Synthesis and characterization of chitosan-based pH-sensitive biofilm: An experimental design approach for release of vitamin B_{12}

Ali Onal[1], Hulya Demir[1]*, Olcay Kaplan Ince[2], Muharrem Ince[3]

[1]Department of Nutrition and Dietetics, Yeditepe University, Istanbul, Turkey; [2]Department of Chemical Technologies, Munzur University, Tunceli, Turkey; [3]Department of Gastronomy and Culinary Arts, Munzur University, Tunceli, Turkey

***Corresponding Author:** Hulya Demir, Department of Nutrition and Dietetics, Yeditepe University, Istanbul, Turkey. Email: hulya.demir@yeditepe.edu.tr

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Abstract

Hydrogels because of their unique potentials, such as highwater content and hydrophilicity, are of interest for the controlled release of drug molecules. In the present study, a biofilm was produced using chitosan, a natural polymer. Characterization analyses of the synthesized biofilm were performed using Fourier-transform infrared spectroscopy (FT-IR), thermogravimetric analysis-differential thermal analysis, x-ray diffraction, and scanning electron microscopy-energy dispersive x-ray spectrometry. The analysis results of the film before loading were compared to the analysis made after adding vitamin B_{12} to the film. The release percentage of vitamin B_{12} was investigated by using ultraviolet-visible spectrophotometery. The FT-IR bands of vitamin B_{12} were in the range of 2,800-3,500 cm^{-1} and 1,000 cm^{-1}-1,750 cm^{-1}. When pH increased from 3 to 7, release of vitamin B_{12} from the biopolymer increased up to pH 5. While examining the release potential, parameters, such as pH, initial concentration of vitamin B_{12}, release time, and solution volume, were optimized by using response surface methodology. It was determined that with the increase in release time and the initial concentration, pH contributed positively to the release of vitamin B_{12}. The following conditions were determined for release of vitamin B_{12} from the biofilm: pH: 4.2, initial concentration of vitamin B_{12}: 54.0 mg L^{-1}, the release time: 117 min, and volume of solution: 2.1 mL. The analysis of variance results showed that the determination coefficients for the use of synthesized biofilm in vitamin B_{12} release were high and R^2 =0.9704.

Keywords: biopolymer, characterization, response surface methodology, vitamin B_{12}

Introduction

Hydrogels are three-dimensional, hydrophilic, and polymeric network structures that can absorb large amounts of water or biological fluids (Ayhan *et al*., 2007). Hydrogels are prepared by the polymerization reaction of one or more monomers and are insoluble because of the presence of chemical or physical cross-links between the main chains. Hydrogels have a wide usage, especially in medicine and pharmacy (Bajpai and Sharma, 2004). They resemble living tissues more than other synthetic biomaterials. This is due to the high water content and soft structure of hydrogels. Owing to the high biocompatibility of hydrogels, they are used in contact lenses, biosensor membranes, artificial skin material, and drug delivery systems. Hydrogels are classified according to their ionic charge, such as neutral, anionic, cationic, or ampholytic. Hydrogels exhibit swelling behaviour that changes depending on the external environment conditions. These polymers are called "physiologically responsive hydrogels" (Gupta *et al*., 2020). Main factors that affect the swelling behavior of physiologically or

environmentally sensitive hydrogels are pH, temperature, ionic strength, and electromagnetic radiation. All pH-sensitive polymers contain acidic groups (e.g., carboxylic acid and sulfonic acid) and basic groups (e.g., sulfonic acids) that are capable of either accepting or donating protons in response to changes in environmental pH (Jin *et al*., 2016). Changes in environmental pH lead to structural changes, as ionizable groups are attached to the polymer structure, and there is a change in the dissolution of polymers and swelling behaviour of hydrogels. The structures of ionic polymers, which exhibit pH-sensitive behavior and are frequently the subject of study are poly(methacrylic acid) (PMAA), poly(acrylamide) (PAAm), poly(acrylic acid) (PAA), Poly(diethylaminoethyl methacrylate) (PDEAEMA), and poly(N,N'dimethylaminoethyl methacrylate) (PDMAEMA). However, polymers containing phosphoric acid derivatives were also examined (Ghorbanloo and Heidary, 2017).

Upon exposure to the aqueous medium of appropriate pH and ionic strength, some groups ionize and develop fixed charges in the polymer network, and pH-dependent swelling or shrinkage of the hydrogel causes electrostatic repulsive forces, resulting in drug release (Ghorbanloo and Heydari, 2017).

For any drugs to be administered orally, some physiological aspects of the body, and more specifically that of the gastrointestinal tract (GIT) must be recognized. For example, pH change occurs throughout the gastrointestinal system (Bajpai and Sharma, 2004); however, more delicate changes occur in various body tissues. Various approaches are used for colon-targeted drug delivery, including pH-dependent swelling-controlled systems (Bajpai and Sharma, 2004), delayed-release delivery systems (Gupta *et al*., 2020), intestinal pressure-controlled colon delivery capsules (Jin *et al*., 2009), and enzymatically degradable systems using a variety of enzymes (Ghorbanloo and Heidary, 2017).

A comprehensive review of the literature available so far on drug release studies has revealed that most *in vitro* release studies involved measuring of release rates from the drug-loaded polymer matrix at different pH environments. In order words, HCl solution was used to simulate gastric juice, and phosphate buffer with pH 6.8 was used to simulate intestinal fluid. Oral administration of therapeutic compounds is the most convenient, mode of administration, although it is not used for many drugs that are not able to overcome their many natural barriers (Dengre *et al*., 2000). The presence of pathological conditions affecting GIT also inhibits the absorption of active ingredients after oral administration. Vitamin B_{12} is an example of such drugs. Deficiency of vitamin B_{12} causes various diseases. Controlled release of vitamin B_{12} increases its concentration in the liver and blood serum level, and helps to prevent or treat cobalt deficiency in the body. Vitamin B_{12} has been preferred as a model drug because of its neutral behaviour and high solubility in water. Patients with pernicious anaemia and other intestinal disorders cannot absorb even a small amount of vitamin B_{12} found in food, causing vitamin B_{12} deficiency, therefore leading to lifelong intake of intramuscular injections of vitamin B_{12} (Bajpai and Sharma, 2004; Sarti *et al*., 2012). Adequate oral absorption of vitamin B_{12} is achieved by using its high daily doses (Gupta *et al*., 2020).

To date, no serious efforts have been made to increase the oral bioavailability of vitamin B_{12} by improving its penetration into the intestinal mucosa. Poly(N-vinyl-2-pyrrolidone) (PVP) is known as a good biocompatible compound. The non-toxic nature and higher hydrophilicity of PVP encourages its use for drug delivery devices. Another important point is that the synthesis of a gel does not require a high temperature or the use of some organic solvents, so the probability of loss of activity during the incorporation of a drug is minimal. The present study aims to close this gap in the field by regulating swelling behavior with the help of a cross-linker, designing drug delivery systems that can deliver the desired amount of vitamin B_{12} for the desired time, and with the experimental data to be obtained.

Polyacrylic acid (PAA) and its copolymers, because of their multifunctional structure, unique properties, and good biocompatibility, are used as carriers in drug delivery systems (Jin *et al*., 2009). Inter-polymer complexes between PVP and PAA are examined to develop new mucoadhesive drug carriers by taking advantage of hydrogen bonding between the carboxyl groups of both PAA and PVP. The adhesion strength and drug release rate of PVP-PAA inter-polymer complexes are controlled by varying the pH values as well as the mole ratios of PVP and PAA. The complex appears as an adequate carrier for the mucoadhesive drug delivery system (Jin *et al*., 2016). The copolymer can form both intrapolymer and interpolymer complexes between PVP and PAA components. PAA-cysteine (PAA-cys) conjugate has been shown to provide a relatively higher absorption of substrates in the rat intestinal mucosa both *in vitro* and *in vivo* (Ghorbanloo and Heidary, 2017; Jin *et al*., 2009). In addition, PAA-cys solution and microparticles have been recently highlighted in *in vitro* studies as having a promising potential for intestinal permeability enhancer of vitamin B_{12} (Ghorbanloo and Heidary, 2017).

The present study aims to synthesize and characterize various pH-sensitive polymers, to examine the release of vitamin B_{12} by loading into the synthesized polymers and to develop hydrogels suitable for vitamin B_{12} release.

Material and Methods

High-purity chemicals, including acrylic acid, chitosan, N, N`-methylene bisacrylamide, acetic acid, ammonium persulfate and vitamin B_{12} were purchased from Sigma Aldrich (ABD).

Use of Central Composite Design (CCD) in Experimental Studies

A CCD, which is a statistical approach, combined with the response surface methodology (RSM), is a suitable system for optimizing the levels of parameters that affect release of drug in the experimental study. This method is a useful method for analyzing the effects of some independent variables on RSM along with a set of statistical and mathematical data. In addition, various estimations and tests, such as estimation and testing of the regression equation with the CCD technique, the factor effect testing, model determination coefficient calculation, optimal level combination estimation, and response surface properties around it, are provided. The CCD is known as a regression analysis and is used to estimate parameters and establish relationships between test indices and periodic variables. The experimental design approach is particularly suitable for optimizing complex synergies or antagonistic effects between variable states (Ince and Ince, 2019). In this study, a CCD containing four and five levels of each of these factors was chosen to examine and optimize the effect of the selected variables. A CCD experiment design (Table 1) was created to measure the effect of each variable, namely pH (X_1), initial concentration (X_2), release time (X_3), and solution volume (X_4), on drug release. Variable levels were coded as -1, 0, and +1, respectively, and were defined as low, medium, and high variables. Also, +2 and -2 star points were defined for each experimental group, corresponding to +α and -α, respectively. The effects of main, interaction, and quadratic variables were modelled using CCD. This experimental design was carried out to minimize the effects of uncontrollable parameters and was based on controllable factors. In addition, for optimizing the process parameters, their interactions were performed using a CCD with a minimum number of experiments. The CCD design in question included the ranges of the independent variables that were preferred in the experimental study and that had an effect

Table 1. The analysis of variance (ANOVA) results of response surface quadratic model.

Source	Sum of squares	Df	Mean square	F-value	*p*-value Prob > F	
Model	6482.93	14	463.07	35.08	< 0.0001	significant
X_1: pH	57.20	1	57.20	4.33	0.0549	
X_2: Initial concentration	226.63	1	226.63	17.17	0.0009	
X_3: Release time(min)	1,155.79	1	1,155.79	87.55	< 0.0001	
X_4: Solution volume	2,596.88	1	2,596.88	196.70	< 0.0001	
$X_1 X_2$	20.36	1	20.36	1.54	0.2333	
$X_1 X_3$	6.06	1	6.06	0.46	0.5083	
$X_1 X_4$	22.68	1	22.68	1.72	0.2097	
$X_2 X_3$	19.91	1	19.91	1.51	0.2383	
$X_2 X_4$	45.39	1	45.39	3.44	0.0835	
$X_3 X_4$	112.10	1	112.10	8.49	0.0107	
X_1^2	959.68	1	959.68	72.69	< 0.0001	
X_2^2	34.11	1	34.11	2.58	0.1288	
X_3^2	115.56	1	115.56	8.75	0.0098	
X_4^2	1,549.08	1	1,549.08	117.34	< 0.0001	
Residual	198.03	15	13.20			
Lack of fit	176.42	10	17.64	4.08	0.0670	not significant
Purity Error	21.61	5	4.32			
Cor Total	6680.96					
R^2	0.9704					
Adj R^2	0.9427	29				
Pred R^2	0.8432					
Adeq precision	22.004					

on adsorption, and the responses corresponding to these variables (Table 2).

Synthesis of Biopolymer Material and Loading of Vitamin B_{12}

Biopolymer production

Acrylic acid, 10 mL, of was taken and filled with ultrapure water to make the final volume up to 50 mL. The solution was mixed in a magnetic heater stirrer until it became homogeneous. Then 0.5 g of chitosan was added, and mixing process was continued for 15 minutes. Mixing was continued by adding 0.6 g of N,N`-methylene bisacrylamide. Then 0.3 g ammonium persulfate (APS) was added and mixed.

Loading the biopolymer with vitamin B_{12}

The films were loaded with vitamin B_{12} according to the concentrations and volumes given in the Table 2. Vitamin B_{12} release was determined by using ultraviolet-visible (UV-Vis) spectrometer.

Results and Discussion

UV-Vis Spectrophotometer Analyses

In present study, UV-Vis spectrophotometer was used to examine the vitamin B_{12} release from biopolymer. By scanning the wavelength in the range of 300-600 nm, the spectrum (Figure 1) was obtained and the maximum absorption wavelength was determined as

Table 2. Independent variables and their levels.

Run	Factor 1	Factor 2	Factor 3	Factor 4	Response
	X_1: pH	X_2: Initial concentration (mgL^{-1})	X_3: Release time (min)	X_4: Solution volume (mL)	Release (%) of vitamin B_{12} (mgL^{-1})
1	3	50	60	1	49.8
2	3	80	120	1	80.8
3	7	50	120	1	78.4
4	7	80	120	1	92.2
5	7	50	60	2.5	78.4
6	3	50	120	1	73.1
7	5	65	30	1.75	78.2
8	3	80	60	1	66.0
9	7	80	120	2.5	99.0
10	5	65	90	1.75	100.0
11	7	80	60	2.5	93.8
12	7	50	60	1	58.8
13	5	65	90	1.75	95.0
14	5	65	90	1.75	98.0
15	9	65	90	1.75	74.2
16	3	50	120	2.5	95.1
17	5	95	90	1.75	95.0
18	5	65	90	1.75	99.0
19	3	80	60	2.5	85.7
20	5	65	90	1.75	95.0
21	5	65	90	0.25	44.4
22	7	80	60	1	67.2
23	5	65	90	1.75	96.7
24	3	50	60	2.5	84.2
25	3	80	120	2.5	93.3
26	7	50	120	2.5	95.0
27	1	65	90	1.75	73.1
28	5	65	150	1.75	100.0
29	5	65	90	3.25	90.1
30	5	35	90	1.75	90.7

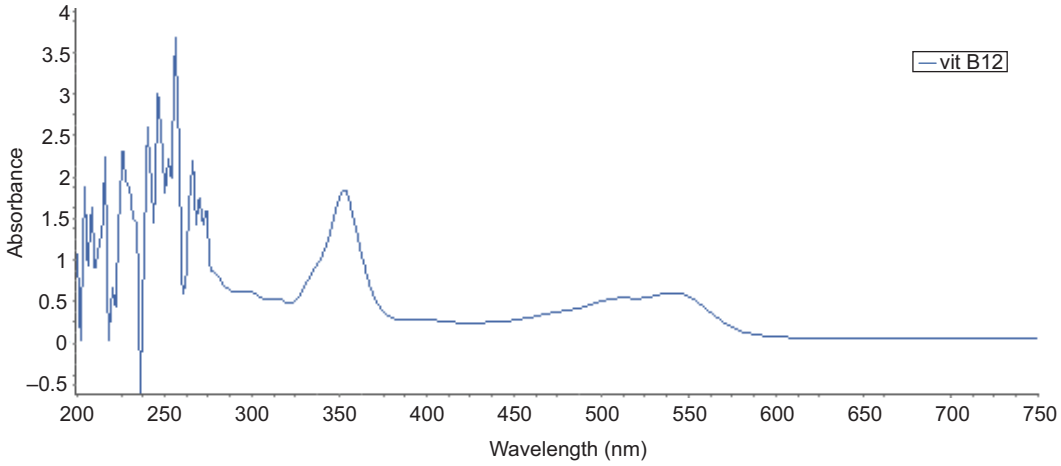

Figure 1. UV-Vis spectrum of vitamin B_{12}.

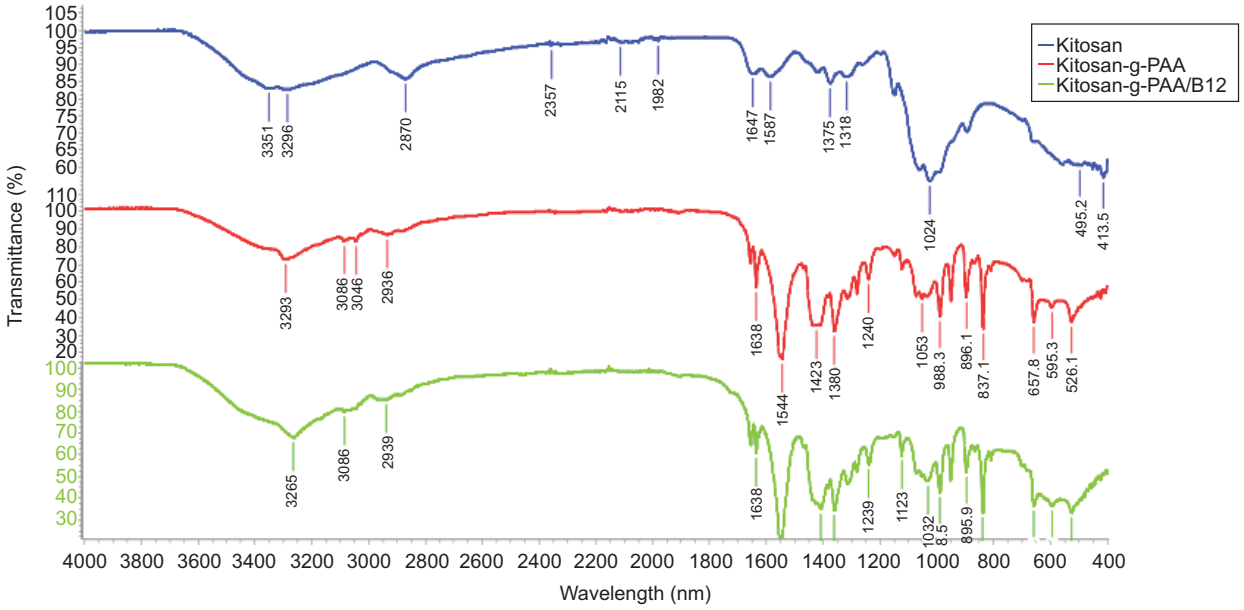

Figure 2. FT-IR spectrum of chitosan, chitosan–grafted PAA, and vitamin B_{12}- loaded chitosan–grafted PAA.

360 nm. Then vitamin B_{12} release was measured at this wavelength.

Fourier-transform infrared (FT-IR) spectroscopy analysis

Fourier-transform infrared (FT-IR) spectroscopy was used for determining the functional groups that helped define the surface chemistry, chemical properties of the synthesized biopolymer. The FT-IR spectrum of chitosan, chitosan–grafted PAA, and vitamin B_{12}- loaded chitosan–grafted PAA were shown in Figure 2.

Chitosan peaks at 3,287 cm^{-1} were assigned to O-H stretching and at 2,864 cm^{-1} to CH stretching. Bands at 1,647 cm^{-1}, 1,590 cm^{-1} and 1,322 cm^{-1} indicated amide I, amide II, and amide III, respectively. Bridge O stretching, C-O stretching, and pyranoid ring stretching were revealed at 1,151 cm^{-1}, 1,061 cm^{-1}, and 890 cm^{-1}. Band at 1,026 cm^{-1} was derived from C-O stretching of the primary -OH. Asymmetric stretching of C–O–C was seen at 1,154 cm^{-1}. After the reaction, these characteristic absorption bands were either weakened or masked by other absorption bands, which meant that these were involved in the co-polymerization reaction. In the spectrum of chitosan-grafted PAA, some new absorption

peaks appeared in addition to the characteristic peaks of chitosan. Peaks at 1,658 cm^{-1}, 1,551 cm^{-1}, and 1,435 cm^{-1} were characteristic peaks of PAA and were stretchings of C-H vibration, asymmetric -COO^- stretching vibration, and C-H bending vibration, respectively. The peak at 1,658 cm^{-1} matched carboxyl absorption by grafted PAA, and the peaks at 809 cm^{-1} and 620 cm^{-1} were also characteristics of PAA. Also, the bands at 1,551 cm^{-1} and 1,409 cm^{-1} matched the sodium carboxyl group. This demonstrated that the acrylic acid in the polymer was grafted onto chitosan and could be assigned to asymmetric and symmetric stretching vibrations of the COO- anion groups. This indicated that the carboxylic groups of PAA dissociated into COO- groups, which formed complexes with protonated chitosan amino groups via electrostatic interaction to form polyelectrolyte complex during the polymerization procedure. The main bands of vitamin B_{12} were in the range of 2,800-3,500 cm^{-1} and 1,000 cm^{-1}-1,750 cm^{-1}. Band widening and intensity reductions in the FT-IR spectra between the unloaded film and the B_{12}- loaded film established chemical bond formation between the vitamin B_{12} molecules and the biopolymer film at 2,940 cm^{-1} and 2,920 cm^{-1} peaks (Zhang *et al.*, 2006). The peak of the -$CONH_2$ group was observed at 1,655 cm^{-1} (Qui *et al.*, 2004). Peaks at 1,555 cm^{-1} represented the peaks of the amide N-H bands (Dengre *et al.*, 2000). There were peaks of CH_2 swing bands at 1,410 cm^{-1} (Tangpasuthadol *et al.*, 2003). Peaks of N-C tensile vibration were observed at 1,295 cm^{-1} (Osman and Aro, 2003). Peak of the C-O stretching vibration in C-O-C bands was observed at 1,160 cm^{-1} (Yuan *et al.*, 2010). A peak of skeletal stress is observed at 1,075 cm^{-1} (Santi *et al.*, 2012). The band at 1,025 cm^{-1} in the FTIR spectrum of chitosan film, corresponding to the wagging vibration of the saccharide structure (Boonsongit *et al.*, 2008).

Thermogravimetric Analysis and Differential Thermal Analysis System (TGA-DTA)

The thermal stability of biopolymers and vitamin B_{12}-loaded biopolymers was examined using TGA from room temperature to 600 °C at a heating rate of N_2 at 4 °C min^{-1}. The percentage of mass loss of chitosan-grafted PAA and vitamin B_{12}-loaded chitosan-grafted PAA is shown in Figure 3. Major mass loss at up to 160 °C was related to the evaporation of water molecules. In the second step between the 160 °C and 275 °C, the decay of -COOH groups was observed. In the third step (275 °C–490 °C), where mass loss was evident, it was assigned to deconstruction. Polymers are the basic inputs used in the production of materials, such as plastic, rubber, fiber, paint, and adhesives that are used frequently in every aspect of daily life. Polymers that occur spontaneously in nature are called natural polymers, and most of the natural polymers are found in living structures (Sacak, 2005). Examples of natural polymers are polysaccharides, such as lignin, cellulose, hemicellulose, alginate, chitin, chitosan, and heparin, and proteins, such as collagen, fibrin, keratin, and eggshell membrane (Mogosanu and Grumezescu, 2013; Sandak *et al.*, 2014). The TGA technique is widely used to study the fundamental stability and properties of thermal decomposition of polymers. The TGA-differential thermal calorimetry (DSC) studies of other chitosan-based polymers reported a decomposition temperature of 310 °C (Cardenas *et al.*, 1992; Kittur *et al.*, 2002). These results were similar to results of the present study.

The DTA results of chitosan-grafted PAA and vitamin B_{12}-loaded chitosan-grafted PAA are shown in Figure 4. In Figure 4, it was observed in Figure 4 that the decomposition was proportionally higher at endothermic peak at

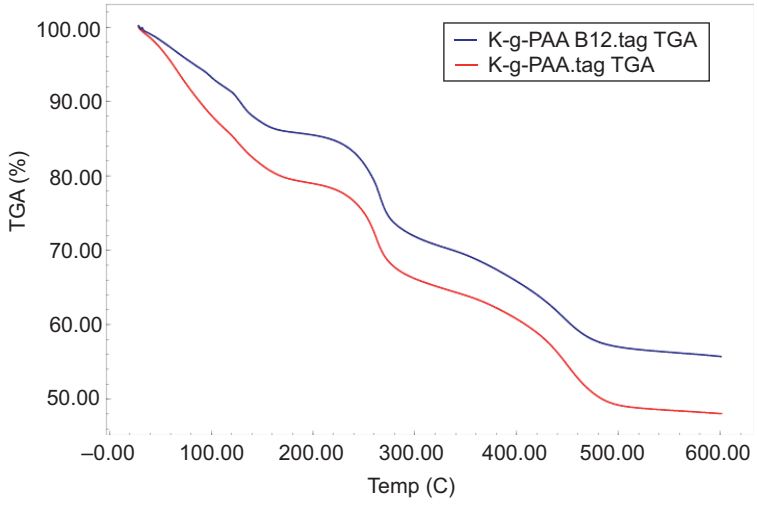

Figure 3. TGA thermograms of chitosan-grafted PAA and vitamin B_{12}-loaded chitosan-grafted PAA.

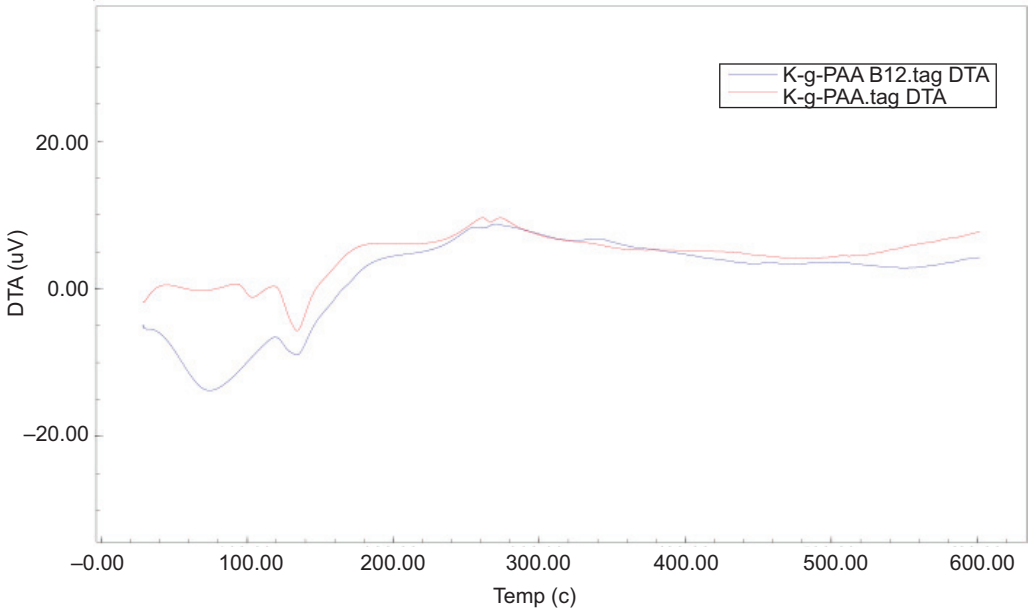

Figure 4. DTA thermograms of chitosan-grafted PAA and vitamin B_{12}-loaded chitosan-grafted PAA.

75°C and at TGA thermogram (Figure 3). This supported that vitamin B_{12} was loaded on chitosan-grafted PAA.

Scanning Electron Microscope (SEM)-Energy Dispersive Spectroscopy (EDS) Analyses

The SEM-EDS images of chitosan-grafted PAA and vitamin B_{12}-loaded chitosan-grafted PAA are shown in Figures 5 and 6, respectively. From the EDS images shown in Figure 6, it was interpreted that vitamin B_{12} was bound on the biopolymer. While the peaks of Co element were not dicovered in the EDS image of chitosan-grafted PAA (Figure 5), the same were checked after vitamin B12 was loaded on chitosan-grafted PAA (Figure 6).

Scanning Electron Misroscopy in all studies and applications involving polymers has been used to examine cracks or scratches on the surfaces, phase boundaries in the materials, agglomeration of the support and additive materials, the attraction between the materials and the surface roughness of materials. Roughnesses, such as indentations and protrusions on the surface, is very important as it affects the wetting feature intensely. This property, which is known as the wettability, which features in solids and is similar to the tendency of fluids to stick to a surface, is directly related to the structure of the surface.

Ideal Model Choice for Vitamin B_{12} Release

In order to optimize the range and levels of variables that affect the vitamin B_{12} release process from biopolymers and to elucidate the nature of the response surface in the experimental design, the CCD model coupled with RSM was selected and developed considering all the important interactions. In the study, it was understood that the CCD model was quadratic and that there was no need for any power transformation. Among the various applications, the use of biopolymers as drug carriers is one of the most promising applications. High loading capacity, encapsulation efficiency, simultaneous application of various treatments, ease of operation, and low cost are the features that make the use of nanofibers produced by electrospinning method attractive in this field (Hu *et al*., 2014). Controlled drug release is a method in which the active substance is designed to be delivered in the desired amount at a certain speed in the system for the desired time (Gur and Taskin, 2004). In classical drug release, the active substance is released suddenly, and the toxic drug concentration in the plasma rises above its value: however, this may cause undesirable adverse effects to the patient. In drug release with nanofibers, the drug substance can be added to the electrospinning solution or it can be produced by encapsulation. Thus, nanofibers are used as both a drug carrier and as a drug delivery system (Celik, 2013). Among the various applications, the use of biopolymers as drug carriers is one of the most promising applications. High loading capacity, encapsulation efficiency, simultaneous application of various treatments, ease of operation, and low cost are the features that make the use of nanofibers produced by electrospinning method attractive to this field (Hu *et al*., 2014). In drug release with nanofibers, the drug substance is added to the electrospinning solution or it is produced by encapsulation. The literature demostrated the release of

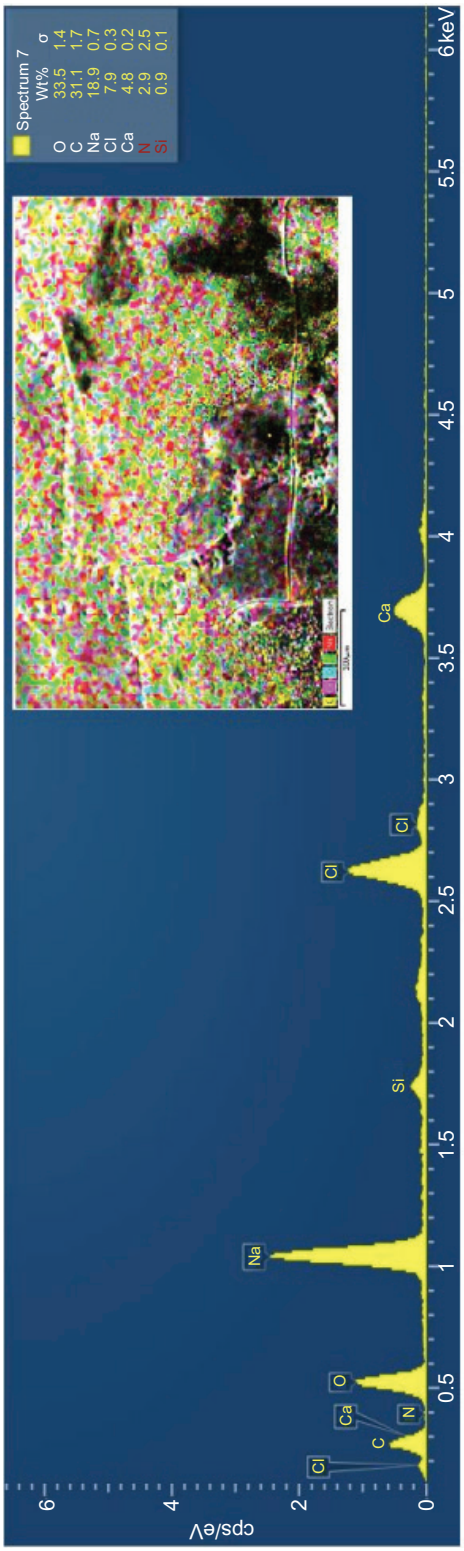

Figure 5. EDS image of chitosan-grafted PAA.

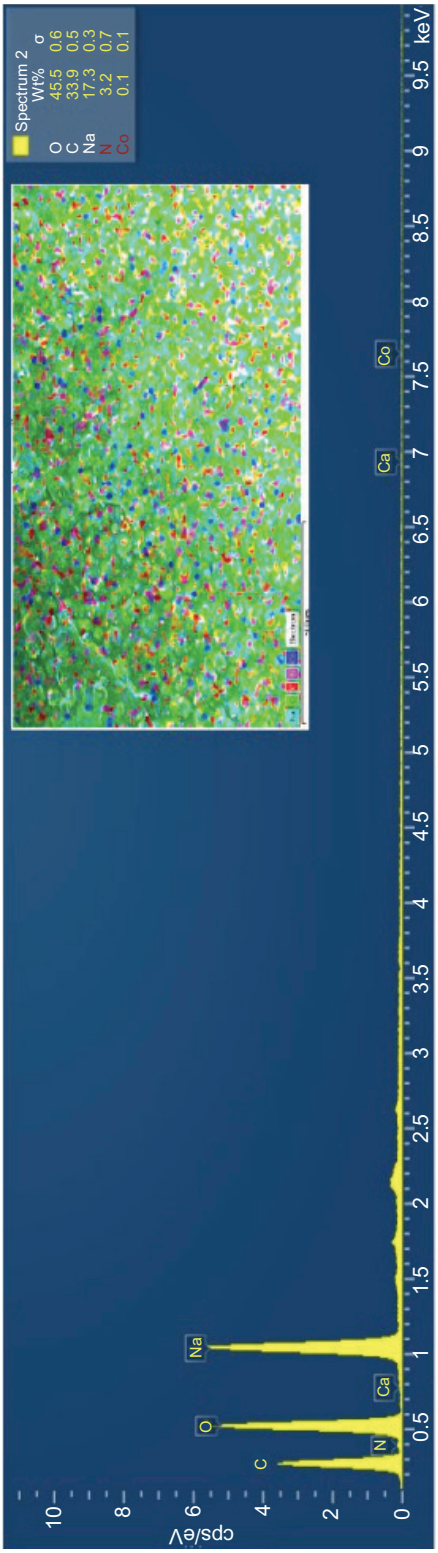

Figure 6. EDS image of vitamin B_{12}-loaded, chitosan-grafted PAA.

vitamin B_{12} are examined, it can be seen that the release of vitamin B_{12} from nanofiber systems developed with different polymers. Today, many studies Show the controlled release of vitamin B_{12} (Baskakova *et al.*, 2016; Mendes *et al.*, 2016).

The ANOVA results and regression coefficients are presented in Table 1 and The results showed that the contribution of a quadratic model was significant (p<0.0001) fort he release of vitamin B_{12}. Fisher's test" shows that larger F-values and smaller *p*-values were more significant than the suggested model terms. In the present investigation, $p < 0.05$ was considered as statistically significant at the 95% confidence level (95% Cl), and F=35.08 indicated that the model was significant. If probe >F <0.05, it showed that the model term was statistically insignificant for the model and probe>F>0.05 showed that term was statistically insignificant fort he selected model. Based on the preferred quadratic, some model terms, such as X_1, X_2, X_3, and X_4, were statistically significant for vitamin B12 release from biopolymer. Also, the variation of the data around the model that matched the experimental data was expressed as the model's lack of fit (LOF). The *p* values of model's LOF measured the fit of the model and indicated that the LOF was not significant with respect to pure error. This value was a sufficient test for the adequacy of model fit without the effects of additional higher-order terms. The LOF value obtained using ANOVA was 0.0670. These LOF *p* values of the vitamin B_{12} release method from biopolymer confirmed the applicability of good reaction settlement. It also showed that the number of experiments performed was sufficient to determine the effects of independent variables on vitamin B_{12} loading for drug release from biopolymer. Since the R^2 and adjusted R^2 values describe the percentage of variation in response, the validity of the polynomial models was evaluated with these values. As the R^2 value of the quadratic model used was obtained as 0.9704, it was concluded that 97.04% of the values estimated by the model matched the experimental values of the release behavior of vitamin B_{12}-loaded biopolymers (Figure 7).

The sensitivity term (AP) measures the signal-to-noise ratio (S/N) and it is desirable that this ratio must be greater than 4. This value was obtained as 22.004, which is a clearly indicated that the model was providing sufficient signal. In the light of preliminary experimental studies, four critical parameters affecting release of vitamin B_{12} from biopolymers were selected as independent variables, and drug release was considered as an dependent variable. As observed in Table 1, the quadratic model with the highest R^2 (0.9704) value was preferred according to the statistical test results of the model used. In order to express the relationship between the independent variables and responses, the experimental data were expressed with a quadratic polynomial mathematical equation. The quadratic mathematical equation (Equation 1) of the model in question was derived the coded factors.

$$\text{Release (\%)} = -122.44 + 13.58X_1 + 1.14X_2 + 0.95X_3 + 84.91X_4 + 0.037X_1X_2 + 0.01X1X3 - 0.79X1X4 - 2.47917E - 003X_2X_3 - 0.14X_2X_4 - 0.11X3X4 - 1.47X_1^2 - 4.95X_2{}^2 - 2.28X_3{}^2 - 13.36X_4{}^2.$$

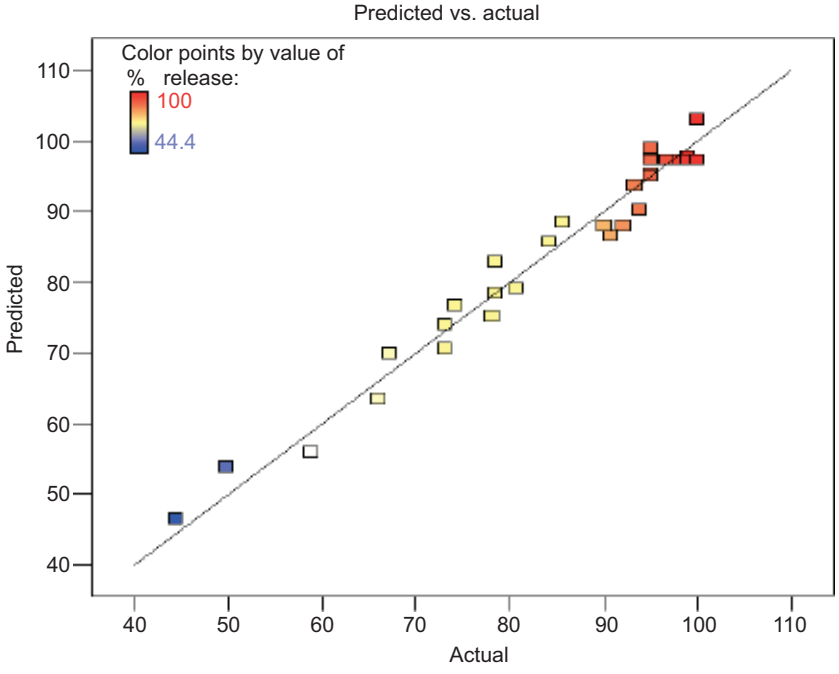

Figure 7. The overlap ratio of the estimated and actual values obtained for vitamin B_{12}-loaded biopolymers.

The best method to determine the optimum point where the parameters selected as independent variables had the maximum effect and to obtain the highest performance were the arrangement of two-dimensional (2D) and three-dimensional (3D) surface graphs of vitamin B_{12} release from biopolymer. The effects and interactions of each parameter on the vitamin B_{12} release biopolymers are presented in Figure 8. The 3D response surface plots derived from CCD were used to define the response maximum, middle, and minimum points of the response.

Figures 8A and 8B represent 3D and 2D response surface plots of the effect of initial concentration and pH on vitamin B_{12} release efficiency from biopolymers. When pH increased from 3 to 7, release of vitamin B_{12} from the biopolymers increased up to pH 5, followed by a partial decrease in vitamin B_{12} release as pH increased to 7. While the initial concentration had a significant effect on vitamin B_{12} release from biopolymers, it was observed that the effect of the interaction between the initial concentration and pH was not statistically significant (p>0.005). In a study conducted by Nath *et al.* (2020), the authors investigated an acrylic acid-grafted gelatin/ layered double hydroxide (LDH)- based biocompatible hydrogel with pH-controlled vitamin B_{12} release. The study reported that a certain increase in the release of vitamin B_{12} from the biopolymers with increased pH values, in parallel with the results of our study, and a partial decrease in the release of vitamin B_{12} from the biopolymers when the pH value increased to 7.

In a study conducted by Bajpai and Dubey (2005), it was determined that vitamin B_{12} released in the environment was 8.6% ± 2.1% and 83.2% ± 4.8% at pH 1.2 and 6.8, respectively.

The 3D and 2D response surface plots of the effect of release time and pH of vitamin B_{12} release efficiency are shown in Figures 9A and 9B. While the increase in the release time caused a significant increase in the release of vitamin B_{12} from the biopolymers, its release from the biopolymers increased with increase in pH increased from 3 to 5, but a partial decrease was observed after pH 5. It was observed that the effect of the interaction between pH and release time was not statistically significant (p > 0.05).

The effect of solution volume and pH on release of vitamin B_{12} is presented in Figures 10A,B. While increase in the solution volume caused a significant increase in the release of vitamin B_{12} from the biopolymers when pH increased from 3 to 5, the release decreased partially with increase in pH above 5. However, the effect of the interraction between pH and solution volume was not statistically significant (p > 0.05). A study conducted by Moradi *et al.,* (2020) reported that the initial volume affected the release of vitamin B_{12}. The study determined the optimal initial concentration of vitamin B_{12} as 1,000 mol m^{-3}.

The 2D and 3D response surface plots of the effect of release time and initial concentration of release of vitamin B_{12} are presented in Figures 11A,B. It was determined that increase in release time and initial concentration positively affected the release of vitamin B_{12}. However, using the ANOVA, it was determined that the interactive effect of both variables was not statistically

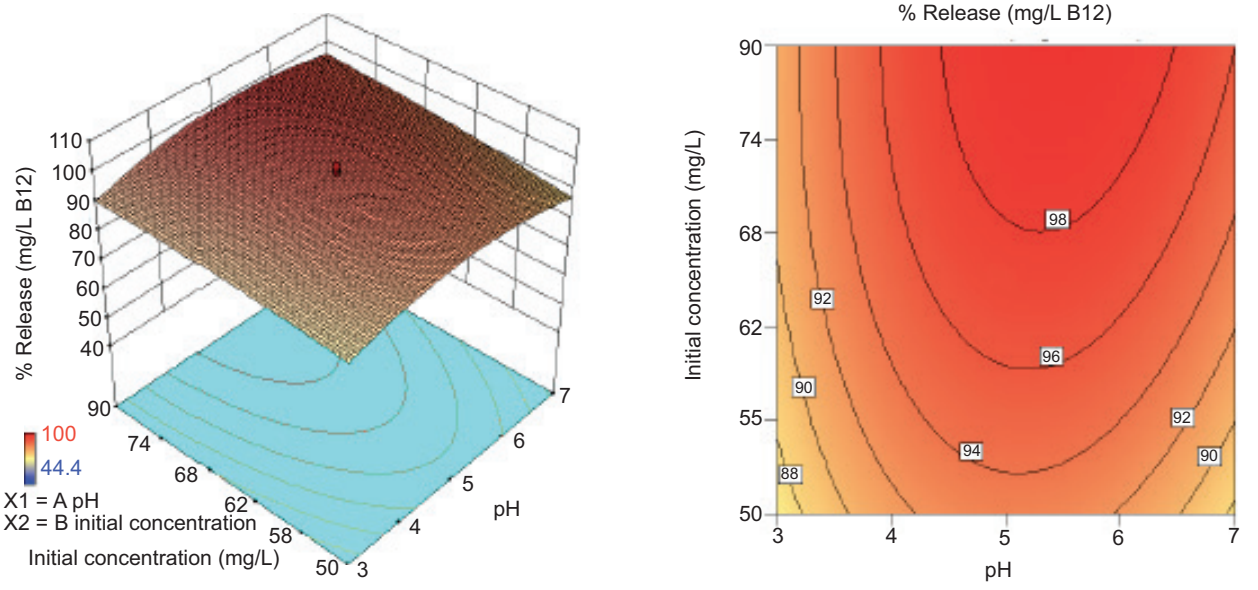

Figure 8. Interaction between pH and initial concentration. (A) 3D graph and (B) 2D graph.

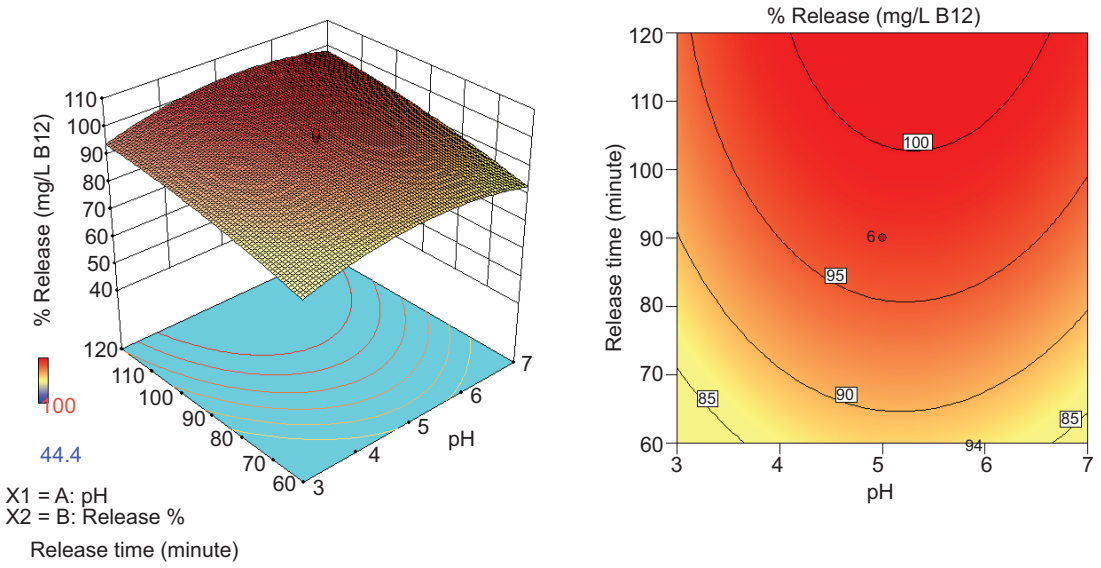

Figure 9. Interaction between pH and release time: (A) 3D graph and (B) 2D graph.

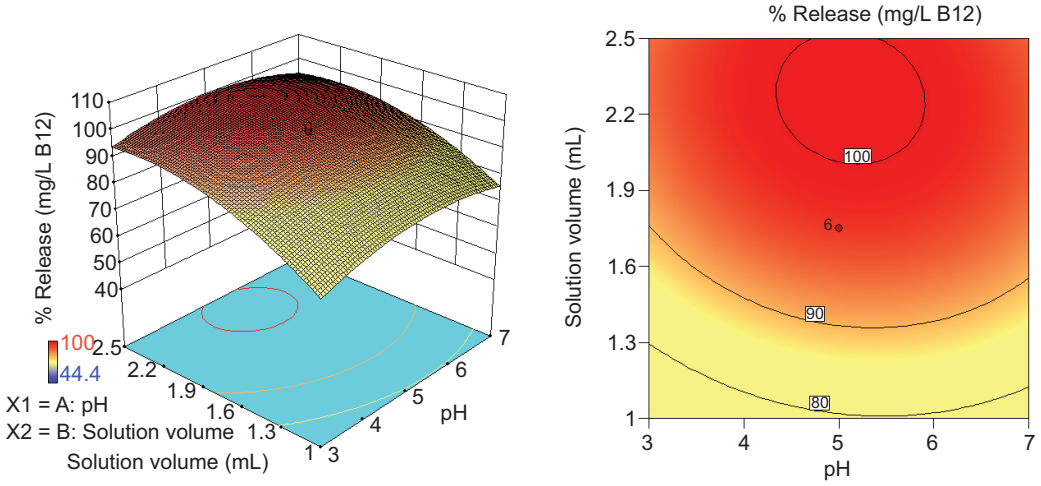

Figure 10. Effect of interaction between pH and solution volüme on vitamin B_{12} release efficiency: (A) 3D graph and (B) 2D graph.

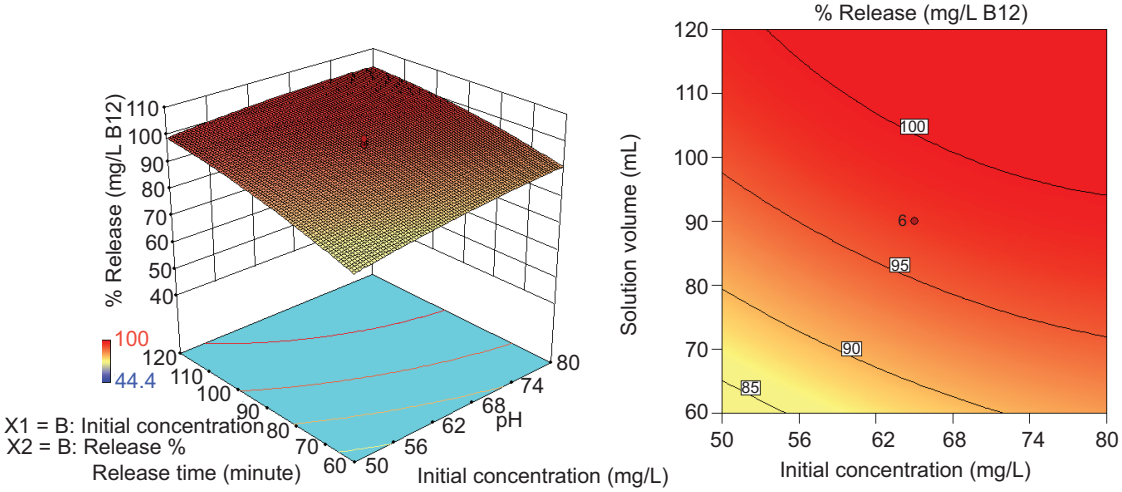

Figure 11. Effect of interaction between release time and initial concentration on vitamin B_{12} release efficiency: (A) 3D graph and (B) 2D graph.

significant (p>0.05). A study determined that the drug concentration loaded on the carrier increased more than the desired amount for a certain time in order to control the optimum drug release from the carrier to the body, and at the same time, the speed of the boundary layer was proportional to the rate of water infusion into the hydrogel. Optimal simulation was obtained with an initial vitamin B_{12} concentration of 1,000 mol m^{-3}, hydrogel membrane thickness of 0.0004 mm, and a constant velocity of 2 x 10^{-11} m s^{-1} (Moradi *et al.*, 2020). In the present study, the time required to achieve equilibrium concentration for a stationary boundary of the system was 1,20, 000 s, while it was 2,10,000 s for a moving boundary with a constant velocity of 2×10^{-11} m s^{-1}.

The effect of solution volume and initial concentration on vitamin B_{12} release efficiency are presented in Figures 12A, B. Both variables contributed positively to vitamin B_{12} release by increasing the solution volume and initial concentration. However, ANOVA results determined that the interactive effect of both variables was not statistically significant ($p > 0.05$). The study conducted by Moradi *et al.,* (2020) reported that the initial concentration was effective on vitamin B_{12} release, and the optimal initial concentration for vitamin B_{12} was determined as 1,000 mol m^{-3}.

The 3D and 2D response surface plots of the effect of solution volume and release time on vitamin B_{12} release efficiency are presented in Figures 13A, B. Increase in solution volume and release time contributed positively to vitamin B_{12} release from biopolymers. Also, ANOVA results demostrated that the interactive effect of solution volume and release time was statistically significant

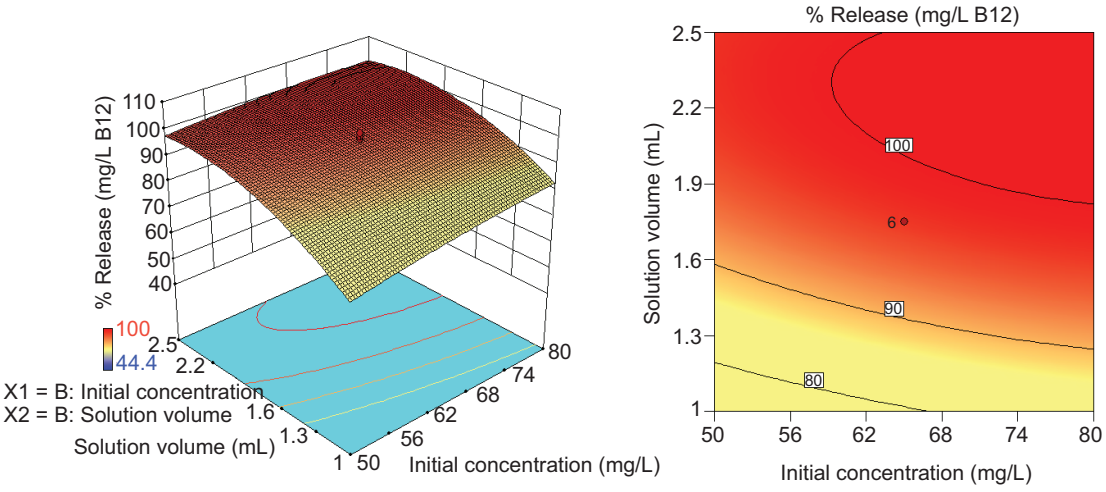

Figure 12. Effect of interaction between solution volume and initial concentration on vitamin B_{12} release efficiency: (A) 3D graph and (B) 2D graph.

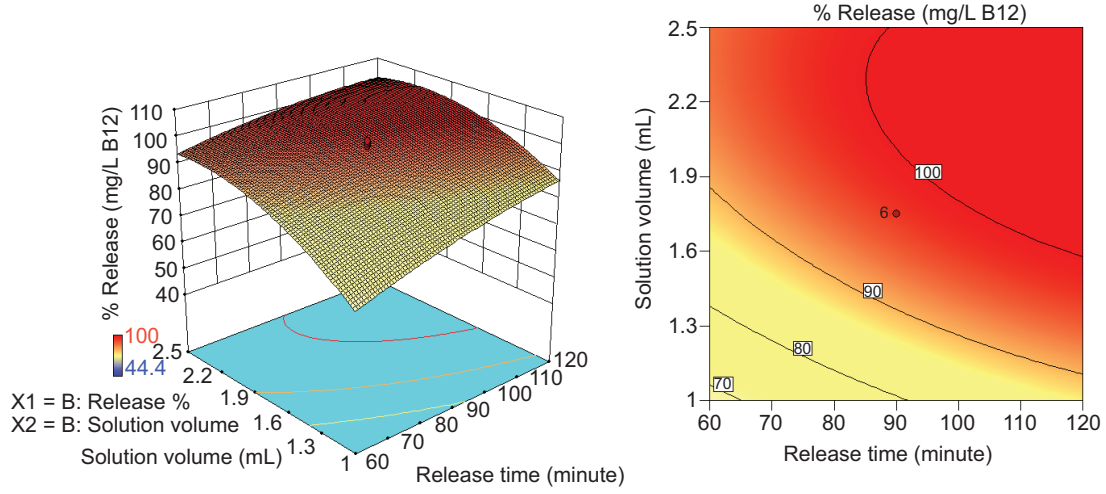

Figure 13. Effect of interaction between solution volume and release time on vitamin B_{12} release efficiency: (A) 3D graph and (B) 2D graph.

($p < 0.01$). The study conducted by de Abreu Figueiredo *et al.* (2020), it was reported that solution volume and release time were associated with release of drug from biopolymers. Nath *et al.* (2020) examined the effect of acrylic acid-grafted gelatin/LDH-based biocompatible hydrogel with the pH-controlled vitamin B_{12} release in their study. The researchers reported that the volume of the solution and the release time were related to the release of vitamin B_{12} from the biopolymers.

In Figure 14A, a Box-Cox graph for vitamin B_{12} release was obtained to control the value of lambda (λ). The Box-Cox plot is a preferred diagnostic plot drawn for estimating any necessary transformation of the experimental value to increase the significance of the model. As observed from the power conversion graph, it was confirmed by the model that no conversion was needed according to the obtained λ value (λ = 1; the most ideal value of λ is 0.78).

A perturbation graph (Figure 14B) was drawn to compare the influence of all factors on the optimum conditions for the release of vitamin B_{12} release from biopolymers. The graph was also used to analyze the variations in factors as well as the combined effect of all factors on a process. In addition, a power conversion plot for vitamin B_{12} release was obtained to control λ value. The power conversion plot is often a preferred diagnostic plot drawn to estimate any necessary conversion of the experimental value to increase the significance of the model. As discovered from the power conversion graph, it was model that there was no need for any conversion according to the obtained λ value, that is, λ = 1.

In order to select the optimal model and the maximum desirability function obtained, the most important statistical metrics, such as the lowest LOF and the highest *p*-value R^2, and F-value, were searched. The desirability value of the optimization process was found as 1.0, and is presented in Figure 15. The lower and upper limits of vitamin B_{12} release of the quadratic model, which were obtained at the end of the experimental study, are presented in Table 1, and the significance level determined was 3.

Based on the possible global solutions (Table 3), validation experiments were performed to support the optimized data from numerical modelling under optimum conditions.

The values obtained from the experimental design were transferred into the CCD system, the ramp chart of the optimum points of the independent variables in vitamin B_{12} release from biopolymers is given in Figure 16. By presenting the ranges of all variables in the figure, the desirability value obtained was as high as 1.0.

When the approval report of the quadratic model used was examined at the 95% Cl, the standard deviation obtained was 0.000, and the model was significant and valid.

Conclusion

In recent years, there has been an increase in studies on the development of systems that can direct the drug to

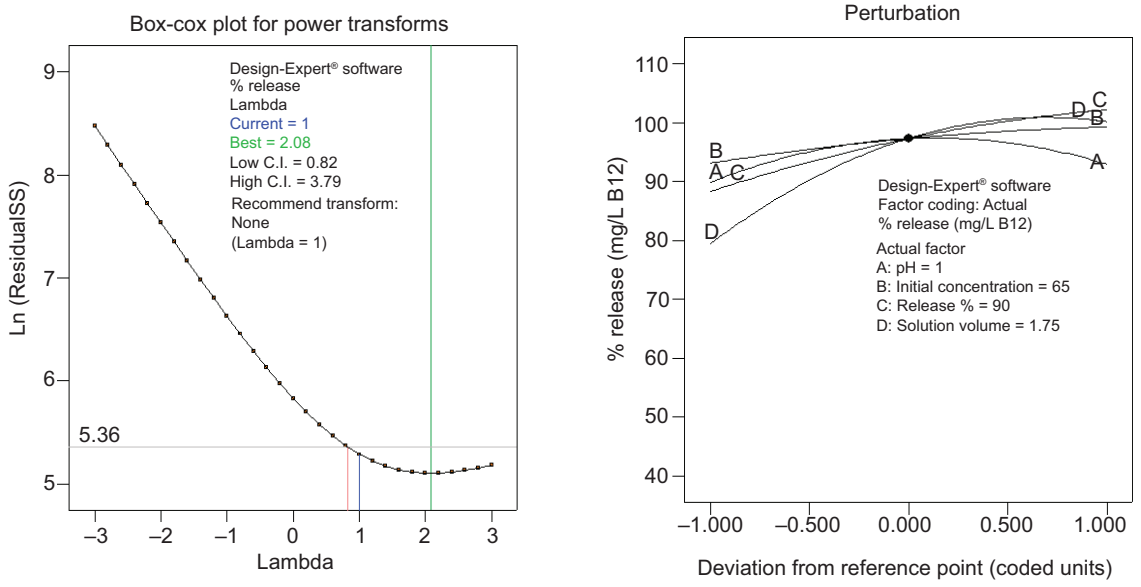

Figure 14. Diagnostic plots: power conversion and disorder plot for vitamin B_{12} release from biopolymers at optimum conditions.

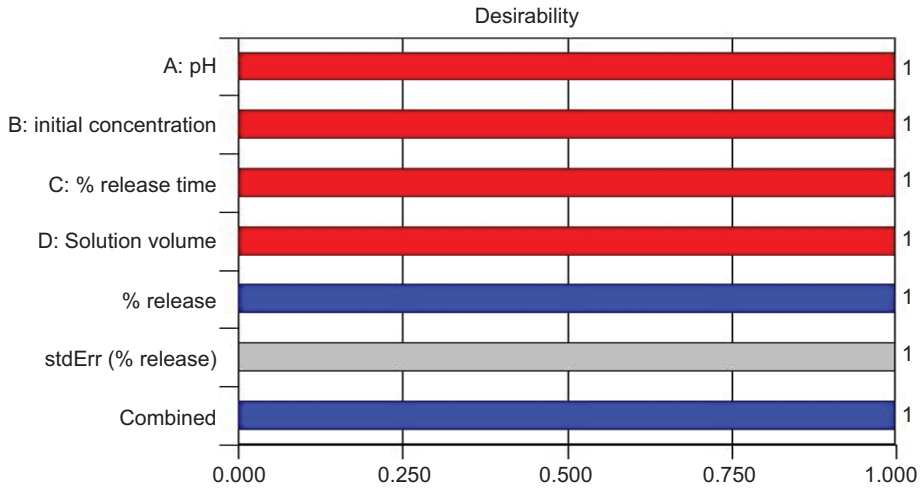

Figure 15. Desirability plot for vitamin B_{12} release from biopolymers at optimum conditions.

Table 3. Possible solutions.

Solutions								
Number	**pH**	**Initial concentration**	**Release time(min)**	**Solution volume(%)**	**Release %**	**Std.Error (Release [%])**	**Desirability**	
1.	4.241	54.039	117.066	2.084	101.258	1.627	1.000	Selected
2.	6.560	74.436	111.860	1.938	101.890	1.597	1.000	
3.	5.273	72.369	83.579	2.138	100.370	1.429	1.000	
4.	6.033	54.903	108.754	2.240	100.587	1.579	1.000	
5.	4.604	72.534	106.784	2.058	102.901	1.436	1.000	
6.	5.536	68.508	113.608	1.892	103.286	1.437	1.000	
7.	5.300	67.250	94.500	1.863	100.094	1.468	1.000	
8.	6.662	77.469	117.750	2.373	100.753	2.172	1.000	
9.	4.079	51.111	119.889	2.489	100.050	2.227	1.000	
10.	5.579	71.394	94.881	2.355	101.804	1.452	1.000	
11.	4.418	57.967	104.873	2.403	101.113	1.518	1.000	
12.	6.752	79.317	116.573	1.899	101.805	1.959	1.000	
13.	5.424	51.930	115.446	2.473	101.011	1.935	1.000	
14.	5.153	69.467	90.213	2.148	101.335	1.436	1.000	
15.	4.446	78.783	111.762	2.429	101.497	1.852	1.000	
16.	5.632	74.078	94.113	1.820	100.441	1.430	1.000	
17.	4.854	75.914	111.612	2.452	102.348	1.712	1.000	
18.	4.429	71.344	117.734	1.871	102.417	1.483	1.000	
19.	4.287	64.830	107.867	2.303	101.983	1.464	1.000	
20.	5.709	68.688	93.893	2.289	101.478	1.433	1.000	
21.	4.090	61.704	106.371	2.386	100.861	1.510	1.000	
22.	6.059	68.664	107.579	1.894	101.891	1.433	1.000	
23.	4.815	71.770	111.034	2.481	102.220	1.615	1.000	
24.	5.171	79.770	91.874	2.372	101.631	1.620	1.000	
25.	5.581	73.361	108.515	2.450	102.446	1.613	1.000	

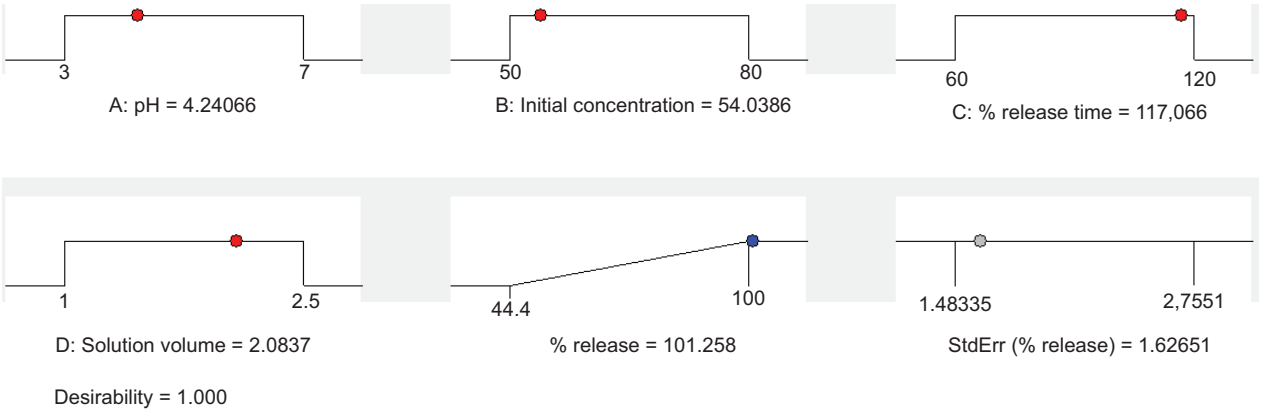

Figure 16. Ramp chart of statistically optimized factors for vitamin B_{12} release from biopolymers.

the target areas of the body and control the long-term drug release rate have increased. These developed systems have been widely used in various fields, such as medicine, physic, biology, biomedicine, and gene engineering. Controlled release systems are the systems that allow the release of active substances to be released to the target areas at a predetermined rate and at specific time intervals. The main purpose of these systems is to maintain the contant concentration of active substances. Polymers are one of the most used materials as a controlled drug system. The release of the active substances is closely related to the properties of the polymer used as well as physiological conditions, such as pH, ionic strength, temperature, and enzyme. In classical applications, possibility exists that the drug concentration in the blood increase above the safe level. Quantity below the mentioned effective level and above the safe level represents the amount of wasted active substance. The main advantages of controlled drug release system over the conventional drug applications are as follows: its abilit to maintain a contant drug level constant at a therapeutic rate; elimination of possible adverse effects in the body because the drug directly affects the desired area; and economical aspect of the drugs used, that is, no need to the use of drugs more than the amount required for treatment.

In addition to these advantages of controlled drug release systems, the following points are worthy of attention: (1) the polymer carrying the drug or the degradation products of this polymer should not have toxicities, that is it should release the drug speedily and must not pose a danger to the body; (2) there must not be any discomfort from the system or the way it is applied to the body; (3) the polymeric material forming the system itself and its preparation should not be expensive; (4) no cracks in the structure of the system that could cause sudden release of the drug; and (5) used polymer's mechanical properties should be good.

In terms of drug delivery, drug delivery systems can be broadly divided into three groups: (1) delayed release system: this system releases drug at the targeted area of the body because of the elimination of the adverse effects of the drug; (2) sustained release system: it ensures that the drug is released continuously and maintains a constant level in the blood plasma; and (3) controlled release system: in this system, drug is released in response to a variable peresent in the external environment (Baker, 2012).

In membrane systems, the polymers used are in the form of sheets, films, capsules, or microcapsules. The drug to be released is placed in these membranes in either dissolved or dispersed form (Blagoeva and Assen, 2006). During the release, the drug diffuses through the membrane to rise to the outer surface of the membrane. Diffusion of the drug across the membrane is the rate-determining step.

Matrix systems are prepared by dissolving the drug in a solid polymer and dispersing it homogeneously. It is easy and inexpensive to produce; however, since there is a first-degree release, the rate of release and thus the amount of drug given to the outside environment decrease constantly. This is a disadvantage of the system (Reddy *et al.*, 2017).

Worn systems in the body indicate hydrolytically or enzymatically degradable bonds within the structure. For this reason, these systems are also called "biodegradable systems". Because of the biodegradation of the said bonds, the polymer is eroded and the drug is released. The biggest advantage of these systems is that these can be eliminated from the body in natural manner without the need for surgical intervention. However, here, the material and its decomposition products should not have any health problems (Lee and Yeo, 2015).

In chain-mounted systems, the drug molecules planned to be sent to the body are bound to the polymer chain by covalent bonds that are broken down by enzymatic

or hydrolytic process and the drug is released in the body. The appropriate designing of the polymers breaks down the bonds, thereby controlling the release rate of drug. These systems are generally used for short-term drug release applications. The biggest advantage of the system, compared to other systems, is that it can contain about 80% drug by weight (Kismir, 2011). In swelling-controlled systems, cross-linked biopolymers form the basis of these systems. Drug molecules in biopolymers diffuse into the external environment by swelling of the biopolymers in the release medium (water or biological fluid). Here, the chemical structure of the biopolymer and the cross-link ratio, in other words the pore size, play an important role and determine the rate and amount of drug released (Kismir, 2011). The controlled drug release systems are prepared by taking advantage of the environmental intumescent biopolymer properties, which can release drugs sensitive to pH, temperature, light, and magnetic and electric fields. Here again, drug release takes place via the swelling mechanism (Kismir, 2011). Osmotic controlled systems consist of a semi-permeable membrane and the drug placed in this membrane. When they are placed in water or a biological fluid, the molecules in the environment penetrate through the pores of the semipermeable membrane and dissolve inside the drug molecules inside. However, to diffuse through the membrane, a hole must be drilled from a suitable part of the system. For this, the laser method is used. Because the drug concentration in these systems is very high (above the saturation limit), the release is usually of zero-order and osmotically controlled (Kismir, 2011).

pH-responsive biopolymers contain side groups and ionizable groups in the cross-link that accept or donate protons in response to the environmental pH. In this case, changing the pH across the pKa threshold of a polymer causes a rapid change in the net charge and hydrodynamic volume of the polymer chains. In other words, the presence of a high-loaded structure causes the mesh to swell because of high-load repellency and ability of biopolymer to change from collapsed to swollen state. The osmotic pressure created by the presence of mobile counterions also helps to explain the increase in the hydrodynamic volume of the biopolymer network chains. It is particularly interesting to exploit pH differences in the human body (e.g.throughout the GIT) to achieve delivery of a drug to the targeted site using polymers that respond to pH changes. In addition, in anticancer drug delivery, the release of drugs can be triggered by the acidic extracellular pH of tumors; this contributes to higher efficacy and lower toxicity to peripheral tissues. PAA is generally used in drug delivery systems targeting the intestine. Owing to the presence of ionizable -COOH groups, this polyelectrolyte structure is protonated so that it is uncharged at low pH and negatively charged at high pH. The presence of a protonated structure at low pH (as in gastric media) eliminates electrostatic repulsive forces and contributes to the formation of a tighter structure (Calejo, 2013).

Formation of a low elasticity network is also promoted by the formation of hydrogen bonds. When the pH rises above the pKa of the biopolymer, the dominant negative charges cause swelling of the network with high charge repellency and release of the drug. Accordingly, because of higher pH of 7.4 in the intestinal environment, the drug is released from PAA polymer network (Calejo, 2013). For other applications, it can also specifically trigger drug release in response to acidic environments. Polybases with amino groups, such as pDMAEMA and poly(amino esters), are deprotonated at neutral pH but charged by acquiring protons at acidic pH. This is particularly effective in the delivery of anticancer. Since the extracellular environment of solid tumors is weakly acidic (pH < 6.5), the pH in endosomes and lysosomes of cancer cells is lower (pH 4.0–6.0). The particles formed by polybases are stable at physiological pH. However, in tumor tissues or their intracellular compartments, the particles are charged positively and dissolved rapidly in response to lower pH, thereby releasing the cytotoxic drug. In normal tissues, the slightly acidic environment of endosomes is used to trigger cargo release in gene delivery applications. Polyethyleneimine (pEI) and poly(L-lysine) (pLL) are two well-known transporters in nonviral gene delivery system (Calejo, 2013). The gels that shrink in gastric pH (pH<2.0) swell in the intestinal environment (pH>7.0) to release the drug. In another application made in the opposite direction with this process, bad-tasting drugs are released with the help of polymers that swell at a low pH. Because the polymer has a low swelling level in at the neutral pH of the mouth (pH 7.0), drug release due to the environment does not take place. Drug release is assured in the acidic medium of the stomach with low pH (Calejo, 2013; Ilmain *et al.,* 1991). pH-sensitive polymers are smart polymers that can respond to pH change as a variable condition. Acidic or basic ionizable groups are present in the structures of these polymers. These ionizable groups in the structure of the pH-sensitive polymers take or donate protons depending on the charge in pH change. This causes changes in the polymer chain to change, resulting in swelling or shrinkage of the polymers (Reyes-Ortega, 2014).

Acidic side groups of pH-sensitive polymers are ionized in neutral or alkaline solutions. The ionization of acidic side groups depends on the logarithmic value of the acid dissociation constant of a polymer, known as pKa. In cases where the pH value of the medium is greater than the pKa value, ionization of acidic groups takes place. Owing to the electrostatic repulsion forces between the ionized groups, the molecular conformation of the polymer changes, resulting in the swelling of the polymer. These polymers are widely used in drug release (Grainger and El-Sayed, 2010).

The second class of pH-sensitive polymers are the polymers containing basic functional groups, that is, cationic groups. Poly(2-(dimethylamino)ethyl methacrylate) and poly(vinyl amine) are widely used pH-sensitive cationic polymers. Unlike polymers containing acidic groups, these polymers show a structure change at pH 8 or a higher pH value. However, as soon as the pH value drops below the pKb (basic ionization constant) value of the cationic group-containing polymer, the cationic groups absorb protons and the polymer swells because the positively charged groups repel each other with electrostatic interaction (Grainger and El-Sayed, 2010; Simsek, 2016).

In the present study, pH-sensitive biopolymers were developed and their use in colon-specific drug release was investigated. Based on these investigations, by loading of vitamin B_{12} onto pH-sensitive biopolymers synthesized with chitosan, and its release throughout the intestinal tract was studied. The synthesized biopolymers were characterized using FT-IR, TGA-DTA, and scanning electron microscopy-energy-dispersive x-ray spectrometry (SEM_EDS) techniques. Biopolymers, whose characterization was completed and produced in a film form, were used for drug release by loading vitamin B_{12}. The following results were obtained by performing the experimental study design and multi-parameter optimization.

The proposed mathematical models (Equation 1) also provided critical analyses for the interactive effects of selected independent variables on the vitamin B_{12} release process from pH-sensitive biopolymers. It was concluded that the values of R^2 values (for chitosan-synthesized biopolymer material: R^2=0.9704, and R^2adjusted=0.9427) obtained using (ANOVA) were of satisfactory level and the overlap ratios of the estimated and actual values were more than 97% of the values predicted by the model match the vitamin B_{12} release values from biopolymers. Based on the 2D and 3D plots, it was observed that the percentage release of vitamin B12 from pH-sensitive biopolymers increased significantly with increase in pH from 3 to along with increase in initial concentration.

The release conditions of vitamin B_{12} from the biofilm were determined at pH 4.2, with initial concentration of vitamin B_{12} being 54.0 mg L^{-1}, the release time being 117 min, and the solution volume as 2.1 mL. ANOVA results showed that the determination coefficients for the use of synthesized biofilm in vitamin B_{12} release were high and the R^2 = 0.9704.

Data from the proposed three-factor CCD combined with RSM confirmed that optimization was an effective approach to model vitamin B_{12} release from pH-sensitive biopolymer, understanding the relationships between independent and response variables and maximizing process efficiency.

Hence, biopolymer materials synthesized, characterized, and used are good candidates for the release of drugs because of their releasing capability.

Author Contributions

Conceptualization, Onal A. and Demir H..; methodology, Onal A., Ince O.K, Ince M., Demir, H.; software, Onal, A.,Ince, M.; validation, Onal,A.,Ince, M.;Demir H.; formal analysis, Onal, A.Ince, O.K., Demir, H.; investigation, Onal, A.; resources, Onal, A.;Ince, O.K., Ince M., data curation, Onal, A., Ince O.K.; writing—original draft preparation, Demir, H.; Ince M.; writing—review and editing, Demir, H., Ince M., Ince, O.K.; supervision, Demir H., Ince, O.K

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Conflicts of Interest

The authors declare no conflict of interest.

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