt-freezing process.[16] Briefly, the proper amount of the previously synthesized chitosan derivative was completely dissolved in acetic acid (2% v/v). Then, one milliliter of PVA aqueous solution with the proper concentration was added and the solution was stirred for 3 h at 100 rpm in the room temperature. Aqueous boric acid solution (2-mL) as the cross-linking agent, with various concentrations, was then added dropwise and the mixture was stirred until a homogenous hydrogel was obtained. For neutralization of the acetic acid, the prepared hydrogel was immersed in the dilute solution of sodium hydroxide (0.05 N) and then was washed three times with doubled- distilled water. To promote crosslinking, the hydrogel was maintained at −20 °C for 24 h and subsequently allowed to thaw at 25 °C for 7 h. This cycle of melt-freezing process was repeated four times. Thereafter, the hydrogel was dried and the structure was examined by FT-IR spectroscopy. Moreover, the surface characteristics of the cross-linked hydrogel were examined using scanning electron microscopy (SEM).

Optimization studies

In this study, statistical optimization was performed using box-behnken response surface methodology. The Design- Expert® software (V.7.0.0; Minneapolis, United States) was used for statistical analysis, mathematical modeling and optimization [17]. Independent variables were considered as the concentration of methylated aminobenzyl chitosan (X_1), concentration of PVA (X_2), and concentration of boric acid (X_3). The appropriate ranges of the independent variables (hereafter referred to as factors) were established on the basis of preliminary screening studies (data not shown). On the other hand, the dependent variables (i.e., responses) were swelling ability (%) in glucose-free PBS (Y_1), swelling ability (%) in PBS supplemented with 5% (*w/v*) glucose (Y_2), and loading efficiency of insulin in the hydrogel structure (Y_3). The ranges and constraints of the variables are indicated in Table 1. According to the software, the experimental preparation of 15 formulations was necessary for further statistical analysis and mathematical modeling. The various prepared formulations are summarized in Table 2.

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Table 1. Ranges and constraints of independent and dependent variables

Independent Variables (Factors)	Levels	
	-1	+1
Concentration of methylated aminobenzyl chitosan (X_1) (mg/mL)	1.0	10
Concentration of PVA (X_2) (mg/mL)	5.0	15.0
Concentration of boric acid (X_3) (mg/mL)	5.0	30.0
Dependent Variables (Responses)	Constrains	
Swelling ability (%) in glucose 0% (Y_1)	Minimized	
Swelling ability (%) in glucose 5% (Y_2)	Maximized	
Drug Loading (%) (Y_3)	Maximized	

Determination of the swelling ability (%) of the hydrogel

To investigate the swelling ability of the hydrogel, a sample of the dried hydrogel was weighed (W_{dry}) and then it was immersed in either 100 mL of glucose-free PBS or PBS containing 5 % (*w/v*) glucose, for a period of 4 h. Then, the hydrogel was collected from the solution, dried using a Whatman filter paper, and weighed (W_{wet}). The value of swelling ability in percent was calculated according to the following equation (Eq.1). The experiment was performed in triplicate.

$$Swelling\ ability\% = \frac{W(wet) - W\ (dry)}{W\ (dry)} * 100$$

(Eq.1)

Table 2. Box-Behnken experimental design (n=3)

Run No.	Independent Variables (Factors)			Dependent Variables (Responses)		
	Concentration of methylated aminobenzyl chitosan (mg/mL) (X_1)	Concentration of PVA (mg/mL) (X_2)	Concentration Boric acid (mg/mL) (X_3)	Swelling ability in glucose 0% (%) (Mean±SD)	Swelling ability in glucose 5% (%) (Mean±SD)	Insulin Loading (%) (Mean±SD)
1	1.0	10.0	5.0	201.3±42.25	867.0±20.85	65.8±7.65
2	5.5	15.0	30.0	52.1±7.86	213.3±29.70	79.7±9.21
3	5.5	10.0	17.5	144.0±39.47	154.7±7.69	81.2±4.46
4	1.0	5.0	17.5	172.5±67.54	231.8±25.38	59.6±5.29
5	5.5	15.0	5.0	236.5±56.72	246.1±12.19	71.6±7.38
6	5.5	10.0	17.5	127.3±47.11	300.8±28.3	93.8±5.89
7	1.0	15.0	17.5	90.1±8.24	59.4±8.17	65.5±6.21
8	10	10.0	30.0	180.7±72.23	210.7±21.35	80.7±9.97
9	5.5	5.0	30.0	204.3±57.88	200.4±15.88	69.9±5.34
10	10	5.0	17.5	292.3±34.72	235.2±66.34	97.4±8.17
11	10	15.0	17.5	169.0±85.73	203.4±74.16	76.3±7.89
12	10	10.0	5.0	237.7±21.42	248.1±25.09	97.1±10.23
13	1.0	10.0	30.0	51.8±7.14	55.7±4.21	63.3±6.97
14	5.5	10.0	17.5	229.7±28.63	161.4±42.56	71.8±9.63
15	5.5	5.0	5.0	73.0±8.25	290.6±62.76	84.4±7.98

Drug loading

The swelling balance method was used for loading insulin into the hydrogel network.[18] Briefly, the hydrogel was immersed in an aqueous solution containing insulin (5 mg/mL) for 72 h, while the temperature was kept constant at 5 ˚C. Thereafter, the swollen hydrogel was collected and dried. The remaining aqueous solution was used for determination of the drug loading by indirect method. For determination of the loading efficiency (LE%),

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the amount of unloaded insulin in the aqueous solution was determined using high-performance liquid chromatography (HPLC), and the loading efficiency was calculated using the following equation (Eq.2).

$$Loading\ efficiency\ (LE\%) = \frac{Initial\ amount\ of\ insulin - the\ amount\ of\ unloaded\ \ insulin}{Initial\ amount\ of\ \ insulin} * 100$$

(Eq. 2)

High Performance Liquid Chromatography (HPLC) Analysis

For HPLC analysis, a Shimadzu® high-performance liquid chromatography system (Kyoto, Japan), equipped with an LC-20AD pump and a UV-visible detector, set at 214 nm, was used. The stationary phase consisted of a VP-ODS Shimadzu®C18 column (250*4.6*5). The mobile phase was a mixture of acetonitrile (30% v/v), phosphate buffer (KH_2PO4; 0.2 N) (69.3% v/v), and triethanolamine (0.7% v/v). Previously, the pH of the phosphate buffer was adjusted to 3.0 using phosphoric acid (85% w/v). The analysis was performed at ambient temperature with a flow rate of 1 mL/min and an injection volume of 20 µl [19]. The method was linear in the concentration range of 1-100 µg/mL (R^2=0.9991). The calculated inter-day and intra-day accuracy and precision were all well-agreed to ICH guidelines (n=3).

***In vitro* release profile**

The insulin-containing hydrogels (equivalent to 150 mg of insulin) were immersed in pre-heated PBS (100 mL) containing 0% (w/v), 3% (w/v), or 5% (w/v) glucose. The mixture was agitated at 100 rpm using a Heidolph® shaker incubator (Schwabach, Germany). The temperature was kept constant at 37 ± 1 °C. Based on the solubility of insulin at pH of 7.4, the volume of the release medium was modified in a manner that establishment of sink conditions was ascertained. At pre-determined time intervals, one milliliter of the medium was collected and replaced by the equal volume of the freshly prepared pre-heated buffer. The amount of insulin in the samples was detected using the previously mentioned HPLC method. The procedure was performed in triplicate. Finally, the release profiles of insulin from the cross-linked hydrogels in various release media were compared using calculation of the similarity factor (Eq.3). The two-release profile is considered to be different when the calculated similarity factor is less than 50.0 [20].

$$Similarity\ factor = 50 * Log \left[\frac{100}{\sqrt{1+\frac{\sum_{t=1}^{n}(Rt-Tt)^2}{n}}} \right]$$

(Eq. 3)

Where:

N: The number of sampling times in the dissolution profile;

R_t: The value of dissolution average for reference product at time t;

T_t: The value of dissolution average for the second product at time t;

Animals

All animal procedures were performed according to appropriate guidelines approved by the Ethical Committee of Hamadan University of Medical Sciences (Approval ID: IR. UMSHA.REC. 1394. 562). In this study, male Wistar rats (weighing 210-240 g) were obtained from the animal house of Hamadan University of Medical Sciences. The rats were kept in standard conditions (i.e., room temperature of 25° C, environmental humidity of 45%, and light/darkness cycles of 12 h). The animals had free access to the standard food diet, and the water was supplied *ad libitum*. After 7 days of incubation, they were divided into five groups (n=5) as follows:

Group I: non-diabetic animals receiving insulin-containing hydrogel;

Group II: diabetic animals receiving insulin-containing hydrogel;

Group III: diabetic animals receiving subcutaneous NPH insulin;

Group IV: diabetic animals receiving insulin–free hydrogel;

Group V: non- diabetic animals receiving insulin–free hydrogel;

Diabetes was induced in the animals of groups II, III, and IV by intraperitoneal (IP) injection of streptozotocin (0.5 mL; 65 mg/mL) dissolved in isotonic citrate buffer. To verify the induction of diabetes, blood samples were collected from the tail

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vein 48 h post-treatment, and blood glucose concentrations were measured with a glucometer. The animals with a glucose concentration of 250 mg/dl or more were selected as diabetic for further studies [21].

***In vivo* studies**

In the appropriate groups (I, II, IV and V groups), the animals were anesthetized using intraperitoneal co-administration of ketamine (87 mg/kg) and xylazine (13 mg/kg). The hydrogels equivalent to 20 mg of insulin were intraperitoneally implanted in the abdominal wall of the specified groups of animals. No surgery was performed in group III of animals. Instead, they received 20 mg of NPH insulin by subcutaneous (SC) injection. After the recovery period of 12 h, the blood samples (0.2 mL) were taken from their tail vein at predetermined time intervals and were collected in the appropriate heparinized tubes. The samples were then immediately centrifuged at 4,000 rpm for 10 min, and the plasma glucose level was determined using a glucometer (Abbot, Portugal). Baseline glucose levels were determined from control samples collected at time zero, immediately after surgery. Finally, the animals were sacrificed by chloroform inhalation.

Statistical analysis

In this study, all experiments were performed in triplicate, unless otherwise stated, and the data were reported as Mean ± SD. The design- expert® software (V.7.0.0) was used for statistical optimization. The experimentally obtained data were analyzed using one-way analysis of variance (ANOVA) and fitted to the appropriate mathematical models. A *P*-value of less than 0.05 was considered statistically significant for the models and parameters.

Results and Discussion

Synthesis of methylated aminobenzyl chitosan

In the present study, a quaternized and aromatized derivative of chitosan (i.e., methylated N-(4-N, N-dimethylaminobenzyl chitosan), designated as methylated aminobenzyl chitosan, was synthesized. The synthesized derivative was characterized by ^{1}H-NMR spectroscopy. The NMR spectrum is depicted in Figure 2. As exhibited in the figure, sharp peaks at 2.3 ppm and 2.7 ppm represent the protons related to aliphatic methyl moieties of -N-(CH_3)- and -N^+-(CH_3)$_2$-, respectively, and indicate the quaternization of aliphatic amines. On the other hand, the quaternization of aromatic amines was confirmed by the appearance of sharp peaks at 3.4 ppm and 3.7 ppm, representing the protons of aromatic methyl moieties of -N-(CH_3)$_2$- and -N^+-(CH_3)$_3$-, respectively. Moreover, the presence of an aromatic ring in the chemical structure of the synthesized derivative is confirmed by the appearance of proton peaks around 7.0 ppm. The degrees of aromatic substitution, aliphatic quaternization and aromatic quaternization were calculated by proportional integration of the relevant peaks and are summarized in Table 3.

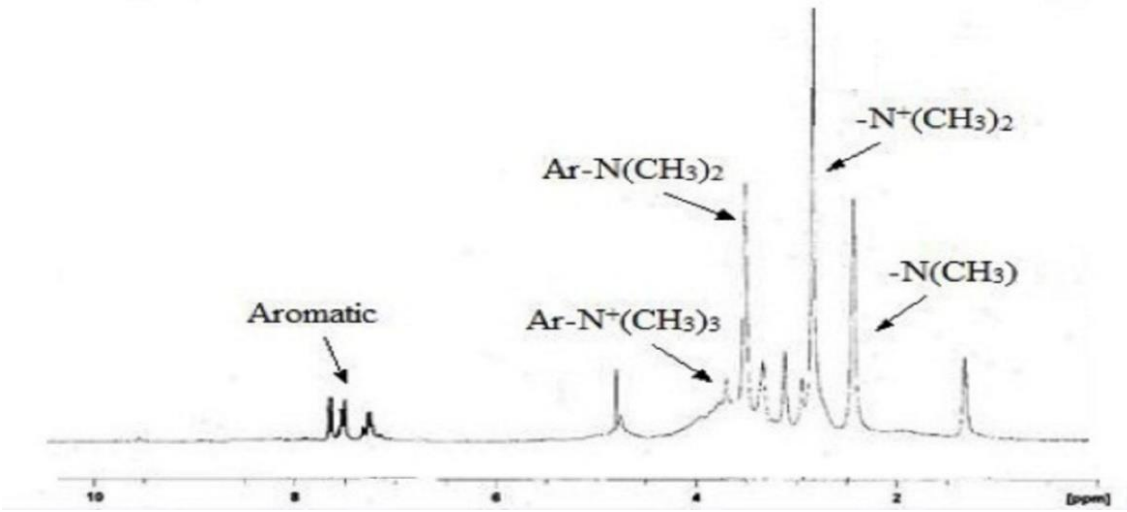


Figure 2. H-NMR spectrum of Methylated N-(4-N, N-dimethylaminobenzyl) chitosan

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Dependent Variables (Responses)	Model Type	R^2	Adjusted R^2	Predicted R^2	Adequate Precision	Lack of Fit (LOF)
Swelling ability (%) in glucose 0% (Y_1)	Linear	0.7225	0.6115	0.6227	8.418	Insignificant ($p>0.2$)
Log Swelling ability (%) in glucose 5% (Log Y_2)	2FI	0.6766	0.5599	0.5431	8.767	Insignificant ($p>0.1$)
Drug loading (%) (Y_3)	Linear	0.7705	0.6453	0.6137	8.048	Insignificant ($p>0.4$)

Table 3. Model characteristics

Previously, Abureesh et al [22]. reported the development of a glucose responsive drug delivery system using chitosan-PVA cross-linked hydrogel. Comparisoned with non-modified chitosan, the synthesized derivative (i.e., methylated aminobenzyl chitosan) is expected to exhibit increased charge density at physiological pH owing to the presence of two quaternized amino-functional groups per unit, thereby increasing the swelling ratio (%) through the Donnan effect [23]. In addition, introduction of two quaternized amino-moieties may increase the hydrophilicity of the polymer and increase the swelling ability (%). Moreover, because each unit of the synthesized chitosan derivative bears two amino-functional groups (i.e., aromatic and aliphatic amines) rather than the single amino-functional group (i.e., aliphatic amine) present in unmodified chitosan, the prepared methylated aminobenzyl chitosan-PVA hydrogel exhibits a higher degree of BA cross-linking. Consequently, this hydrogel demonstrates a greater capacity for glucose incorporation, and therefore an improved glucose-responsive release behavior is expected. Furthermore, although the pH of the medium can exert a significant effect on the swelling ratio (%) of chitosan–PVA hydrogels [22, 24], the permanent positive charges on the quaternized amino-functional groups of the synthesized derivative, which persist over a wide pH range, are expected to render the pH less influential on the swelling ratio (%) of the prepared hydrogel composed of methylated aminobenzyl chitosan.

In this study, the structure of methylated N-(4-N,N-dimethylaminobenzyl) chitosan (designated as methylated aminobenzyl chitosan) was confirmed by ^{1}H-NMR spectroscopy. As shown in the spectrum, the protons relevant to the aliphatic rings of the chitosan units were appeared in the range of 3-5 ppm. Due to being displaced by deuterium element existing in D_2O, four protons, among 11 protons available in each unit of chitosan, did not develop any peaks in ^{1}H-NMR spectrum. These protons were relevant to hydroxyl- and amino-functional groups of chitosan units [25]. Peaks corresponding to the methyl protons of the methylated and quaternized aliphatic amines of chitosan appeared in the range of 2–3 ppm, indicating the formation of the quaternized derivative. On the other hand, the protons relevant to quaternization of aromatic amines were appeared in the range of 3-4 ppm. Finally, presence of the aromatic ring in the structure of the synthesized polymer was confirmed by appearance of the aromatic ring protons between 7-8 ppm. It was found that due to unlocalization of the protons relevant to aromatic amino-functional groups, the degree of quaternization in aliphatic amines was higher than the relevant degree in aromatic amines. These findings are in line with the study carried out by Mahjub et al [14].

Preparation and characterization of the hydrogel

In the present study, a BA-crosslinked hydrogel, composed of methylated aminobenzyl chitosan and PVA, was prepared by freeze-thaw method. The insulin was loaded into the hydrogel using swelling method. The prepared hydrogels were characterized and were statistically optimized using box-behnken response surface methodology.

The experimentally achieved data for characterization of the suggested formulations were analyzed using design-expert® software and are summarized in Table 2. Using regression technique

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and one way analysis of variance (ANOVA), the data were fitted to the appropriate mathematical model. The fitted models were characterized by calculation of R^2, adjusted-R^2, predicted-R^2 and adequate precision. For explaining the correlation between factors and responses, the models were explained by polynomial mathematical equations. Finally, the optimized value for each factor was determined.

Swelling ability (%) in the glucose-free PBS

As shown in Table 2, the swelling percentages of the prepared hydrogels in the glucose-free PBS solution were varied from 51.8 ± 7.14 % (i.e., run No. 13) to 292.3 ± 34.72 % (i.e., run No. 10). For identifying the best significant model for prediction of swelling ability (%), the achieved results were statistically analyzed by one way ANOVA. For better predictability, the step-wise method was used for model reduction and elimination of non-significant factors.

The characteristics of the best-fitted 2-factorial interaction (2-FI) proposed model is summarized in Table 3. According to Table 3, the proposed model was found to be statistically significant ($p<0.05$), while the lack of fit (LOF) was non-significant ($p>0.05$), indicating the model's proper predictability. Analysis of variance for the proposed model revealed that the effect of the concentration of methylated aminobenzyl chitosan (X_1), as one of the main factors, is significant ($p<0.02$). Moreover, the binary interaction between the two other factors, including the concentration of PVA (X_2) and the concentration of BA (X_3), is also found to be significant ($p<0.005$). The coefficients of the significant variables on the swelling ability (%) in the glucose-free medium are shown in Eq.4 as follows:

$$Y_1 = -7.65375 + 9.07250 * X_1 + 17.16555 * X_2 + 9.9945 * X_3 - 1.25776 * X_2.X_3 \quad \text{(Eq. 4)}$$

Where:

Y_1: Swelling ability (%) of the hydrogel in PBS;

X_1: Linear co-efficient for the concentration of methylated aminobeznyl chitosan;

X_2: Linear co-efficient for the concentration of PVA;

X_3: Linear co-efficient of the concentration of boric acid;

$X_2.X_3$: The binary interaction coefficient of X_2 and X_3;

The 3-D response surface plots for changes in swelling ability (%) of the hydrogel in PBS are presented in Figure 3. As shown in Figure 3 (a), the swelling ability (%) of the hydrogel in glucose-free PBS was significantly increased by elevation of the concentration of methylated aminobenzyl chitosan.

The significant binary interaction between the concentration of PVA (X_2) and the concentration of boric acid (X_3) is shown in Figure 3 (b). As shown in the figure, at the lowest boric acid concentration (5 mg/mL), the swelling ability (%) of the hydrogel in PBS increased with increasing PVA concentration. However, at the highest concentration of boric acid (30 mg/mL), as the concentration of PVA increased, the swelling ability (%) dramatically declined. Furthermore, as represented in Figure 3 (b), at the lowest concentration of PVA (5 mg/mL), the swelling ability (%) of the hydrogel in PBS was sharply increased by rising the concentration of boric acid. However, at the highest concentration of PVA (15 mg/mL), increasing the concentration of boric acid resulted in a sharp decline in the swelling ability (%) of the hydrogel.

Similar to other chitosan derivatives, in aqueous media, methylated aminobenzyl chitosan forms a 3-D network structure which can swell and absorb high amounts of water [26]. Moreover, water molecules can also adsorb to the abundant pores present on the surface of the polymer [8, 27]. Based on the above findings, the swelling ability (%) is expected to increase sharply with increasing methylated aminobenzyl chitosan concentration.

The PVA, due to its hydrophilic characteristics, enhances the absorption of water molecules into the structure of the hydrogel network [28]. Moreover, the OH-functional groups present in the chemical structure of PVA can establish strong hydrogen bonds with H_2O molecules. Therefore, as it is obvious in glucose-free medium, in the low levels of boric acid, the swelling ability (%) was increased by PVA increase [22, 29]. However, in the highly cross-linked polymer (i.e., high levels of boric acid), increasing the concentration of PVA caused a decline in the swelling ability (%). This phenomenon can be justified by considering the establishment of hydrogen bonds between PVA and boric acid in

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PVA-BA complexes. In high concentrations of boric acid, the most hydroxyl-functional groups in the PVA structure were involved in hydrogen bonding with BA molecules. Therefore, the capacity of the hydroxyl-functional groups in the PVA structure for the establishment of hydrogen bonds with water molecules was reduced. The phenomenon leads to a reduction in water absorption and consequently resulted in the shrinkage of the hydrogel in the higher concentrations of boric acid [26]. In addition, a high degree of cross-linking reduces the number of pores on the hydrogel surface, thereby decreasing the adsorption of water molecules and consequently leading to hydrogel shrinkage.

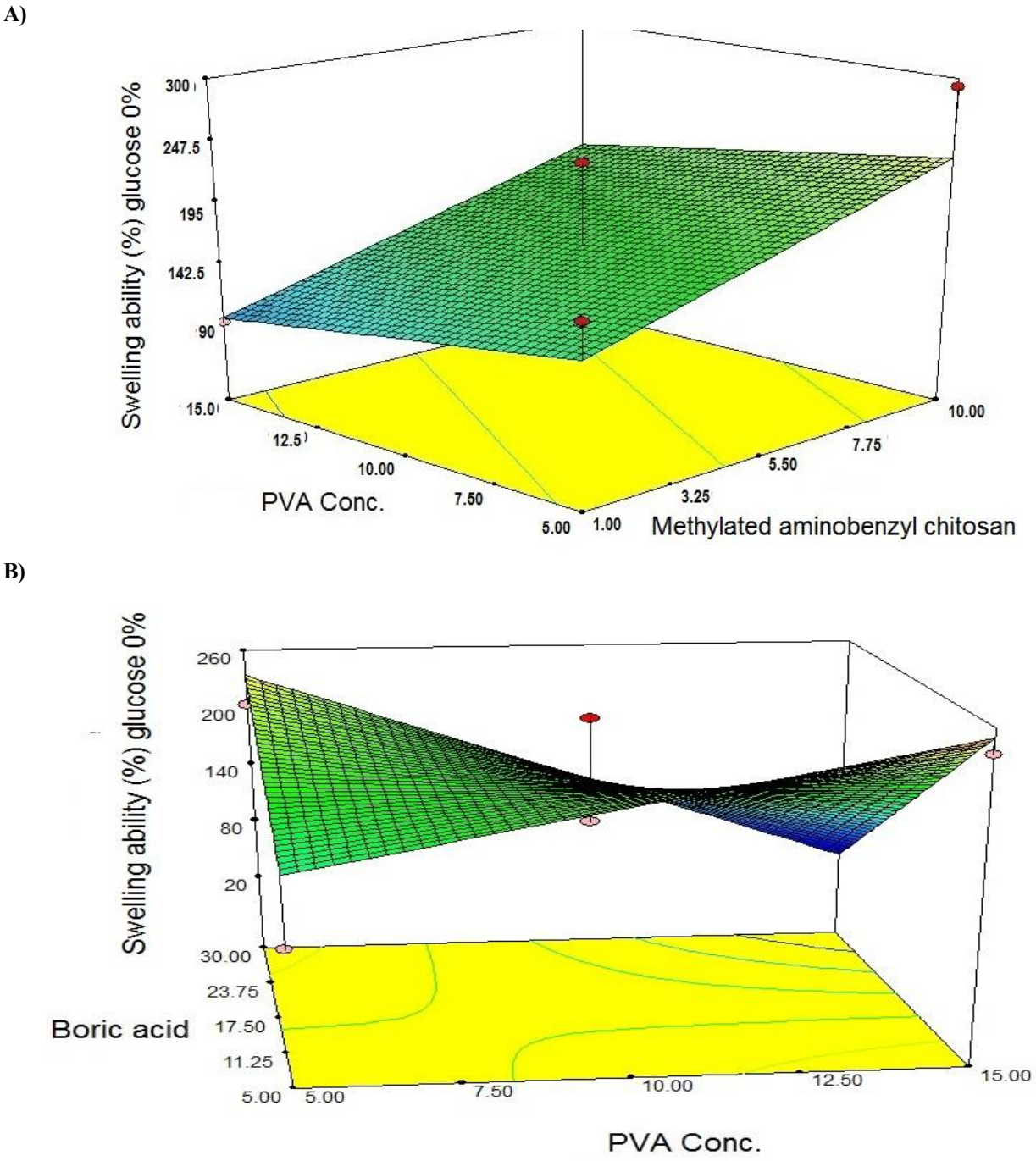


Figure 3. 3-D response surface plot for alteration in swelling ability (%) in glucose 0% due to changes in: A) Concentration of methylated aminobenzyl chitosan; B) Concentrations of PVA and Boric acid.

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Swelling ability (%) in PBS supplemented with glucose (5%)

As summarized in Table 2, the swelling percentages of the prepared hydrogels in the PBS solution containing glucose (5% w/v) varied from 55.7 ± 4.21 % (run No. 13) to 867.0 ± 20.85 % (run No. 1). For better statistical modeling and regression, the obtained data for swelling ability (%) were transformed to base-10 logarithm, and the results were statistically analyzed by ANOVA. For better predictability, the step-wise method was used for model reduction and elimination of non-significant factors.

The characteristics of the best-fitted 2-factorial interaction (2-FI) proposed model is summarized in Table 3. As presented in Table 3, the proposed significant model ($p<0.005$) exhibited proper characteristics while the LOF was found non-significant ($p>0.2$). The findings demonstrate proper predictability of the model. Analysis of variance for the proposed model revealed that the main factor of concentration of BA (X_3) is significant ($p<0.03$). Moreover, the binary interaction between the two factors, including concentration of methylated aminobeznyl chitosan (X_1) and concentration of BA (X_3) is also found significant ($p<0.01$). The co-efficients of the significant variables on the swelling ability (%) in PBS supplemented with glucose (5%) are shown in Eq.5 as follows:

$$\text{Log } Y_2 = +2.88659 - 0.064132 * X_1 - 0.037388 * X_3 + 0.004496 * X_1.X_3 \quad \text{(Eq.5)}$$

Where:

Y_2: Swelling ability (%) of the hydrogel in glucose solution (5%);

X_1: Linear co-efficient for concentration of methylated aminobeznyl chitosan;

X_3: Linear co-efficient of concentration of BA;

$X_1.X_3$: The binary interaction coefficient of X_1 and X_3;

The 3-D response surface plot representing the binary interaction between concentration of methylated aminobenzyl chitosan (X_1) and concentration of boric acid (X_3) on logarithm of swelling ability (%) of the prepared hydrogel in PBS solution containing glucose (5%) is shown in Figure 4. As shown in the figure, at the lowest concentration of boric acid (5 mg/mL), the logarithm of swelling ability (%) of the hydrogel was slightly decreased by increasing the concentration of methylated aminobenzyl chitosan from 1 mg/mL to 10 mg/mL. Otherwise, at the highest concentration of boric acid (30 mg/mL), rising the concentration of methylated aminobenzyl chitosan resulted in a sharp increase in the logarithm of swelling ability (%).

Moreover, as shown in Figure 4, at the lowest concentration of methylated aminobenzyl chitosan (1 mg/mL), increasing the concentration of boric acid caused the logarithm of swelling ability (%) in glucose (5%) to decline sharply. However, at the highest concentration of methylated aminobenzyl chitosan (10 mg/mL), increasing the concentration of boric acid caused a slight increase in the logarithm of swelling ability (%).

The achieved results revealed that in 5% glucose, the swelling ability (%) of the prepared hydrogel was affected by both the concentration of the chitosan derivative and BA concentration. Despite the glucose-free medium in which the swelling ability (%) of the hydrogel was increased by increasing the methylated aminobenzyl chitosan concentration in either low or high BA concentrations, in the presence of glucose (5%) and in low concentrations of BA, increasing the concentration of methylated aminobenzyl chitosan caused a slight decrease in swelling ability (%) of the hydrogel. However, in the high levels of BA, by elevation of methylated aminobenzyl chitosan concentrations, the swelling ability (%) was sharply increased. In this study, it is postulated that in the presence of glucose, the glucose molecules can compete with PVA to form a complex with boric acid. The glucose-BA complex can then lead to elevation in osmotic pressure which consequently results in an increase in the hydrogel's swelling ability (%) [30]. It is suggested that, at low BA concentrations, increasing the concentration of methylated aminobenzyl chitosan diminishes the ratio between boric acid molecules and the units of the chitosan derivative. It is assumed that swelling of the hydrogel in glucose 5% is mainly due to complexation of glucose with BA molecules and formation of glucose-BA complexes. Arum et al. [30] suggested that complexation between boric acid and glucose, increase the hydrophilicity in the hydrogel network and consequently leads to an elevation in

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swelling ability (%). Therefore, in reduced concentrations of BA, less glucose molecules may be incorporated in the BA-glucose complex, which then results in reduction in swelling ability (%). On the other hand, in the high levels of cross-linking agent (boric acid), the more glucose molecules can attach to the boric acid structures via both covalent and hydrogen bonding. This phenomenon leads to the generation of greater osmotic pressure and consequently increases the swelling ratio (%) of the hydrogel.

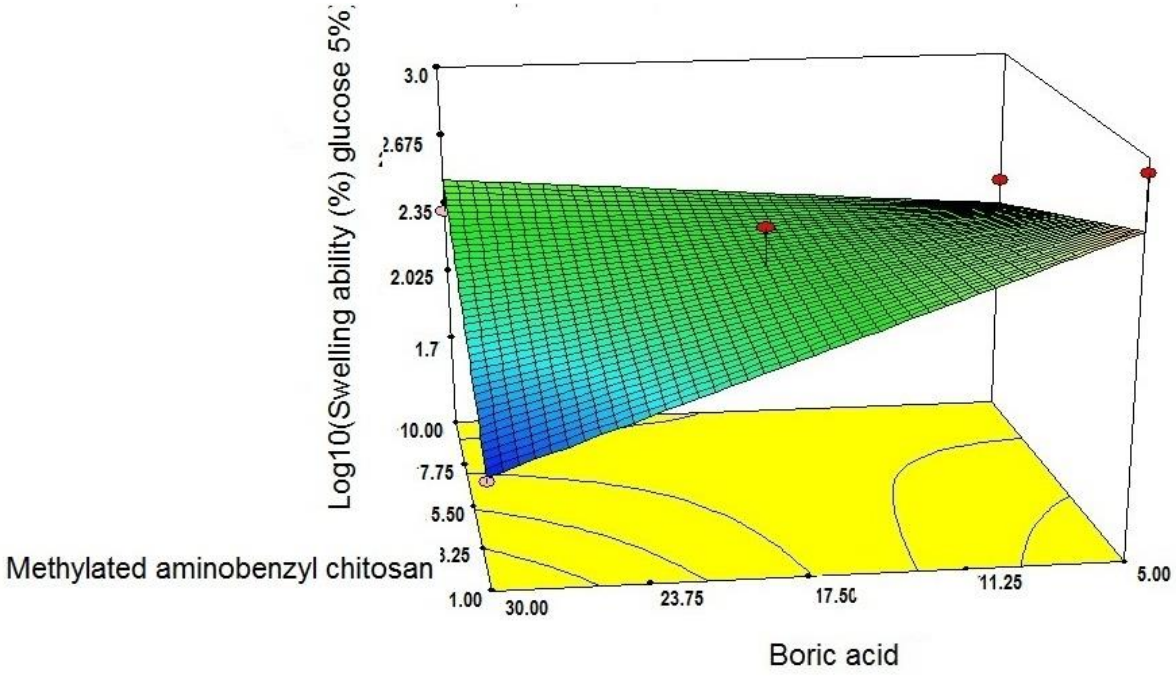


Figure 4. 3-D response surface plot for alteration in swelling ability (%) in glucose 5%

As it is presented in the manuscript, at low levels of methylated aminobenzyl chitosan, elevation of the boric acid concentration caused a sharp reduction in the swelling ability (%) of the hydrogel in 5% glucose. However, at high concentrations of the chitosan derivative, the swelling ability (%) in a glucose (5%)-containing medium was increased by BA concentration.

Since, in the presence of glucose, sugar molecules tend to form complexes with BA molecules through either covalent or hydrogen bonding, thereby causing hydrogel swelling, and given that BA molecules form covalent bonds with the amino-functional groups of methylated aminobenzyl chitosan units, a favorable ratio between boric acid and the chitosan derivative appears to be required to achieve adequate swelling in glucose-containing media [26].

At low concentrations of the chitosan derivative, increasing the boric acid concentration disrupts the proper ratio between boric acid and the amino groups of the chitosan derivative. Consequently, the excess boric acid cannot bind to the chitosan units; although these excess BA molecules may bind to glucose molecules, they cannot contribute to hydrogel swelling. Therefore, at low concentrations of the chitosan derivative, the swelling ability (%) of the hydrogel decreases with increasing boric acid concentration. On the other hand, at high concentrations of the chitosan derivative, sufficient amino-functional groups are available to complex with the excess boric acid molecules. Therefore, as the boric acid concentration increases, all cross-linking molecules can bind to the chitosan backbone, resulting in the incorporation of more glucose molecules into the hydrogel structure and consequently increasing the swelling ratio (%) through greater osmotic pressure. Similarly, Rohindra et al. [31] and Bidianto et al. [26] suggested that the swelling ability (%) of the hydrogel is mainly affected by the amount of cross-linking agent.

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Drug Loading (%)

As depicted in Table 2, the percentages of insulin loading in the prepared hydrogels were differed from 59.6 ± 5.29% (run No. 4) to 97.4 ± 8.17% (run No. 10). The obtained results were statistically analyzed by ANOVA for identifying the best significant model for predicting drug loading. For better predictability, the step-wise method was used for model reduction and elimination of non-significant factors.

The characteristics of the best-fitted linear proposed model are summarized in Table 3. As presented in the Table, the proposed significant model ($p<0.001$) exhibited proper characteristics and non-significant LOF ($p>0.8$). Analysis of variance for the proposed linear model revealed that, among all of the main factors, only concentration of methylated aminobenzyl chitosan exhibited a significant effect on the drug loading (%) ($p<0.001$). The co-efficient of the significant variable on the drug loading is summarized in Eq.6 as follows:

$$Y_3 = 65.24767 + 2.411 * X_1 \quad \text{(Eq.6)}$$

Where:

Y_3: Drug loading (%);

X_1: Linear co-efficient for concentration of methylated aminobenzyl chitosan;

The 3-D response surface plot of the variation in drug loading (%) due to changes in concentration of methylated aminobenzyl chitosan is shown on Figure 5. As it is exhibited in the figure, by elevation of the concentration of methylated aminobenzyl chitosan, the percentage of insulin loading in the hydrogel network was significantly increased.

Optimization and model validation

The optimization of the characteristics in the prepared hydrogel was performed using statistical and mathematical analysis. The suggested optimized factors as well as their corresponding predicted responses are depicted in Table 4. As shown in the Table, the suggested optimized values for concentration of methylated aminobenzyl chitosan (X_1), concentration of PVA (X_2) and concentration of BA (X_3) were 6.4 mg/mL, 5.2 mg/mL and 5.0 mg/mL, respectively. It is predicted that in the optimized conditions, the prepared hydrogel exhibits the minimized swelling ability (%) in glucose-free PBS solution, the maximized swelling ability (%) in PBS solution containing glucose (5%) and the maximized drug loading (%).

In order to determine the predictability and reliability of the proposed models, the optimized hydrogel was experimentally prepared and characterized (n=5). For each response, the prediction error (%) was calculated according to Eq. 7, as follows:

$$Prediction\ Error\ (\%) = \frac{Observed\ Response - Suggested\ Response}{Observed\ \ Response} * 100 \quad \text{(Eq.7)}$$

Table 4. Suggested Optimized Formulation and Predicted Responses

Suggested Optimized Factors			Predicted Responses		
Concentration of Methylated Aminobenzyl Chitosan (X_1) (mg/mL)	Concentration of PVA (X_2) (mg/mL)	Concentration of Boric Acid (X_3) (mg/mL)	Swelling ability (%) in glucose 0% (Y_1)	Log Swelling ability (%) in glucose 5% (Log Y_2)	Drug loading (%) (Y_3)
6.4	5.2	5.0	105.83	2.66 (Y_2=457.08)	87.57

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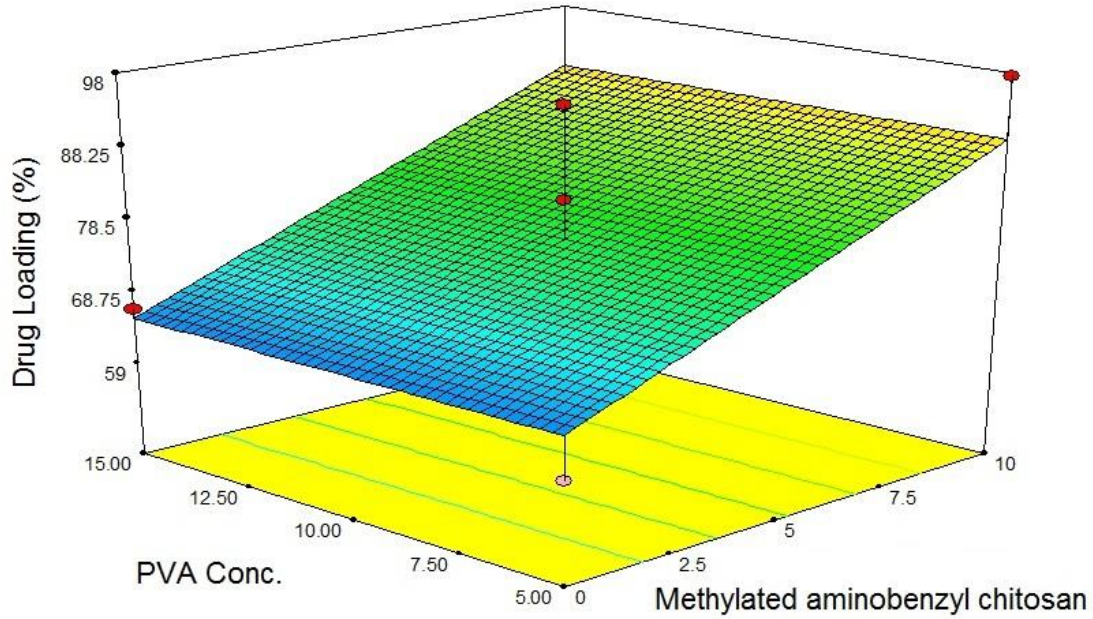


Figure 5. 3-D response surface plot for alteration in drug loading (%)

The experimentally observed responses for characterization of the optimized formulation, as well as the appropriate calculated prediction errors (%) are summarized in Table 5. As shown in the Table, all calculated prediction errors (%) were well below 15%, indicating high predictability and reliability of the fitted models.

Table 5. The experimentally obtained responses and the calculated Prediction Error (%) (n=5)

Dependent variables (Responses)					
Swelling ability (%) in glucose 0% (Y_1)		**Log Swelling ability (%) in glucose 5% (Log Y_2)**		**Drug loading (%) (Y_3)**	
Observed (Mean±SD)	Error%	Observed (Mean±SD)	Error%	Observed (Mean±SD)	Error%
93.43± 8.72	- 13.26	2.90± 0.76 (Y_2=794.32±86.43)	+8.49	83.61±8.3	-4.73

FT-IR Studies

The chemical structure of the optimized hydrogel was studied by FT-IR spectroscopy. For better comparison, the spectra of methylated aminobenzyl chitosan and PVA were also examined. The FT-IR spectra are presented in Figure 6. As shown in the Figure, in methylated aminobenzyl chitosan, the main peaks appeared at 3404 cm^{-1} (vibration of OH and NH), 2960cm^{-1} and 2937 cm^{-1} (vibration of CH), 1649 cm^{-1} and 1551 cm^{-1} (vibration of amide), and 926 cm^{-1} (vibration of carbohydrate ring). In the FT-IR spectrum of PVA, the main peaks appeared at 3425 (vibration of OH), 2919 $cm^{-1,}$ and 1448 cm^{-1} (vibration of CH). In the prepared hydrogel, the main peaks were identified at 3412 cm^{-1} (vibration of OH and NH), 2921 cm^{-1} and 2852 cm^{-1} (vibration of CH), 1422 cm^{-1} (vibration of B-O), 836 cm^{-1} (vibration of B-OH), and 663 cm^{-1} (vibration of O-B-O). The last three peaks in the FT-IR spectrum of the prepared hydrogel indicate cross-linking and formation of the covalent complex between BA and PVA.

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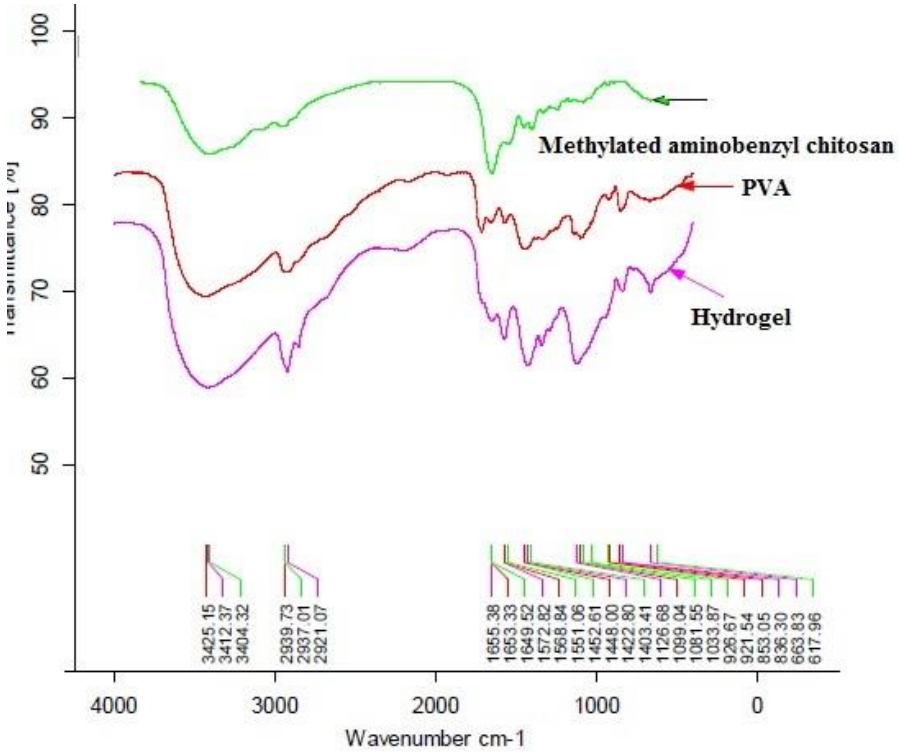


Figure 6. FT-IR spectra of methylated aminobenzyl chitosan, PVA, and the optimized hydrogel

Morphological Studies

The optimized hydrogel was examined by SEM, and the obtained images are illustrated in Figure 7. As presented in Fig. (a), the drug-free hydrogel exhibits a porous, rough, and coarse surface, whereas the insulin-containing hydrogel displays a scalier and flakier surface (Figure 7 (b)). Moreover, the visualized strips of insulin molecules on the surface of the insulin-containing hydrogel revealed surface adsorption of the drug molecules (Figure 7 (b)).

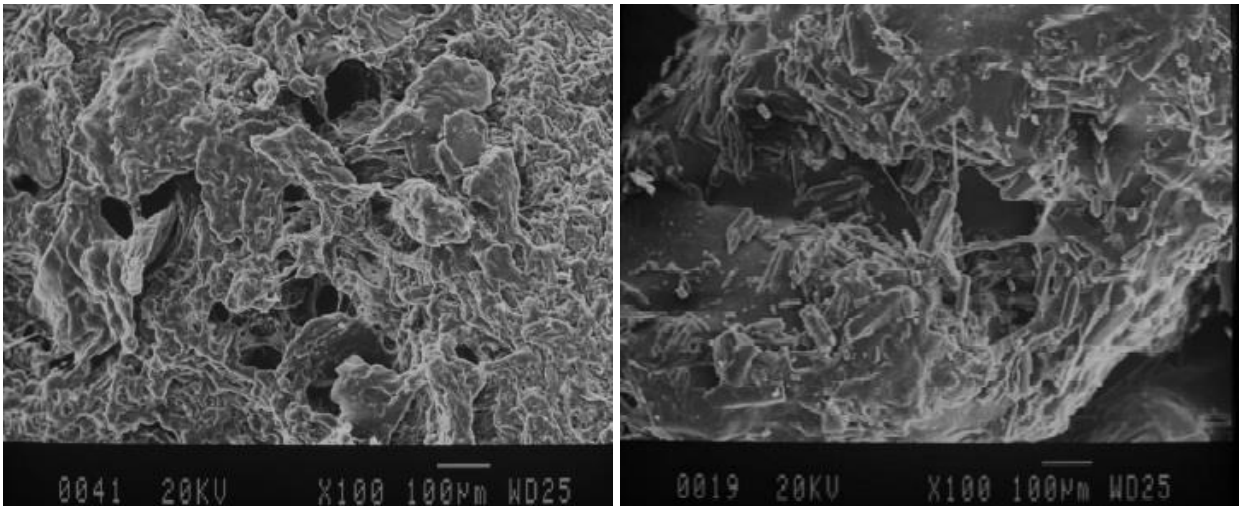


Figure 7. SEM images of: A) Insulin-free optimized hyrogel; B) Insulin- containing optimized hydrogel

The SEM images of the cross-linked methylated aminobenzyl chitosan- PVA hydrogel revealed presence of porous, coarse, and irregular surfaces. These findings are in agreement with the previous studies [22, 25]. In addition, as shown in the figure, loading of insulin molecules caused significant changes in the surface morphology of the hydrogel and it became scalier and flakier. It is suggested that although most of insulin molecules were entrapped inside the hydrogel system, some insulin molecules

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can still adsorb to the surface of the hydrogel and change the surface morphology. As it is visualized in the figure, strips of the insulin molecules which were connected to the surface of the loaded hydrogel can be obviously observed. The adsorption of the drug molecules in the surface of hydrogel was also reported by Mohamed et al. [32] Additionally, the changes in surface morphology could result from partial collapse of the polymer network during drying [22].

In vitro Release Study

The *in vitro* release profile of insulin from the optimized hydrogel was studied in PBS containing different concentration of glucose, including either 0% (w/v), 3% (w/v) or 5% (w/v). The release profile is shown on Figure 8. Although an initial burst release of insulin from hydrogels was observed in the first 15 min of incubation, in further time intervals, all *in vitro* release profiles showed sustained and prolonged release behavior. As depicted in the figure, the release rate of insulin from the optimized hydrogel was glucose-dependent and it was increased by increasing the concentration of glucose. Although, after 480 min of incubation, only 26.1±1.84% of insulin was released in glucose-free phosphate buffer, in the same period of time, the percentage of insulin release was 52.7 ± 3.11% and 78.9 ± 3.85% in the phosphate buffer containing 3% and 5% of glucose, respectively. The differences between the *in vitro* release profiles were examined by calculation of the similarity factor. All calculated similarity factors were less than 32, indicating the significant difference between the obtained three release profiles. The observed glucose-dependent release behavior indicates development of a glucose-responsive intelligent drug delivery system.

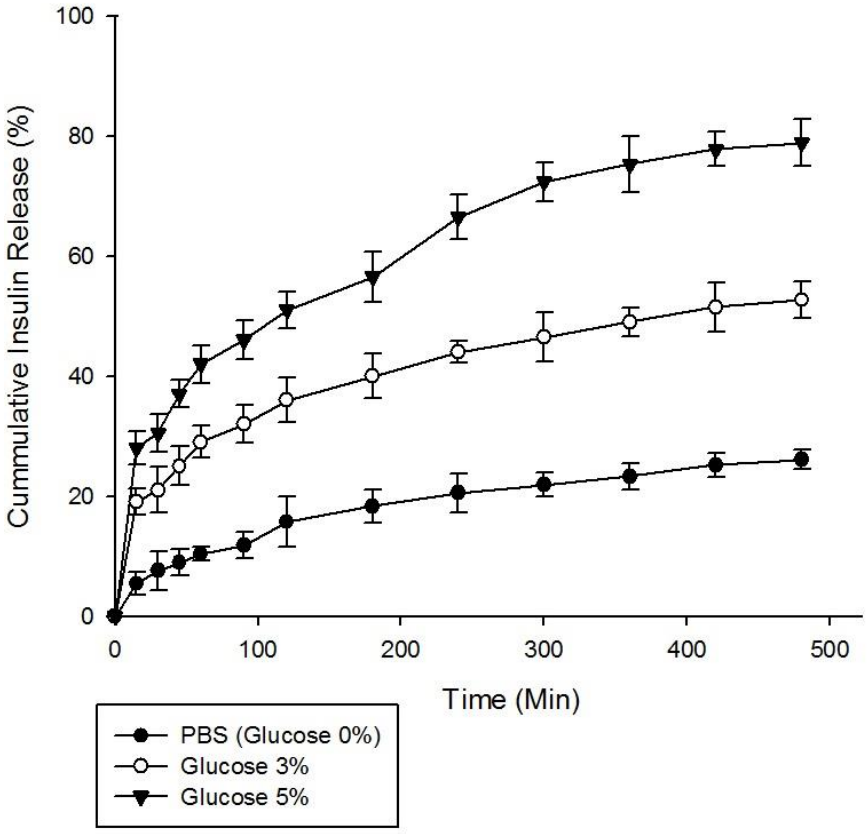


Figure 8. *In vitro* release studies in various release media containing glucose 0%, 3%, and 5% (n=3).

It is postulated that the observed burst release is related to the release of insulin molecules that had accumulated and adsorbed onto the surface of the prepared hydrogels. However, the sustained release behavior is suggested to result from the release of insulin molecules entrapped within the 3-D structure

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of the hydrogel. As shown in the release profile, the insulin release from methylated aminobenzyl chitosan-PVA hydrogel was glucose-dependent in a manner that increasing the glucose concentration in the medium caused an elevation in the release rate. As previously discussed, glucose molecules are suggested to disrupt the interactions in PVA-BA complexes by forming either covalent or hydrogen bonds with BA. This phenomenon can lead to the incorporation of glucose molecules into the hydrogel structure, which in turn causes swelling of the system through osmotic pressure and consequently accelerates insulin release in the presence of glucose.

Animal Studies

The changes in blood glucose of the studied animals are depicted in Figure 9. In non-diabetic rats that were treated with the insulin-containing hydrogels (group I), the implantation caused only a slight decrease in the blood glucose concentration, and it reached 89.6 ± 6.54% of its initial value after 36 h of post-surgery. This finding indicated the extreme slow release of insulin in non-diabetic subjects. On the other hand, it was revealed that in diabetic animals treated with the insulin-containing hydrogels (group II), the level of glucose declined and reached a plateau level of approximately 50%, indicating a higher release rate of insulin from the hydrogels. However, in group III, which received subcutaneous injection of NPH insulin, a single insulin injection caused a dramatic decrease in glucose concentration, with plasma glucose levels reaching 31.5 ± 7.43% of baseline at 18 h post-injection. It was shown that, unlike group II, in which plasma glucose levels reached a plateau, glucose levels increased in group III after 18 h post-injection, indicating plasma elimination of the subcutaneously injected insulin.

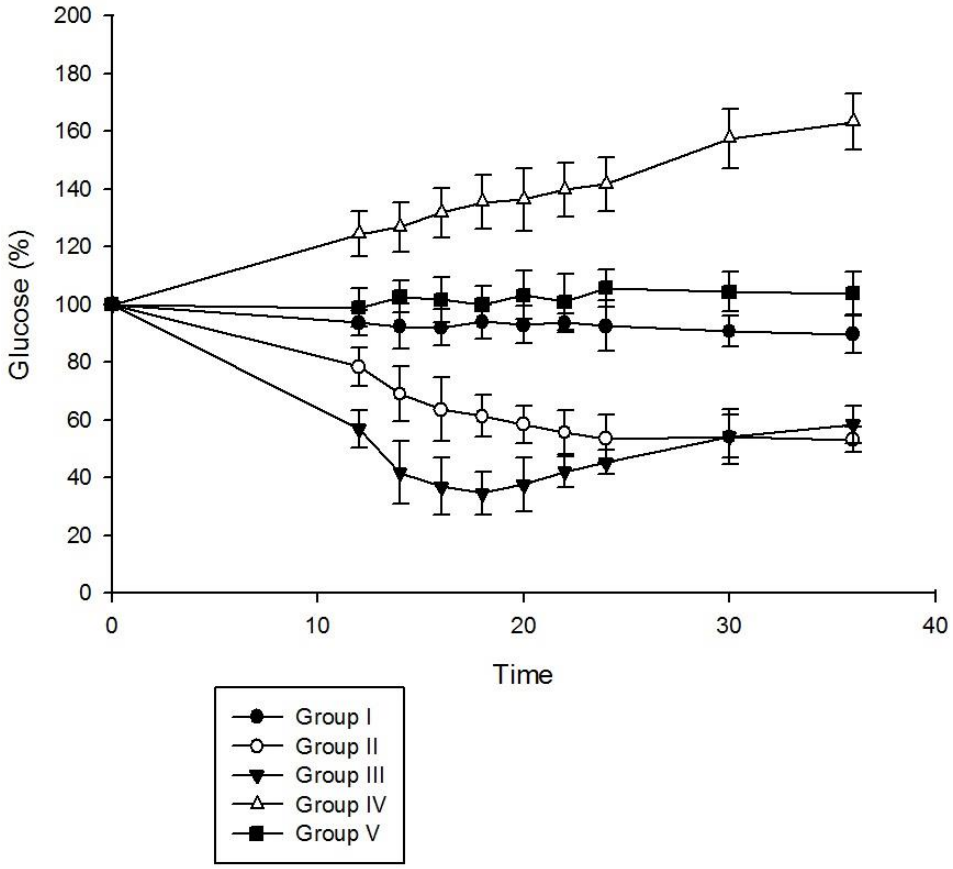


Figure 9. *In vivo* blood glucose level in the studied animals (n=3); Group I: non-diabetic animals receiving insulin-containing hydrogel; Group II: diabetic animals receiving insulin-containing hydrogel; Group III: diabetic animals receiving subcutaneous NPH insulin; Group IV: diabetic animals receiving insulin–free hydrogel; Group V: non-diabetic animals receiving insulin-free hydrogel.

As depicted in the figure, in group IV, which consisted of diabetic animals treated with insulin-free hydrogels, the blood glucose levels were dramatically increased over time, reaching 163 ± 12.83% of the

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initial value. On the other hand, in non-diabetic animals receiving glucose-free hydrogels (Group V), non-significant changes in blood glucose level were observed over time. The data for the two latter groups of animals (groups IV and V) confirmed that in both non-diabetic and diabetic animals, the procedure of implantation as well as the hydrogel structure had no significant impact on the blood glucose level.

In the present study, the *in vivo* efficacy of the prepared glucose-responsive hydrogel was studied using male Wistar rats. As summarized in the figure, a significant reduction in blood glucose level was observed in diabetic rats which received the insulin-containing glucose-responsive hydrogel. On the other hand, the intraperitoneal implantation of the hydrogel in non-diabetic animals revealed only a slight decrease in blood glucose level. This phenomenon can justify the glucose-responsive behavior of the cross-linked methylated aminobenzyl chitosan-PVA hydrogel. According to the experimental data, there was no evidence that the chemical structure of the hydrogel or the implantation procedure affected blood glucose levels.

In the current research, one group of diabetic animals received subcutaneous injection of a commercially available sustained release formulation of insulin (i.e., NPH). It was shown that in this group of animals, insulin injection caused a dramatic decline in blood glucose level in early time intervals. However, despite the insulin-containing hydrogels which posed sustained efficacy in reducing blood glucose level over time, the efficacy of injection formulation was diminished in further time periods.

Conclusion

Based on the findings obtained, an intelligent, glucose-sensitive hydrogel composed of methylated 4-(N,N-dimethyl aminobenzyl) chitosan, designated as methylated aminobenzyl chitosan, was developed for glucose-responsive delivery of insulin. Firstly, the methylated aromatic derivative of chitosan was synthesized and the structure was confirmed using 1H-NMR spectroscopy. The methylated aminobenzyl chitosan-PVA hydrogel was prepared by cross-linking using boric acid molecules. The preparation of the hydrogel was optimized using Box-Behnken response surface methodology. The independent variables were the concentrations of the synthesized derivative, PVA, and boric acid, while the dependent variables were defined as the swelling ability (%) in glucose-free PBS, the swelling ability (%) in PBS supplemented with glucose (5%), and drug loading. The optimized hydrogel showed minimized swelling ability (%) in glucose-free medium, while the swelling ability (%) in glucose-containing medium and drug loading (%) were maximized. The FT-IR spectroscopy revealed formation of covalent bonds between boric acid and PVA in the chemical structure of the hydrogel. Furthermore, although SEM morphological studies of insulin-free hydrogel showed porous and rough surfaces, the loading of insulin molecules was revealed to cause morphological changes and formation of scaly and flaky surfaces. It seems that, although the insulin molecules were mainly entrapped inside the hydrogel, surface adsorption insulin molecules also occurred to some extent. The in vitro release profile exhibited a glucose-responsive release of insulin from the hydrogel. Finally, in the in vivo study, intraperitoneal implantation of the prepared hydrogel into the abdominal wall of the diabetic rats demonstrated greater efficacy in lowering blood glucose than in non-diabetic rats, indicating the glucose-sensitive behavior of the developed system.

However, although the developed system was shown to exhibit proper glucose-responsive behavior in the studied male Wistar rats, further investigation is required before it can be considered suitable for clinical application at the bedside. As there are some boric acid derivatives such as phenyl boronic acid (PBA) that can be incorporated as the cross-linker in the structure of the hydrogel; therefore, the efficacy of these derivatives on glucose-dependent release behavior of the system should be studied. In addition, the stability the system upon storage should be determined. Furthermore, although chitosan itself is classified as a "generally regarded as safe (GRAS)" compound, limited data are available on the toxicity of the synthesized derivative. Therefore, for clinical translation, the safety and toxicity profile of the synthesized derivative as well as the prepared hydrogel and its degradation products should be investigated. On the other hand, the efficacy of the hydrogel in maintaining normoglycemia should be investigated in diabetic patients. Furthermore, for

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commercial preparation, the fabrication procedure should be modified, and the effects of the sterilization process on the properties of the hydrogel should be investigated.

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Conflict of Interest

No conflicts of interest were reported by the authors.

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