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ORIGINAL ARTICLE / ÖZGÜN MAKALE

DEVELOPMENT AND CHARACTERIZATION OF ASCORBIC ACID LOADED POLYELECTROLYTE CHITOSAN-GELATIN HYDROGELS

ASKORBİK ASİT İÇEREN POLİELEKTROLİT KİTOZAN-JELATİN HİDROJELLERİN GELİŞTİRİLMESİ VE KARAKTERİZASYONU

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ABSTRACT

Objective: *Aim of study was to formulate chitosan-gelatin hydrogels containing ascorbic acid, an antioxidant, with/without polyelectrolyte-complex.*
Material and Method: *Effect of formation polyelectrolyte-complex, gelatin concentration (10-20%) and chitosan:gelatin ratio(1:1, 1:2, 2:1w/w) on the rheological properties, in-vitro release, encapsulation efficiency of hydrogels were investigated. Dissolution rates were also compared using area under dissolution curve (AUC), mean dissolution time (MDT), mean residence time (MRT). Also, the potential for topical use of the hydrogel was evaluated by examining the 24-and 72-hours cytotoxic and proliferative effects on L929 cell line using MTT test.*
Result and Discussion: *Polyelectrolyte complex formation led to improved drug release and increased viscosity. Cell viability of the free and drug-loaded polyelectrolyte-hydrogels was over 70% at the end of the 72h in all formulations (except formulations with chitosan:gelatin ratio of 1:2w/w) showed that ascorbic acid and hydrogels did not cause cellular toxicity and could be used safely. It has been demonstrated that the gelatin ratio should be at most 50%, and excess gelatin reduces cell viability. F6-coded-polyelectrolyte-hydrogel (20% gelatin; 2:1 chitosan:gelatin w/w) was ideal formulation as it led to best sustained drug release with high MDT and AUC values, and cell viability >80%. In conclusion, polyelectrolyte-complex formation is more superior, and chitosan:gelatin ratio and gelatin concentration can be manipulated to obtain the desired properties.*
Keywords: *Ascorbic acid (vitamin C), chitosan, gelatin, hydrogels, polyelectrolyte complex chitosan-gelatin*

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ÖZ

Amaç: *Çalışmanın amacı, güçlü bir antioksidan olan askorbik asit içeren, polielektrolit kompleksi olan ve olmayan kitozan-jelatin hidrojelleri formüle etmektir.*

Gereç ve Yöntem: *Polielektrolit kompleksi oluşumunun, jelatin konsantrasyonunun (%10-20) ve kitozan:jelatin oranının (1:1, 1:2, 2:1 a/a) reolojik özellikler, in vitro salım ve enkapsülasyon etkinliği üzerindeki etkisi araştırılmıştır. Salım sonuçları AUC, MDT ve MRT kullanılarak karşılaştırılmıştır. Ayrıca MTT testi kullanılarak L929 hücre hattı üzerindeki 24 ve 72 saatlik sitotoksik ve proliferatif etkileri incelenerek geliştirilen hidrojelin topikal kullanım potansiyeli değerlendirilmiştir*

Sonuç ve Tartışma: *Polielektrolit kompleksi oluşumu, ilaç salımının gelişmesine ve viskozitenin artmasına yol açmıştır. Boş ve ilaç yüklü polielektrolit hidrojellerin hücre canlılığının 72 saat sonunda tüm formülasyonlarda (kitozan:jelatin oranı 1:2 a/a olan formülasyonlar hariç) %70'in üzerinde olması, askorbik asit ve hidrojellerin hücresel toksisiteye neden olmadığını ve güvenle kullanılabilir olduğunu göstermektedir. Jelatin oranının en fazla %50 olması gerektiği ve fazla jelatinin hücre canlılığını azalttığı kanıtlanmıştır. Sonuç olarak F6 kodlu polielektrolit hidrojel (%20 jelatin; 2:1 a/a kitozan:jelatin), yüksek MDT ve AUC değerleri ve >%80 hücre canlılığı ile en uzun kontrollü ilaç salınımına yol açtığı için ideal formülasyondur. Sonuç olarak, polielektrolit kompleks oluşumu daha uygundur ve istenen özellikleri elde etmek için kitozan:jelatin oranı ve jelatin konsantrasyonu manipüle edilebilir.*

Anahtar Kelimeler: *Askorbik asit (vitamin C), hidrojeller, jelatin, kitozan, polielektrolit kompleks kitozan-jelatin*

INTRODUCTION

Ascorbic acid (vitamin C) (AA) is vital for the performance of many important metabolic and physiological functions. There are many studies on topical formulations containing AA and its derivatives to strengthen skin tissues, increase collagen synthesis, reduce pigmentation loss and induce enhanced growth and health activities. AA is a popular antioxidant and due to this effect, it is used as anti-aging and photoprotective agent in cosmetic formulations, as well as being added to various formulations as an antioxidant [1]. Antioxidants can scavenge toxic free radicals and other reactive oxygen species (ROS) formed in cell metabolism. With their talent to avoid the negative effects of free radicals, they are crucial to protect the structural integrity and functions of cells and tissues [2-4]. Although AA has been widely studied, it is still important to prepare different formulations and to investigate its potential contribution to many different aspects of cell metabolism, not only for its antioxidant properties but also for many different purposes, including its anticancer effect [5].

Chitosan is a cationic, hydrophilic, natural copolymer with biocompatibility and biodegradability properties, which are also important for biological devices. Chitosan-based hydrogels have the ability to serve many purposes, including tissue engineering and wound healing. In addition, the fact that chitosan is suitable for use as a controlled release system makes chitosan-based hydrogels suitable drug delivery systems for use in medical and pharmaceutical applications [6,7]. Chitosan is often used with other polymers to improve its biological and mechanical properties. The use of chitosan in combination with gelatin contributes to the enhancement of the biological activity of chitosan by increasing cell adhesion, cell migration and forming a polyelectrolyte complex. Gelatin also contributes to the improvement of the wettability and water absorption abilities of chitosan [8,9].

Gelatin is a partially denatured derivative of collagen, a protein that is the main component of the extracelluler matrix in skin, bone and connective tissue. Gelatin is a suitable polymer for use as a biomaterial due to being biocompatible, biodegradable and non-immunogenic [9,10]. A polyelectrolyte complex is formed by the electrostatic interactions of the negatively charged carboxylic groups of gelatin and the positively charged amino groups of chitosan, and the intermolecular hydrogen bonds [7,11]. Since gelatin is known to have a negative charge above pH 4.7 (isoelectric point) [12], it is essential to raise the pH of chitosan >4.7 to form a polyelectrolyte complex between the positively charged chitosan and gelatin.

There has been numerous studies to show films/scaffolds containing chitosan and/or gelatin are

suitable for tissue engineering [7-10,13]. However, there are limited studies to explain drug-loaded polyelectrolyte complex formed chitosan-gelatin hydrogel/film/scaffold. In a study conducted by Mathew and Arumainathan (2022), dopamine loaded chitosan/gelatin nanocomposite were produced and pH-related cross-linking were confirmed [14]. In addition, to our knowledge, no prior studies have examined AA-loaded chitosan-gelatin drug delivery system. In a study in which chitosan-gelatin sponge was produced for wound healing, AA was used to enhance the mechanical properties of the product by increasing the solubility of chitosan. In the study of Lu et al. (2016), firstly chitosan was dissolved in AA solution and then acetic acid was added [15]. However, AA was not used for drug loading as in present studies and were not evaluated in this sense. Therefore, our study will be first in the literature in terms of producing a polyelectrolyte complex structured chitosan-gelatin hydrogel and producing an AA-loaded chitosan-gelatin drug delivery system.

L929 cells are mouse fibroblast cells and are frequently preferred to figure out cytotoxic concentrations of various samples [16]. They are also designated as reference cells for cytotoxicity testing by international standards [17,18].

The aim of this study was to produce chitosan-gelatin hydrogels containing AA, an antioxidant drug, with and without polyelectrolyte complex between chitosan and gelatin, and to determine the rheological properties, *in vitro* release, encapsulation efficiency and cytotoxicity and proliferation on L929 cell lines. Dissolution rates were also compared using area under the dissolution curve (AUC), mean dissolution time (MDT) and mean residence time of the drug substance molecules in the dosage form (MRT).

MATERIAL AND METHOD

Materials

Chitosan (medium molecular weight, 200-800 cP, 1wt% in 1% acetic acid), 3-(4,5-dimethyldiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) for cell culture were purchased from Sigma (USA). Gelatin (bloom 250-270 g) was gifted from Halavet (Turkey). AA was purchased from Riedel-de Haën (Germany). Eagle's Minimum Essential Medium (EMEM) (ATCC® 30-2003™) was provided from Biochrom, Germany. The NCTC clone 929 [L cell, L-929, connective mouse tissue) was provided from the American Type Culture Collection (ATCC® CCL-1™), USA. Plates and cell culture flasks were purchased from Corning® Cedex (France). Smart Slides and Trypan Blue solution were purchased from Roche (Switzerland). All other chemicals were analytical grade.

Quantification of AA

Amount of AA was measured using UV-spectrophotometer (Shimadzu 1800, Japan). The maximum spectrum of AA was scanned between 200 nm and 600 nm. AA solutions (5-50 µg/ml) were prepared using a stock solution of AA in the dark. The standard curve was calculated by linear regression, according to the following formula: y=ax+b (x: the concentration of AA as µg/ml, y: the absorbance).

Preparation of Chitosan-gelatin Hydrogels

In our study, polyelectrolyte chitosan-gelatin hydrogels (PCGHs) were produced by making some revisions in the preparation of chitosan-gelatin biomaterials produced by 3D printing by Ng et al. and Fischetti et al. [7,19]. Firstly, chitosan and gelatin solutions were prepared. 1% w/v chitosan was dissolved in acetic acid (2% v/v). 10% and 20% w/v gelatin was dissolved in phosphate buffer saline (pH:7.4) at 40°C. They were stirred on a magnetic stirrer for 6 hours. Since the pH of the chitosan solution needed to be adjusted above 4.7 for the chitosan and gelatin to form a polyelectrolyte complex, the pH was adjusted to 4.8 using 0.5 M sodium hydroxide. AA was added to the gelatin solution at a concentration of 100 µg/ml in each hydrogel and stirred for 30 minutes. AA solutions and formulations containing AA were stored in the dark. Consequently, gelatin and chitosan solutions were added dropwise in different ratios (1:1, 1:2 and 2:1 w/w) during mixing and they were stirred for 2 hours. AA-loaded chitosan-gelatin hydrogels prepared in the study are given in Table 1. In the study, non-pH

adjusted chitosan-gelatin hydrogels (NPCGHs) were also produced without adjusting the pH of chitosan. NPCGHs were designated F1A-F6A.

Table 1. Compositions and characterization parameters of ascorbic acid-loaded chitosan-gelatin hydrogels[a]

	F1	**F2**	**F3**	**F4**	**F5**	**F6**
Chitosan (%) (w/v)	1	1	1	1	1	1
Gelatin (%) (w/v)	10	10	10	20	20	20
Chitosan:Gelatin Ratio (w/w)	1:1	1:2	2:1	1:1	1:2	2:1
Viscosity of PCGHs[b*] (mPa.s)	63.0±0.4	73.4±1.5	42.2±0.1	402.8±1.9	776.9±0.7	281.3±3.5
Viscosity of NPCGHs[c*] (mPa.s)	48.6±0.2	56.1±1.1	48.6±0.4	271.9±0.2	578.1±1.8	97.7±0.2
Encapsulation efficiency of PCGHs (%)	97.4±2.1	94.6±2.0	91.3±1.8	82.4±1.6	81.8±1.2	80.5±1.1

[a]Values are expressed as mean ± standard deviation, $n = 3$.
[b]Polyelectrolyte chitosan-gelatin hydrogels
[c]non-pH adjusted chitosan-gelatin hydrogels
[*]Viscosities at 200 rpm are given.

Characterization of Formulations

Viscosity

The viscosity of the solutions was performed with Brookfield DV3T Rheometer (USA) at 25°C and 37°C at 50-250 rpm (spindle: CP-52). Results were expressed as mean ± standard deviation (n=3).

Encapsulation Efficiency

AA-loaded hydrogels (2 ml) and blank hydrogels were diluted into 40 ml pH 7.4 phosphate buffer. They were centrifuged for 1 h at 25°C (Thermo Scientific SL16R, USA) and supernatants were collected (n=3). The supernatant collected from the blank hydrogels was taken as a blank and the amount of AA in the supernatants was analyzed by UV spectrophotometer at 287 nm and the encapsulation efficiency was calculated [20]. Data were expressed as mean ± SD. The encapsulation efficiency was calculated using the following equation.

$$\text{Encapsulation efficiency (EE\%)} = [(E_{initial} - E_{supernatant}) / E_{initial}] \times 100$$

In Vitro Release

In vitro release study was performed by placing a 12,000 Dalton pore size dialysis membrane between the receptor and donor chambers of Franz diffusion cells for 24 hours at 37°C. One ml of hydrogel was placed in the donor chamber and 2 ml of pH 7.4 phosphate buffer in the receptor chamber and stirred on a magnetic stirrer at 400 rpm during the experiment (n=3). At predetermined time intervals (2^{nd}, 5^{th}, 8^{th} and 24^{th} hours), 2 ml samples were collected and 2 ml of fresh buffer was added. The amount of released AA was determined by UV spectrophotometer. Drug release from the AA solution at the same concentration as the drug in the hydrogel was also determined.

For dissolution rate comparison, AUC, MRT and MDT were calculated by DDSolver Software and equations of dissolution rate parameters were given in Table 2 [21,22].

Cell Culture Studies

L929 cells were grown in an incubator under 5% CO_2 atmosphere at 37ºC with a medium consisted of EMEM containing 25 mM glucose, 1% gentamicin, 5 mM glutamine supplemented with 10% horse serum, and 7.5% sodium bicarbonate. A microscope was used to control the presence of a

confluent monolayer. The medium was replaced with fresh EMEM every 48 hours.

Table 2. Equations of dissolution rate parameters

Dissolution Rate Parameter	Equation
AUC	$AUC = \sum_{i=1}^{n} \frac{(t_i - t_{i-1})(y_{i-1} + y_i)}{2}$
MDT	$MDT = \frac{\sum_{i=1}^{n} \overline{ti} \,.\, \Delta Mi}{\sum_{i=1}^{n} \Delta Mi}$
MRT	$MRT = \frac{\int_0^t t\ (100 - y).dt}{\int_0^t (100 - y)\,.dt}$

n number of sampling points; t*i* *i*th time point; y_i percentage of drug dissolved at time t_i; y percentage of drug dissolved at time t; $\overline{ti}$ time at the midpoint between i and i–1; ΔM_i additional amount of drug dissolved between i and i–1; k order of the moments of dissolution times

Cytotoxicity Assay

MTT test was used for cell viability on L929 cells. L929 cells were seeded (10.000 cells/well) in three different 96-well culture plates [4]. Plates were stored for 24 hours at 37ºC to adherence of cells. Then, the cells were treated with blank and AA-loaded formulations for 24 hours in parallel with *in vitro* release study and for 72 hours to evaluate the proliferation effect. After incubation, the medium was replaced with 13 µl MTT solution (5 mg/ml in phosphate-buffered saline) and 100 µl fresh medium. Then it was incubated for 4 hours at 37°C and 100 µl of DMSO was added to each well to dissolve the formazan precipitate. The colour density was measured at 570 nm with a multi-well ELISA reader (Biotech Synergy HT, USA). The control group, wells containing medium only, were considered 100% cell viability, and the viability of the samples was calculated as a percentage using the control group values.

Statistical Analysis

SPSS 20.0 for Windows (SPSS, Chicago, IL) were used for statistical analysis. The significance was evaluated with one-way ANOVA followed by Tukey's post hoc test (SPSS 20.0). $p < 0.05$ is considered statistically significant.

RESULT AND DISCUSSION

Quantification of AA

The maximum wavelength of AA was determined as 287 nm. The standard curve was determined by linear regression (y=0.0212x+0.014). The calibration curve was linear with a high correlation coefficient (r^2 =0.9998).

Preparation and Characterizations of Chitosan-gelatin Hydrogels

Compositions and characterization parameters of AA-loaded PCGHs were given in Table 1.

Encapsulation Efficiency and Drug Release From Chitosan-gelatin Hydrogels

Encapsulation efficiencies of PCGHs were between 80.5 and 97.4%, while they were between 82.1 and 95.8% for NPCGHs. The increase in gelatin concentration led to a decrease in encapsulation efficiency. However, all hydrogels have been shown to have favorable encapsulation efficiency (≥81%) (Table 1).

It was shown that all hydrogels provide a more sustained release compared to AA solution. While 92.7% of the drug was released from the solution at the 4th hours and completely finished at the 8th hours,

the drug release in the hydrogel could be prolonged for 24 hours (Figure 1 and 2). AA release from its solution and PCGHs was given in Figure 1.

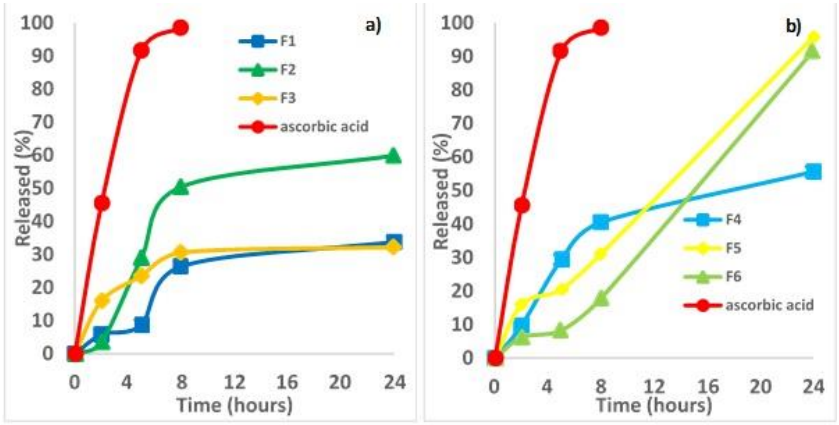

Figure 1. Ascorbic acid release from its solution and polyelectrolyte chitosan-gelatin hydrogels contains a) 10% gelatin and b) 20% gelatin

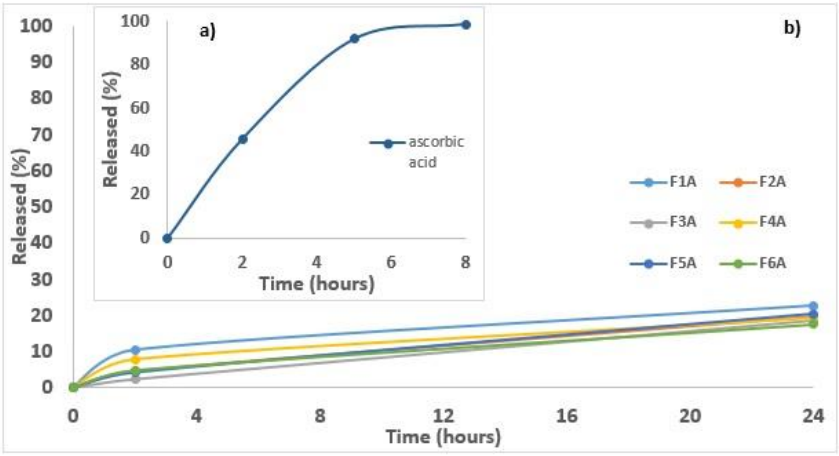

Figure 2. Ascorbic acid release from a) its solution and b) non-pH adjusted chitosan-gelatin hydrogels

AA release from NPCGHs was shown in Figure 2. Drug release was reduced compared to the solution ($p<0.05$). The AA released within 24 hours was only between 18.5% and 22.7% (Figure 2). Drug release in PCGHs was better compared to NPCGHs (Figure 1 and 2) ($p<0.05$). The low cumulative release of AA from NPCGHs was about the anionic character of AA at physiologic pH of 7.4 [23]. In uncomplexed gels (NPCGHs,) the negatively charged chitosan prevented the release of negatively charged AA since chitosan was free in the gel and there was a strong interaction via hydrophobic and/or electrostatic interaction or hydrogen bonding between chitosan and AA at pH 7.4. Sun et al. (2020) claimed that the interaction between the functional group density and the group was the most crucial factor for the drug release from chitosan films [24]. As a result, forming a polyelectrolyte complex had a significant effect on drug release and led to better sustained release profile and improved drug release.

AUC, MRT and MDT results of PCGHs, which were calculated by DDSolver Software, were given in Table 3.

AUC values of F2 and F5, hydrogels with a chitosan:gelatin ratio of 1:2 w/w, were the highest (Table 3). So, hydrogels with higher gelatin content (chitosan:gelatin ratio of 1:2 w/w) showed advanced release characteristics compared to lower gelatin content (chitosan:gelatin ratio of 1:1 and 2:1 w/w)

(Figure 1). This is due to the quicker hydration rate of gelatin and its superior water absorption ability compared to chitosan [8,25].

Table 3. Dissolution rate results (n=3)

Parameter	F1	F2	F3	F4	F5	F6
AUC	561.67	1055.42	659.53	943.49	1164.20	944.34
MRT	10.96	9.67	11.40	9.98	6.18	6.65
MDT	7.37	6.40	3.56	7.05	11.85	13.70

The slower release rate is characterized by high MRT and low MDT. As seen in Table 3, the hydrogel with the slowest drug release rate was the F4-coded hydrogel among the 20% gelatin-containing hydrogels (F4-F6), while the F3-coded hydrogel among the 10% gelatin-containing hydrogels (F1-F3) (Figure 1).

Higher MRT values and lower AUC values at low gelatin concentration for hydrogels containing the same ratio of chitosan:gelatin proved that, the increase in gelatin concentration caused an increase in the amount of AA released in 24 hours (Table 3) (Figure 1) (p<0.05). Therefore, it has proven that higher gelatin concentration led to higher drug release.

As a result, hydrogels coded F5 and F6 were determined as ideal PCGHs as they led to the sustained release with 11.85 and 13.70 MDT values (Table 3), and the cumulative release rate of AA reached 95.8% and 91.7% of AA at 24 h (Figure 2), respectively.

Rheology of Chitosan-gelatin Hydrogels

Viscosities of the hydrogels as a function of shear rate at both room temperature (25°C) and body temperature (37°C) were evaluated. Temperature plays an inhibitory role in the formation of intermolecular attraction forces. According to Figure 3, increasing the temperature from 25°C to 37°C resulted in a decrease in the viscosity of PCGHs ranging from 20-88% (Figure 3) (p<0.05). As the gelatin ratio increased, there was a more significant decrease in the viscosity of the PCGHs with increasing the temperature to 37°C (p<0.05). While PCGHs with a chitosan:gelatin ratio of 1:2 w/w had a viscosity reduction of 62.8% (F2) and 88.1% (F5), they were of 20% (F3) and 74.8% (F6) in PCGHs with a chitosan:gelatin ratio of 2:1 w/w, respectively (Figure 3).

As a result, it was concluded that the increase in concentration and ratio of gelatin led to a higher reduction in the viscosity of the hydrogels as a function of increasing temperature. Because higher temperatures accelerate the destruction of the biopolymer components of gelatin and the formation of low molecular weight fractions [26]. Desbrieres found that the viscosities of chitosan solutions were not dependent on temperature [27]. In addition to literature data, our findings proved the change in viscosity with temperature was dependent on the amount of gelatin, not the amount of chitosan.

The viscosity of PCGHs containing 10% gelatin ranged from 42-89 mPa.s (Figure 3a), while the viscosity of PCGHs containing 20% gelatin ranged from 263-777 mPa.s (Figure 3b). The viscosity of hydrogels containing 20% gelatin was higher than that of containing 10% gelatin, due to the increase in the viscosity of the gelatin solution as the gelatin concentration increased from 10% to 20% (Figure 3 and 4) (p<0.05). The viscosity of PCGHs at 25°C increased 7-9 fold by increasing the gelatin concentration from 10% to 20% (Figure 3).

As can be seen in Table 1 and Figure 3-4, the increase in the gelatin ratio also caused an increase in viscosity (p<0.05). This was probably due to the viscosity of gelatin solutions being more viscous than chitosan solution.

Rheology studies have shown that the viscosities of PCGHs were higher than those of NPCGHs (Figure 4) (p<0.05). This was due to the fact that the polyelectrolyte complex formed by electrostatic interactions and intermolecular hydrogen bonds between chitosan and gelatin resulted in denser zones in the gel network which was described by Nicolay et al. [11]. Ma et al. found that hydrogen bonding increases viscosity and strength [28].

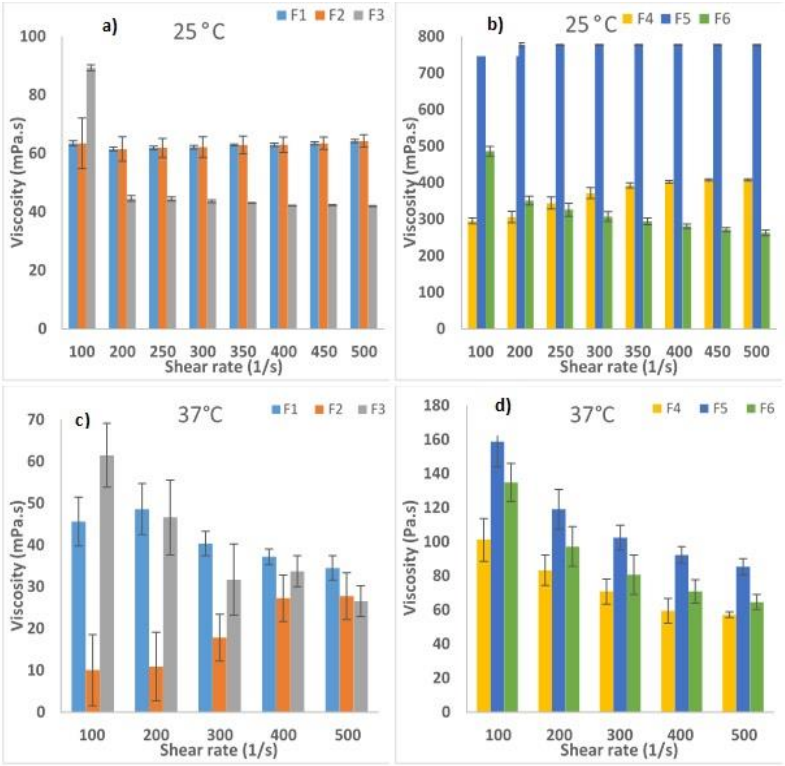

Figure 3. Viscosity as a function of shear rate at different temperatures for PCGHs which containing different ratios of 1% chitosan and a) 10% gelatin at 25°C, b) 20% gelatin at 25°C, c) 10% gelatin at 37°C, d) 20% gelatin at 37°C

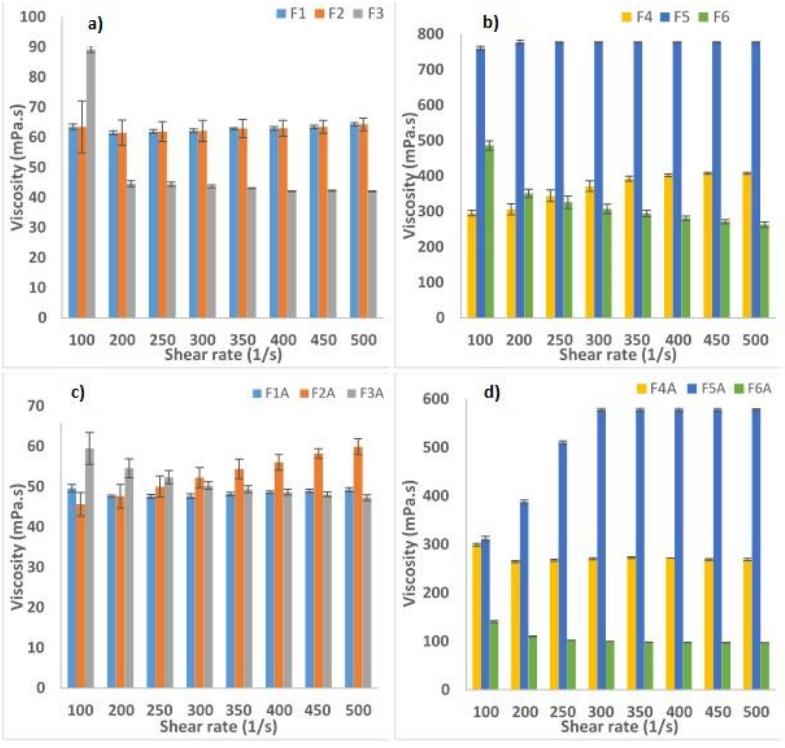

Figure 4. Viscosity as a function of shear rate at 25°C for PCGHs containing a) 10% gelatin, b) 20% gelatin, and for NPCGHs containing c) 10% gelatin and d) 20% gelatin

It is known that pseudo-plastic behaviour is associated with a decrease in viscosity with an increase in shear rate, while dilatant behaviour is associated with an increase in viscosity. In newtonian fluids, the viscosity is constant [29,30]. Hydrogels with high chitosan content (F3, F3A, F6 and F6A) exhibit pseudo-plastic behaviour, while hydrogels with high gelatin content (F2, F2A, F5 and F5A) exhibit dilatant behaviour at 25°C (Figure 3). This may be due to the fact that gelatin exhibits a dilatant and shear-thickening behavior [30], while chitosan exhibits a pseudo-plastic and shear thinning behavior [29].

In addition, newtonian flow was observed in F1, F1A and F4A, while dilatant flow was observed in F4 with a chitosan:gelatin ratio of 1:1 w/w at 25°C (Figure 3). Although the chitosan:gelatin ratio was the same, the PCGH with 20% gelatin (F4) had more gelatin than the PCGH with 10% gelatin (F1). Thus, F4 could exhibit the same flow behaviour as gelatin, while F1 could not exhibit either the gelatin or chitosan flow behaviour. Unlike viscosities at 25°C, all PCGHs had plastic behaviour (except F2) at 37°C, regardless of their chitosan or gelatin ratio.

Cell Culture

The effect of formulation components developed with AA was determined on L929 cell viability. The results are given in Figure 5.

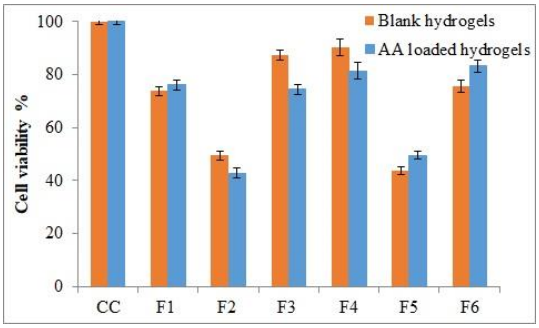

Figure 5. Percentage of cell viability of L929 cells incubated with blank hydrogels and AA-loaded hydrogels for 72 h (CC is cell control, values are expressed as mean ± standard deviation, $n = 6$)

No significant difference in cell viability was observed between the blank hydrogels and the AA-loaded hydrogels (Figure 5) ($p>0.05$).

We measured the effect of formulations on cell viability with the most commonly used method, the MTT test. While the effects of the developed formulations on L929 cell viability were around 50% at 24 h, 70% and above were obtained at the end of the 72 h. The fact that the viability at 72 h was higher than the viability at 24 h showed that the formulations were effective on cell proliferation. According to the literature, the IC_{50} value of AA is 114 µg/ml [31]. In our formulations, 100 µg/ml was used as the initial dose.

According to MTT test results, it was observed that AA and formulations did not cause cellular toxicity. The fact that cell viability was above 70% at the end of the 72 h in all formulations showed that they could be used safely. Also, increasing the gelatin concentration from 10% to 20% enhanced cell viability ($p<0.05$). F4 and F6 coded PCGHs containing 20% gelatin were found to have cell viability over 80%. (Figure 5)

Previous studies have shown that the presence of gelatin increases the cyto-compatibility of chitosan [10]. In our study, while cell viability was over 70% in PCGHs at 72 hours, the viability decreased to 43% (F2) and 50% (F5) with the increase in gelatin concentration to 66.6% (chitosan:gelatin ratio to 1:2 w/w). Adding more gelatin than necessary for complex formation prevented the induction of the cells by electrostatic interaction with the negatively charged L929 cell due to the negative structure of gelatin [32]. In addition, it was found for the first time in our study that the gelatin ratio should be at most 50% for the formation of a suitable chitosan-gelatin polyelectrolyte complex,

and that excess gelatin required for complex formation (>50% gelatin, 1:2 w/w chitosan:gelatin ratio) reduces cell viability.

To sum up, AA-loaded PCGHs with favorable properties such as high encapsulation efficiency, sustained drug release and enhanced cell viability were successfully produced. The effect of gelatin concentration and chitosan:gelatin ratio on the rheological behaviour, *in vitro* release, encapsulation efficiency and cytotoxicity and proliferative effects of chitosan-gelatin hydrogels were determined in detail. AUC, MDT and MRT were also calculated to compare dissolution rates of hydrogels. In the study, NPCGHs were also produced without the polyelectrolyte complex to determine the effect of forming a polyelectrolyte complex on chitosan-gelatin hydrogels. The results of the study confirmed that polyelectrolyte complex formation has proven to be more favorable. It is well known that the addition of gelatin to chitosan has a positive effect on cell viability. However, the study first concludes that there is a limit to the gelatin ratio (≤ 50%) for the formation of a suitable chitosan-gelatin polyelectrolyte complex, and excess gelatin reduces cell viability. As a result of this study, F6 coded hydrogel containing 20% gelatin and a chitosan:gelatin ratio of 2:1 w/w was determined as the ideal formulation as it provided best sustained drug release with high MDT and AUC values. Also, the cell viability of F6 was found to be over 80% (83.2%) at a concentration of 74 µg/ml obtained from the release experiment. In conlusion, it is possible to say that the properties such as drug release, viscosity and cell viability of well-characterized hydrogels that allow topical application of AA and many antioxidants and provide a significant advantage in increasing their effectiveness, can be affected by gelatin concentration and chitosan/gelatin ratio.

AUTHOR CONTRIBUTIONS

Concept: T.E.B., Ç.Y.; Design: T.E.B., Ç.Y.; Control: T.E.B., Ç.Y.; Sources: T.E.B., Ç.Y.; Materials: T.E.B., Ç.Y.; Data Collection and/or Processing: T.E.B., Ç.Y.; Analysis and/or Interpretation: T.E.B., Ç.Y.; Literature Review: T.E.B.; Manuscript Writing: T.E.B., Ç.Y.; Critical Review: T.E.B.; Ç.Y.; Other: -

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

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