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In vitro characterization of alginate-chitosan hydrogels prepared with pH modification

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ABSTRACT: Hydrogels are biomaterials frequently used as carrier systems for wound care, tissue engineering, and local drug applications. Our study aimed to prepare and characterize hydrogels using chitosan and sodium alginate through pH modification. Mechanical properties (hardness, adhesiveness, cohesiveness, compressibility, and elasticity), viscosity, surface morphology, and cytotoxicity of polyion complex hydrogels containing different ratios of sodium alginate and chitosan were investigated. Mechanical properties were determined with a texture analyzer. The viscosity values of hydrogels varied between 12235 and 40743.3 cP. Hydrogel samples absorbed water up to 1000 – 1400% of their weight. The effect of pH and polymer concentration on the structural and mechanical properties of alginate-chitosan hydrogels was demonstrated. The findings showed that the decrease in pH improved the mechanical properties of alginate-chitosan hydrogels and increased the viscosity. The concentrations of chitosan and sodium alginate also altered the properties of hydrogels depending on pH. The formulation H5, which had the highest polymer ratio (3%) and lower pH, showed the highest hardness (0.285 ± 0.018 N), adhesiveness (0.824 ± 0.042 N. s), and compressibility (1.334±0.020 N.mm) values. The results showed that the prepared alginate-chitosan hydrogels are not cytotoxic (cell viability of over 80% in L-929 cell line) and safe for use in living organisms.

KEYWORDS: chitosan; sodium alginate; hydrogel; TPA analysis; polyion complex.

1. INTRODUCTION

Hydrogels are three-dimensional structures made of cross-linked hydrophilic polymer chains [1]. Hydrogels provide a medium for cellular adhesion and migration because of their high-water content and physical properties resembling various tissues [2]. Hydrogels should have appropriate rheological and mechanical properties for ease of application to the area of interest, spreadability, adherence to the application area thanks to their adhesive properties, and enough retention time [3]. In addition, it is expected that the hydrogels could encapsulate active ingredients inside due to their porous structures and swell owing to their water absorption ability to allow the release of active ingredients by diffusion [4].

Polysaccharides are widely used polymers as drug carriers in the preparation of pharmaceutical formulations because of their properties like biocompatibility, bioadhesiveness, and biodegradability [5]. Hydrophilic polymers have been used to increase swelling capacity and improve the adhesive properties of hydrogels [4, 6]. Chitosan is a polymer that is produced from natural resources and has repeating units of β-(1-4) linked D-glucosamine [7]. Chitosan is obtained from chitin through deacetylation and is a biocompatible, mucoadhesive, and cationic polymer [8]. Chitosan has positively charged amino ($-NH_2$) groups that are protonated in a slightly acidic medium [9]. Because of its polycationic nature, it can form a polyion complex hydrogel through ionic interaction with a polyanion [10].

Sodium alginate is a preferred polymer for hydrogel preparation due to its biocompatibility, low immunogenicity, water retention ability, and degradability [11]. Alginates are anionic polysaccharides that are produced from brown algae and bacteria and contain blocks of (1,4)-linked βD-mannuronate (M) and α-L-guluronate (G) residues in their structures [12]. Alginates contain various free hydroxyl (-OH) and carboxyl (-COOH) groups [13]. They can form gels via ionic interaction with polycations like chitosan due to negatively charged groups of alginates [1, 14, 15].

Mixing polycation and polyanion solutions generally causes nonhomogeneous precipitation [14]. When the number of charged ion pairs is limited in the formation of a polyion complex, the density of cross-linking decreases. This problem was resolved by polymerization of oppositely charged polymers at a charge ratio of

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1:1 [16]. Therefore, when hydrogels were prepared, chitosan and alginate were used with a ratio of 1:1 to provide an equal charge ratio and gelation was obtained through pH modification.

Although there are hydrogels prepared by using different cross-linking methods with alginate and chitosan in the literature, polyion complex alginate-chitosan hydrogels were prepared by adjusting the pH of hydrogels with glacial acetic acid and NaOH without any cross-linking agent in our study. Furthermore, their usability as biocompatible materials were investigated by examining their viscosity, swelling properties, texture profiles, and cytotoxicity.

2. RESULTS

2.1. Determination of pH

The sodium alginate and chitosan must have oppositely charged ions for cross-linking by ionic interaction. To this aim, the chitosan was protonated and positively charged by decreasing pH value of the polymer mixture with glacial acetic acid. pKa value of amino groups of the chitosan is 6.5 and chitosan can dissolve in water at lower pH [17]. The pH values of hydrogels should be in the range that will enable a polyion complex formation between alginate and chitosan and will not harm cell viability. Therefore, the pH values of the prepared hydrogels were first measured. The prepared alginate-chitosan hydrogel formulations and their pH values are presented in Table 1.

Table 1. Alginate-chitosan hydrogel formulations and pH values.

Formulation	Chitosan (w/v)	Sodium Alginate (w/v)	Acetic acid (v/v)	1 M NaOH (v/v)	pH (Mean ±SD)
H1	% 2	% 2	% 1.5	% 20	5.59 ± 0.07
H2	% 2	% 2	% 1.5	% 25	6.34 ± 0.05
H3	% 2.5	% 2.5	% 1.5	% 20	5.98 ± 0.05
H4	% 2.5	% 2.5	% 1.5	% 25	6.42 ± 0.11
H5	% 3	% 3	% 1.5	% 20	6.21 ± 0.04
H6	% 3	% 3	% 1.5	% 25	6.50 ± 0.13

2.2. Viscosity of hydrogels

The viscosity of sodium alginate and chitosan solutions was also measured together with the hydrogels to examine the change in viscosity because of the formation of the gel structure. Viscosity values of 2%, 2.5%, and 3% (w/v) sodium alginate solutions were determined as 1.56 ± 0.01 cP, 5.64 ± 0.02 cP, and 19.77 ± 0.02 cP at room temperature, respectively. Viscosity values of 2%, 2.5%, and 3% (w/v) chitosan solutions in 1.5% (v/v) glacial acetic acid were determined as 15.21 ± 0.19 cP, 17.30 ± 0.11 cP, and 75.47 ± 0.09 cP, respectively. It was seen that the viscosities of the hydrogels were considerably higher than those of alginate and chitosan solutions at the same concentration. As presented in Table 2, increasing the concentration of chitosan and alginate caused an increase in the viscosities of the hydrogels. In addition, the viscosity increased as the pH value decreased in hydrogels with the same polymer concentration.

Table 2. Viscosity values of alginate-chitosan hydrogels.

Formulation	Viscosity (cP) ± SD
H1	17566.7 ± 208.2
H2	12235.0 ± 97.3
H3	31716.7 ± 225.5
H4	18140.0 ± 36.1
H5	40743.3 ± 190.1
H6	19243.3 ± 250.3

2.3. Mechanical properties of hydrogels

The hardness, adhesiveness, cohesiveness, compressibility, and elasticity of the hydrogels were determined using force-time curves obtained with the measurements (Figure 1). Data on the mechanical properties of hydrogels are presented in Table 3. According to the texture profile analysis (TPA) results, the mechanical properties of the hydrogels changed depending on the pH and polymer concentration.

The hardness value of the hydrogel with lower pH was higher at the same polymer concentration, but the difference was not statistically significant. Although the increase in polymer concentration increased the hardness value in H1, H3, and H5 hydrogels with the same glacial acetic acid and sodium hydroxide ratio, only the difference between H1 and H5 was significant ($p<0.01$). The difference between the hardness values of H2, H4, and H6 hydrogels was not significant.

The decrease in pH value of hydrogels with the same polymer concentration increased the adhesiveness values (H1>H2, H3>H4, and H5>H6). In H1, H3, and H5 hydrogels with the same glacial acetic acid and sodium hydroxide ratio, the increase in polymer concentration significantly increased the adhesiveness value ($p<0.01$). However, the difference between the adhesiveness values of H2, H4, and H6 hydrogels was not statistically significant. Cohesiveness decreased with increased polymer concentration. While the compressibility of hydrogels increased with increasing polymer concentration, it decreased with increasing pH value. Since there was no statistically significant difference between hydrogels in terms of elasticity values, the elasticity of hydrogels was not affected by pH and polymer concentration.

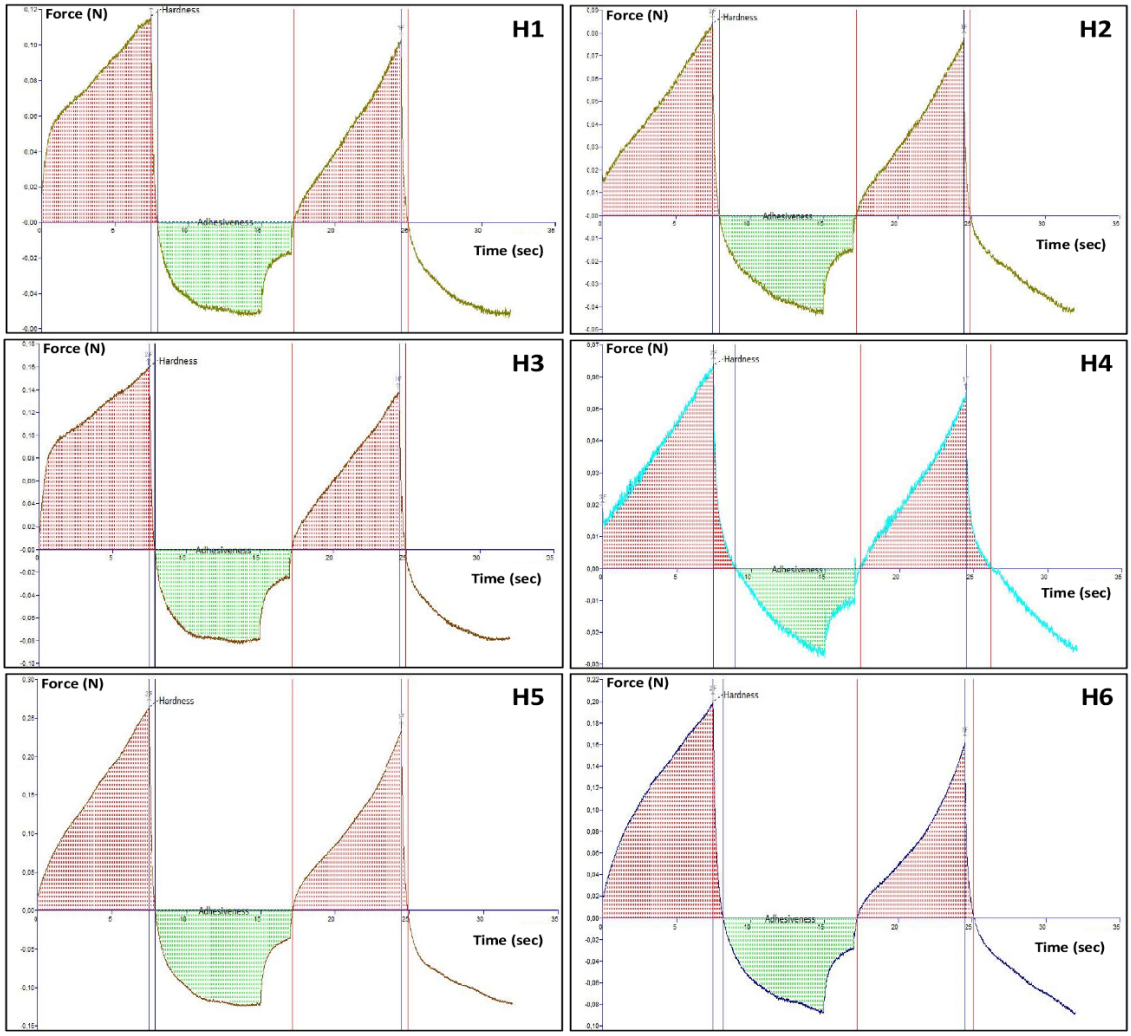

Figure 1. TPA graphs of the hydrogels.

Table 3. TPA results of alginate-chitosan hydrogels.

Formulation	Hardness (N)±SD	Adhesiveness (N.s) ±SD	Cohesiveness ±SD	Compressibility (N.mm) ±SD	Elasticity ±SD
H1	0.119 ± 0.035	0.349 ± 0.087	0.718 ± 0.087	0.529±0.014	0.969 ± 0.003
H2	0.103 ± 0.046	0.281 ± 0.014	0.740 ± 0.050	0.422±0.017	0.958 ± 0.017
H3	0.165 ± 0.021	0.526 ± 0.047	0.673 ± 0.094	0.781±0.015	0.974 ± 0.006
H4	0.099 ± 0.031	0.216 ± 0.087	0.614 ± 0.043	0.439±0.014	0.957 ± 0.009
H5	0.285 ± 0.018	0.824 ± 0.042	0.626 ±0.089	1.334±0.020	0.970 ± 0.006
H6	0.168 ± 0.033	0.381 ± 0.012	0.552 ± 0.008	0.749±0.018	0.960 ± 0.006

2.4. Swelling properties of hydrogels

The swelling ratios (%) of lyophilized hydrogels were examined in PBS (pH 7.4). Hydrogel samples were able to absorb water nearly 1000 – 1400% of their weight. The hydrogels with the lowest polymer concentration, H1 and H2 containing 2% alginate and chitosan had higher swelling ratios (%) (Figure 2). Hydrogels with higher pH values at the same polymer concentration had higher water absorption capacity (H2>H1, H4>H3, H6>H5). When glacial acetic acid and NaOH were used at the same rate in the preparation of the hydrogels, the swelling ratios of the hydrogels (H3 and H5, H4 and H6) containing 2.5% (w/v) and 3% (w/v) polymers were found to be close to each other.

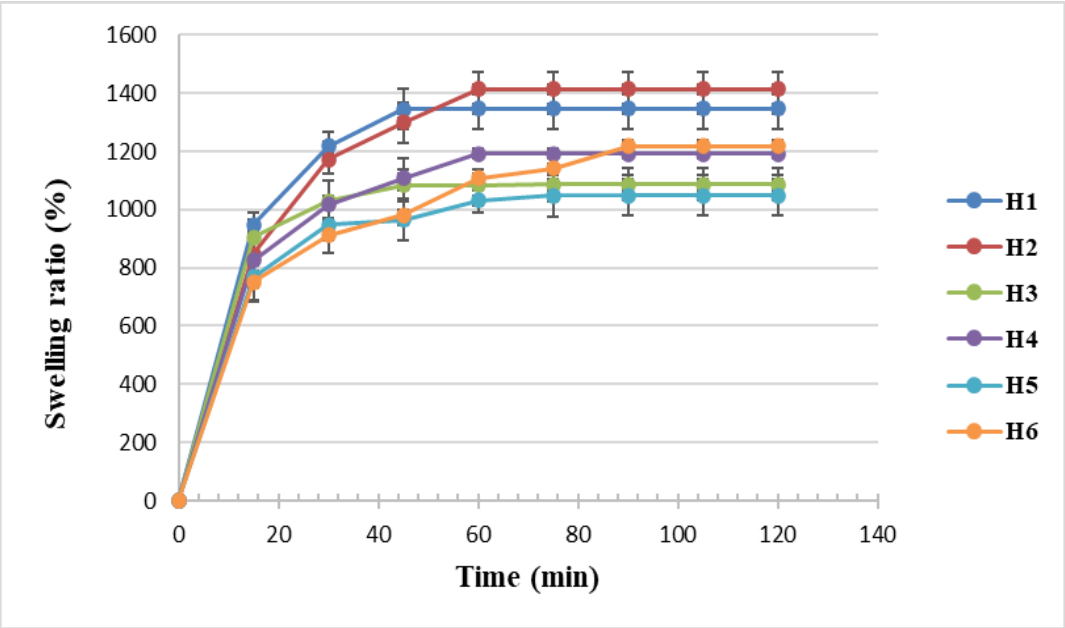

Figure 2. Swelling profiles of hydrogels.

2.5. Fourier transform infrared spectroscopy (FTIR) analysis

The chemical structures of chitosan, sodium alginate, and alginate-chitosan hydrogels and the interactions between polymers were investigated by FTIR spectroscopy. The FTIR spectra of polymers and hydrogels are shown in Figure 3 and the peaks of the groups in their structures are presented in Table 4.

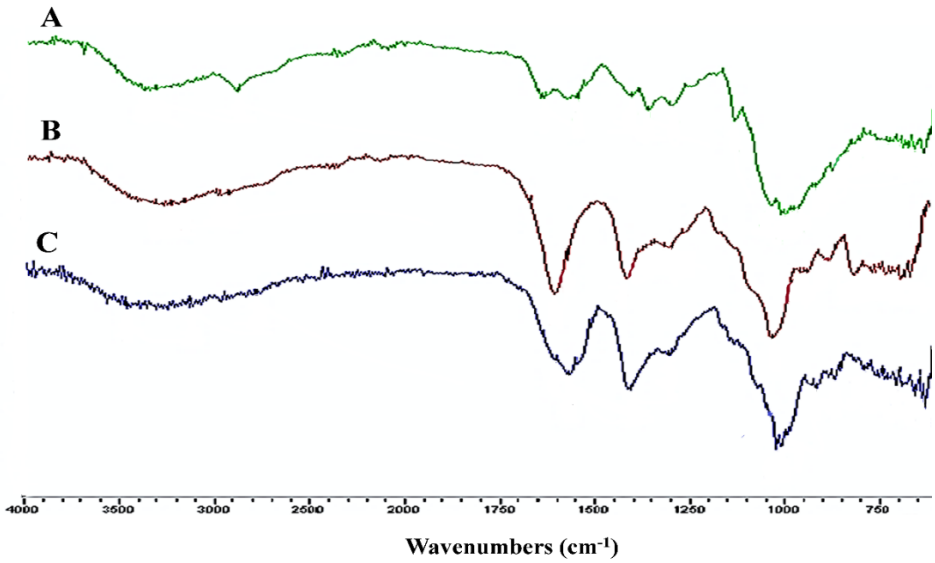

Figure 3. FTIR spectrum of chitosan (A), sodium alginate (B), and alginate-chitosan hydrogel (C).

Table 4. FTIR spectral data.

Functional group	Wavelengths (cm^{-1})		
	Chitosan	Sodium alginate	Alginate-chitosan hydrogel
O-H (stretching)	3346.61	3259.81	3354.32
C-H (stretching)	2868.24	2929.97	-
C=O (stretching)	1645.33	-	-
N-H (bending)	1558.54	-	1558.54
COO- (asymmetric stretching)	-	1593.25	1595.18
CH_2 (bending)	1417.73	-	-
COO- (symmetric stretching)	-	1393.44	1393.44
C-O-C (asymmetric stretching)	1153.47	-	-
C-O (stretching)	1058.96	1024.24	1031.95

The absorption peak at 1593.25 cm^{-1} indicated the peak of the carbonyl (C=O) group in the spectrum of sodium alginate. The peak of the carbonyl group was presented at a higher wavelength in the spectrum of the hydrogel. The absorption peak at 1645.33 cm^{-1} referred to the characteristic amide I peak of the chitosan. Amide I peak was not found in the spectrum of the hydrogel. The amide II peak of chitosan shown at 1558.54 cm^{-1} became more intense in the spectrum of the hydrogel. The absorption bands that were presented in the spectrum of the hydrogel confirmed the formation of a polyion complex between sodium alginate and chitosan.

2.6. Scanning electron microscopy (SEM)

The morphologies of the alginate-chitosan hydrogels were examined by SEM. SEM images of the hydrogels are presented in Figure 4. The hydrogels had a porous structure with a compact and rough surface. It was seen that the hydrogels had various pore sizes.

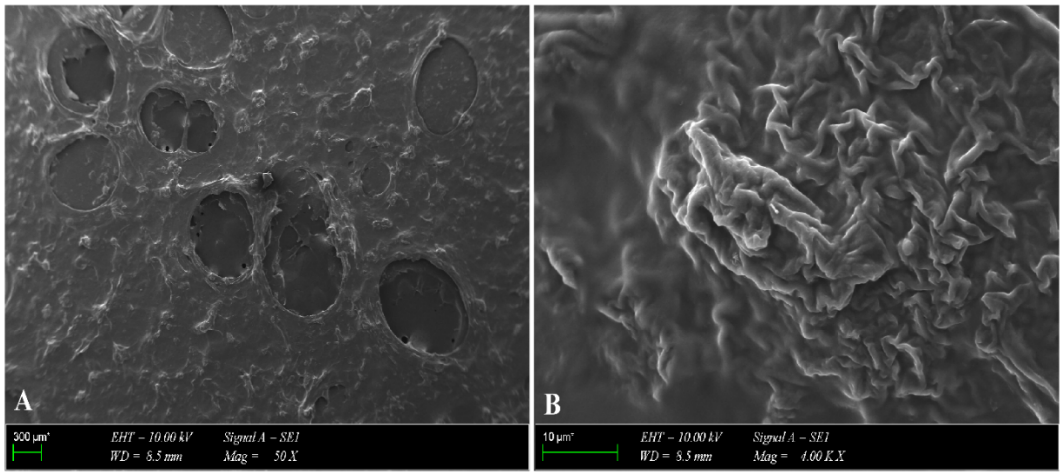

Figure 4. SEM images of alginate-chitosan hydrogels. A. H5 hydrogel (50X) B. H3 hydrogel (4kX)

2.7. Cell viability

Cell viability (%) in L-929 cells treated to alginate-chitosan hydrogels for 72 hours was determined by MTT assay. Compared to the control group, cell viability (%) of cells treated with H1, H2, H3, H4, H5, and H6 hydrogels were 82.7% ± 4.3, 81.3% ± 5.3, 94.7% ± 8.7, 93% ± 5.3, 87.5% ± 8.7 and 86.3% ± 9.2, respectively (Figure 5). There was no statistically significant difference between the cell viability (%) of hydrogels and the control group ($p>0.05$). The highest cell viability was obtained with H3 hydrogel containing 2.5% (w/v) sodium alginate and chitosan, while the lowest cell viability was obtained with H2 hydrogel containing 2% (w/v) sodium alginate and chitosan.

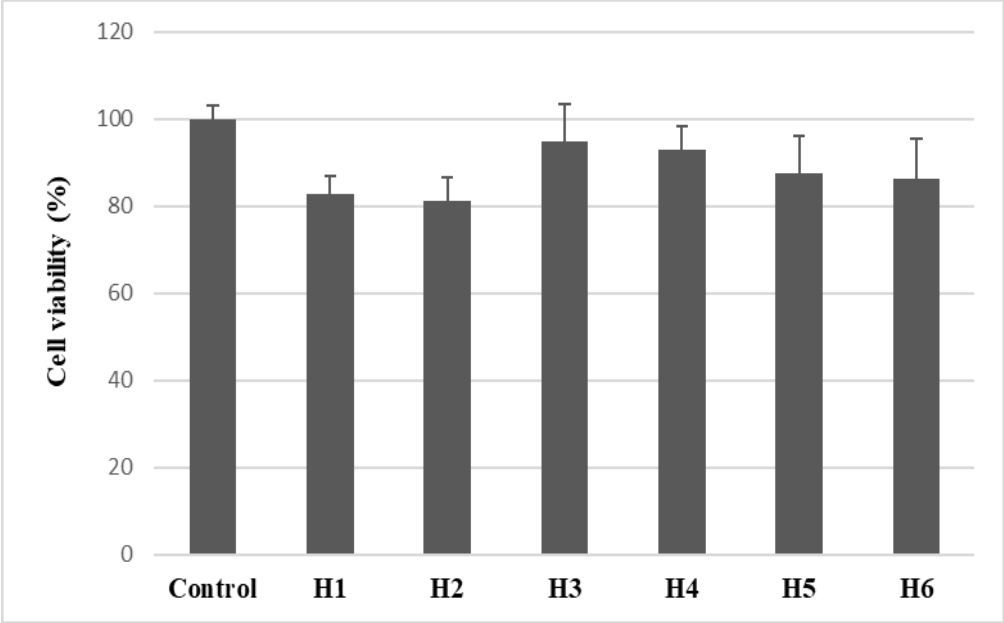

Figure 5. Effects of alginate-chitosan hydrogels on cell viability of L-929 cell lines.

3. DISCUSSION

In our study, alginate-chitosan hydrogels were prepared with sodium alginate and chitosan without any cross-linking agents only by pH modification, and pH values were measured. Chitosan (pKa 6.5) can become positively charged by protonation and form hydrogel through ionic interaction with a polyanion if dissolved in a slightly acidic solution [1, 14, 17]. In addition, pH values should not cause irritation or toxic effects on living tissues to allow alginate-chitosan hydrogels to be used safely in living organisms [18, 19]. Increased chitosan concentration caused an increase in the pH values of hydrogels. The dissolution of chitosan as a result of the protonation of amino groups causes an increase in pH by decreasing hydrogen ions in the solution. Therefore, pH also increases monotonically as the concentration of chitosan increases [20].

The hydrophilic nature and water solubility of a polymer are important in the adhesion to mucosal tissues, but at the same time, polymer solutions should have enough viscosity to be retained in the application area [5]. The viscosity of the hydrogels increased compared to the chitosan and alginate solutions prepared at polymer concentrations of the hydrogels and a gel-like structure was formed. A formulation with low viscosity might diffuse at the application area and leak out. On the other hand, very high viscosity might cause difficulties in the application of the hydrogel [21]. Viscosities of alginate-chitosan hydrogels were affected by polymer concentration and pH values. The formulation viscosities increased as the polymer concentration of the hydrogels increased. In hydrogels with the same polymer concentration, the hydrogel with a lower pH value had higher viscosity. In previous studies, it has been reported that increasing the polymer concentration increases viscosity [22-24]. The pKa value of the amine groups of chitosan is 6.5, while the pKa value of the carboxyl groups of alginates is 3.5. The amine groups of chitosan become positively charged by protonation and interact ionically with the negatively charged alginate in the pH range of 4-6. Since the number of positively charged amino groups increases at lower pH values, a stronger interaction occurs between alginate and chitosan, increasing viscosity [25]. The viscosity values of the hydrogels were found in the range of 12235 ± 97.3 cP and 40743.3 ± 190.1 cP. Sezer et al. suggested that the retaining times of fucoidan-chitosan hydrogels with viscosity values greater than 10000 cP are more appropriate [24]. All prepared alginate-chitosan hydrogels had viscosity values of >10000 cP.

Texture profile analysis has been used to determine the mechanical properties of semi-solid dosage forms like hydrogels [26]. Gel formulations should have acceptable mechanical properties for easy removal and application of the gel, long-term adhesion at the application site, and subsequently structural recovery of the gel [27].

Hardness is described as the maximum force required to achieve a certain deformation [28]. Adhesiveness is the work required to overcome the attractive forces between the surface of the sample and the surface of the probe [29]. Decreasing the pH value increased the hardness and adhesiveness values of hydrogels with the same polymer concentration. The reason for this is the increase in the number of cross-links formed through ionic interaction with carboxyl groups of sodium alginate as a result of the increase in the number of positively charged amino groups of chitosan with decreased pH and consequently the formation of more mechanically durable hydrogels [1, 14]. The increased polymer concentration in hydrogels containing an equal ratio of glacial acetic acid and NaOH increased adhesiveness and hardness. High adhesiveness means that formulations have long residence times at the application site [30]. A low hardness value indicates that the gel can be easily applied [18]. The hardness values of alginate-chitosan hydrogels were found between 0.099 ± 0.031 N and 0.285 ± 0.018 N. Cevher et al. reported that gel formulations with hardness values lower than 0.396 N are suitable for application on mucosal epithelium [31].

Compressibility defines the work necessary to compress a product over a certain distance. Compressibility indicates the easy removal of a prepared gel from the container and its spreadability to an application site [28]. The compressibility value of a gel should be low to allow its easy removal from the container and spread on application sites like skin, and mucosal epithelium [27]. Among the alginate-chitosan hydrogels, the lowest compressibility value belonged to the H2 hydrogel containing 2% polymer. The compressibility values of hydrogels with higher pH decreased in hydrogels with the same concentration. Consistent with previous studies, increasing polymer concentration in prepared alginate-chitosan hydrogels increased the compressibility of hydrogels [3, 28, 31].

Cohesiveness defines the structural reconstitution of the gel after application [32]. Formulations with low cohesiveness values spread easily over the application area [3]. The cohesiveness of hydrogels decreased with increased polymer concentration. In previous studies, it has been reported that cohesiveness decreases with increased polymer concentration [18, 27, 31].

Elasticity means the recovery rate of a deformed sample to its original state after the removal of deforming force [23]. Elasticity values of alginate-chitosan hydrogels varied between 0.957 ± 0.009 and 0.974 ± 0.006. The elasticity values of the hydrogels were close to each other and there was no significant difference between them.

After drying with lyophilization, the main factor that affected swelling properties was the change in pH value. In hydrogels with the same concentration of alginate and chitosan, the hydrogel with a higher pH absorbed more water. The swelling property of hydrogels mainly depends on the medium, cross-linking density, and porosity of hydrogel networks [12]. It is thought that an increase in the pH of the hydrogel decreases the number of cross-links, causing the hydrogel to have a looser network structure and larger pores. The increased number of cross-links tightens the hydrogel network structure and improves strength while making it difficult for hydrogel swelling and water penetration into spaces between polymer chains [33]. H1 and H2 hydrogels with the lowest polymer concentration had the highest water absorption capacity. Rençber

et al. reported that the increased amount of chitosan prevents swelling of the hydrogel matrix by causing a denser network structure in their inter-polymeric complex hydrogels [22].

FTIR analysis was performed to investigate the interactions between chitosan and sodium alginate in the hydrogel. The carbonyl bond peak of sodium alginate shifted to the higher wavelength in the alginate-chitosan hydrogel. The amide I peak of chitosan disappeared, while the amide II peak intensified in the spectrum of the hydrogel [14, 34]. All changes suggested electrostatic interaction occurred between sodium alginate and chitosan.

SEM images of alginate-chitosan hydrogels showed that the hydrogel structure has pores of various sizes. A large surface area facilitates cell adhesion and growth, while a large pore volume is required to accommodate enough cells and enable cell transfer [35].

The alginate-chitosan hydrogels were observed not to have cytotoxic effects on L-929 cells. According to the results of the MTT assay, cell viability rates of hydrogels ranged as H2 (81.3% ± 5.3) <H1 (82.7% ± 4.3) <H6 (86.3% ± 9.2) <H5 (87.5% ± 8.7) < H4 (93% ± 5.3) < H3 (94.7% ± 8.7), compared to the control group. Although the cell viability (%) of those with lower pH in hydrogels with the same polymer concentration was slightly higher, the difference between them was not statistically significant. Cell viability of more than 70% indicates that the tested sample is not cytotoxic [36].

4. CONCLUSION

It was demonstrated that the viscosities, mechanical properties, and swelling behaviors of alginate-chitosan hydrogels prepared with only pH modification using biocompatible polymers derived from natural resources change depending on the polymer concentration and pH value. Increasing polymer concentration and decreasing pH value increased viscosity, adhesiveness, and compressibility. It was observed that the hydrogels at the specified concentrations of alginate and chitosan have the appropriate morphological structure to allow the adhesion of living cells, mechanical properties, and high-water absorption ability. Since alginate-chitosan hydrogels were non-toxic on L-929 cell lines, they were deemed safe for use in living organisms.

5. MATERIALS AND METHODS

5.1. Materials

Chitosan (Low molecular weight, 75-85 % degree of deacetylation) was purchased from Sigma-Aldrich (USA). Sodium alginate was provided by Ilko Pharmaceuticals (Türkiye). Cell Proliferation Kit I (MTT) was purchased from Roche Diagnostics (Germany). Dulbecco's Modified Eagle Medium (DMEM) (PAN Biotech, Germany), fetal bovine serum (FBS) (PAN Biotech, Germany), Gibco™ L-Glutamine (Life Technologies, USA), and Gibco™ Penicillin/Streptomycin (10000 U/ml) (Life Technologies, USA) were used for cell culture. All other reagents used were of analytical grade.

5.2. Preparation of hydrogel formulations

The concentrations of chitosan and sodium alginate were determined to be 2% (w/v), 2.5% (w/v), and 3% (w/v) in the hydrogels. Sodium alginate was dissolved in bidistilled water. Then, chitosan powder was evenly dispersed in sodium alginate solution. Gelation was achieved by adding 1.5% (v/v) glacial acetic acid to the mixture. The pH values of the hydrogels were adjusted by adding 1 M NaOH solution at the ratio of 20% (v/v) and 25% (v/v) in the hydrogel.

5.3. Determination of pH

pH values of the hydrogels were measured with a pH meter (Mettler Toledo, USA) at 25°C to investigate the compatibility of the hydrogels for application to living cells and tissues.

5.4. Viscosity measurements of hydrogels

Viscosities of sodium alginate solutions, chitosan solutions, and hydrogels were measured using spindle 7 at 20 rpm by a rotational viscometer (Brookfield DV-E, CA). All measurements were performed in triplicate.

5.5. Texture profile analysis

Mechanical properties of the hydrogels were determined using TA.XT Plus Texture Analyzer (Stable Micro Systems, UK) at 25°C with 5 kg load cell. Gel samples were compressed twice by immersing a 10 mm diameter Perspex probe into the hydrogel to a depth of 15 mm at a defined rate of 2 mm/sec with a 15-second

delay between two compressions [29]. Texture Exponent 4.0.4.0 software was used for the calculation of mechanical parameters. Five parameters (hardness, adhesiveness, cohesiveness, compressibility, and elasticity) were used to characterize the hydrogels. Each sample was measured in triplicate.

5.6. Swelling behavior

The hydrogels were taken into pre-weighted dishes and then lyophilized. After the dry weights of the samples were measured, the samples were immersed in PBS (pH 7.4). The samples were periodically removed from the solution, wiped with tissue paper to remove excess fluid, and weighed. Each sample was studied in triplicate. Swelling ratios of hydrogels were calculated according to the equation below [37].

(Eq.1) $$\text{Swelling ratio (\%)} = ((W_0-W_t)\ /W_0)\ x100$$

where W_t is the weight of the swollen samples at time t and W_0 is the weight of the dry sample.

5.7. FTIR analysis

FTIR analysis was performed to observe the structural differences of alginate-chitosan hydrogel compared to chitosan and sodium alginate. The FTIR spectra of chitosan, sodium alginate, and lyophilized hydrogel sample were recorded with FTIR-8400S Spectrophotometer (Shimadzu Scientific Instruments, Japan).

5.8. SEM observation

The surface morphology of alginate-chitosan hydrogel was analyzed by SEM (Zeiss EVO MA10, German). The lyophilized hydrogel sample was coated with a mixture of gold and palladium under vacuum and scanned at an accelerating voltage of 10 kV.

5.9. Cell proliferation study

The cytotoxicity of the hydrogels was examined by MTT (3,4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium) assay. L-929 cells (ATCC CCL-1™, USA) were seeded into a 96-well plate with a density of $5x10^3$ cells/well and allowed to grow in DMEM containing 10% FBS and antibiotic solution at 37 °C in a humidified atmosphere with 5% CO_2. Afterward, the medium was removed from wells, and 10 mg of hydrogel samples were applied to the cells with 100µl of fresh medium, except for the control group. After 72 hours of incubation, 10 µl of MTT (0.5 mg/ml) was added to each well and incubated for 4 hours. 100µl of 0.01 M HCl solution containing 10% SDS was added into each well to dissolve the formazan crystals formed by living cells. After overnight incubation, the absorbance of the samples was measured at 570 nm. Cell viability (%) was calculated according to the absorbance of the control group (taken as 100%).

5.10. Statistical analysis

Statistical analysis was performed using one-way ANOVA (Tukey's multiple comparisons test) in GraphPad Prism 8 (GraphPad Software, Canada). Differences were considered significant if $p < 0.05$.

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