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Original Article

Formulation and Evaluation of Growth Hormone-Carrying Hydrogel Containing Chitosan and Polyvinyl Alcohol for Wound Healing

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Abstract

Wound healing is an important issue related to health. Due to lifestyle changes, population growth, and disease patterns, it is necessary to propose methods for efficient wound healing. In this study, a novel hydrogel dressing is offered containing Chitosan (CN) and Polyvinyl alcohol (PVA) polymers chemically cross-linked by Genipin (GN) and using Glycerol (G) as a softener. Somatropin is used to directly affect collagen formation and the acceleration of the healing process. The morphology was evaluated using SEM, and the effect of each component on properties (e.g., softness, roughness, and crystallinity) was examined. Evaluating mechanical properties showed the effect of PVA and G on the hydrogel's elongation, strength, and elastic modulus. The most similar sample to skin was also introduced. Differential scanning calorimetry (DSC) was performed, and the effect of components on crystal structure and thermal properties was investigated. FTIR was conducted to identify organic compounds and their functional groups to ensure the compatibility of CN, PVA, and somatropin. In vitro, degradation tests showed the biodegradability of samples, and the most appropriate degradation rate was determined. Findings show the direct effect of PVA and G on water absorption rate. Investigating the pH changes during degradation revealed that this rate positively affects the wound-healing process. In the drug-release test, G-containing samples exhibited better performance and had a higher release rate in the first four days of healing, when the wound needed the highest growth hormone. MTT assay evaluated the cytotoxicity of samples. According to the results, cell growth and survival percentages were higher in the drug-containing samples.

Keywords: Chitosan**,** Genipin, Growth hormone, Polyvinyl alcohol, Somatropin, Wound dressing.

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1. Introduction

Wound healing is a complex and dynamic process, as a change in the wound condition leads to a change in the person's health. There is an interaction among the wound, blood cells, extracellular matrix, and cells involved in the healing process. The wound healing process

consists of 4 phases: hemostasis, inflammation, proliferation, and remodeling [1, 2]. Hydrogels can absorb wound exudates, thereby increasing fibroblast cell proliferation and the migration of keratinocytes. These two processes are very effective for complete epithelialization and wound healing. In addition, the network structure of the hydrogel protects the wound against infection and the invasion of microorganisms and bacteria. This structure allows bioactive molecules such as antibiotics and drugs to enter the wound area. As a result, these molecules are trapped in the hydrogel network during the gelation process. Thus, when they come in contact with the wound, they are moved along with the wound exudates. Since the amount of water in hydrogels is the same as that in the tissue, they have enough flexibility to adapt to different body parts [3]. As the most abundant natural amino polysaccharide, chitin is a protective substance in crustaceans and insects. This polymer has high biocompatibility, low toxicity, biodegradability, and antibacterial properties. Chitin and its other derivative, chitosan, have a variety of medical applications, including drug release, tissue engineering, wound healing, blood cholesterol reduction, and as an anticoagulant and antitumor [4, 5, 6]. CN is soluble in acidic media because of a free amino proton group. It is naturally antibacterial and has other benefits, such as analgesic effects and hemostatic activity for wound dressing. However, it should be used as cross-linked due to the lack of appropriate mechanical properties for wound dressing. Cross-linking occurs when a substance or chemical compound (called a crosslinker) creates intermolecular covalent bridges between polymer chains. Chemical crosslinkers include glutaraldehyde, Genipin (GN), glyoxal, dextran sulfate, and diisocyanate. One of the relatively new cross-linking agents is a natural substance called GN, an excellent crosslinker for polymers that contain amine groups. A blue gel is created after the spontaneous reaction of GN with amine groups [7]. GN is extracted from Gardenia fruit and used in traditional Chinese medicine. It has been reported to be an effective cross-linking agent that can react with amine groups. It is much less toxic than glutaraldehyde [8-11]. The cross-linking of CN with GN leads to the opening of the dihydropyran ring and the formation of the tertiary amine structure. Moreover, polymerization occurs between GN molecules and the CN amine group. The cross-linking mechanism involves the nucleophilic attack of the amine group of CN on the olefinic carbon No. 3 of GN, followed by the opening of the dihydropyran ring. The formation of a secondary amide bond and a heterocyclic amino acid leads to CN cross-linking [7]. PVA is among the most widely used water-soluble polymers, and its monomer does not exist in its free form. This substance is obtained from the hydrolysis of polyvinyl acetate and is generated with different degrees of hydrolysis and different molecular weights up to 98%. PVA is a non-toxic polymer, soluble in water, and highly hydrophilic. It is added to CN to increase the hydrophilicity of the hydrogel. Although PVA is a synthetic polymer, it is used as a base material in many biomedical applications because of its non-toxicity, non-carcinogenicity, biocompatibility, and favorable physical properties (e.g., natural elasticity and ability to create strong films) [8]. In this study, the

mechanical properties of the samples were matched with human skin by adding Glycerol (G) to some of them. Glycerol or glycerin is a three-factor alcohol soluble in water at any ratio. Glycerin has a simple structure and is made from mono-, di-, and triglycerides that naturally exist in the body. This compound is used as a carrier for many drugs. Glycerin is quickly absorbed in body fluids, has no toxicity, and is metabolized in the food chain. In addition, it plays an important role in skin hydration and elasticity and repairing epidermal barriers [12-14]. Growth hormone has been widely tested in many studies and reported to be effective in accelerating the wound-healing process, and its availability has led to its clinical use [15, 16]. Growth hormone accelerates the proliferation of fibroblast cells, which form the basis of the wound-healing process. Research has shown that the presence of Growth hormone locally in the wound site increases the germination and protein expression of the insulin-like growth factors of the tissue [17]. Growth hormone can create bioactive systems when added to bone cement, ceramics, and polymers [16]. This study attempts to evaluate the physical and chemical properties of different ratios of CN and PVA polymers in the presence and absence of G and investigate the release rate of the drug and cell viability after somatropin loading.

2. Materials and Methods

2.1. Materials

Chitosan (CN) has a 75-85% deacetylation degree and an average molecular weight of 190-310 KDa (Sigma-Aldrich Company). Polyvinyl alcohol (PVA) has a hydrolysis degree of 98% and an average molecular weight of 72000 gr/mol (Sigma-Aldrich Company). Genipin (GN) is a natural crosslinker with a molecular weight of 226.3 gr/mol and LD50 of 382 mg/Kg (rat) (from Aprin Advanced Technologies Development Company, Iran). 100% acetic acid with a molecular weight of 60.05 gr/mol (Dr.Mojalali Industries Chemical Company, Iran). Glycerol (G) (Kimia Tehran Acid Company, Iran). Somatropin (Homa Pharmed Pharmaceutical Company, Iran). The PBS solution was prepared using the DNAbiotech Company's powder for a concentration 1X in one liter of water.

2.2. Methods

2.2.1. Preparing the CN, PVA, and GN solutions

To make 4wt% solutions of each polymer (CN & PVA), we placed the CN powder in the oven to reach a constant weight. Then, it was dissolved in 0.1 molar acetic acid solution for 4 hours at medium speed and 60℃ on a magnetic stirrer. The PVA was dissolved in preheated double distilled water at 100℃ for 5 hours. Next, both solutions were placed at ambient temperature for 2 hours to remove their bubbles. A 0.5 wt.% solution of GN was prepared by placing it in double distilled water for 1 hour at 50℃ and the medium speed of the stirrer.

2.2.2. Preparing of CN/PVA and CN/PVA/G films

We combined different amounts of these two solutions to get different proportions of CN and PVA solutions, some containing G. Samples with weight percentages of C100 (100:0), C70P30 (70:30), and C80P20 (80:20) without

G. In contrast, those with the weight percentages of C65P30G5 (65:30:5) and C68P30G2 (68:30:2) contained it. G-free samples were prepared by adding PVA dropwise to the CN solution on the stirrer at 94℃ and medium speed. Then, it was dissolved for 30 min, and 0.5 wt% of the GN solution (as a natural crosslinker) was added to the mixture in the last 5 min. The G-containing samples were prepared using the same method, except we added G to the dropwise CN solution. Finally, the samples were placed at 50℃ for 12 hours until their color turned green [18, 19, 20].

2.2.3. Preparation of the somatropin-containing sample

First, 4 IU of somatropin was dissolved in 10 mL of 0.1% NaCl solution in distilled water at ambient temperature and on a stirrer at medium speed. This research selected the effective drug concentration after reviewing some related articles [15, 16]. Then, 25 mIU of the drug solution was added to the samples after the polymer solution temperature was reduced on the stirrer (at ambient temperature).

2.2.4. Morphological properties

The microstructure of the samples was analyzed using the electron microscope imaging method according to the ASTM F561 standard and a KYKY-EM3200 electron microscope. This test was performed on the C80P20, C70P30, and C65P30G5 samples. Before the test, the samples were coated with Au to increase their conductivity.

2.2.5. Fourier-transform infrared (FTIR)

FTIR was carried out to identify specific chemical groups in the structure. For this purpose, the ATR capability of the FTIR device (Frontier model produced by Pekin-Elmer Company) was employed to calculate the adsorption of the samples. All spectra were recorded in the 1650-4000 cm^{-1} range with a resolution of 4.000. All measurements were done in a dry medium at ambient temperature (25±5℃). Moreover, the ATR spectrum of the C68P30G2 sample was examined with and without drug loading.

2.2.6. Water absorption rate

The study of the liquid absorption rate is significant for analyzing the biodegradability of substances. In this study, the liquid absorption rate was measured by weighing the samples before immersion in the PBS solution and then placing them in an incubator at 37℃ for 10 hours. After wiping off the extra water on the samples, their weights were measured at 0, 15, and 30 min and 1, 2, 3, 4, 8, and 10 hours to reach a constant weight. The experiment was repeated three times for each sample, and their average weights were considered. Finally, the swelling rate (S_i) was calculated using the following equation:

$$Si = \frac{Wf - W0}{W0} \times 100 \qquad \textbf{Eq. 1}$$

W_f is the weight of the swollen sample, and W_0 is the weight of the dry sample.

2.2.7. In-vitro degradation rate

Three samples with identical compositions were prepared as strips with approximate dimensions of 2 mm × 20 mm × 10 mm to obtain the degradation rate (Di) according to ISO 13781. Next, they were placed in the incubator in 20 mL of phosphate-buffered saline (PBS) with an approximate pH of 7.4 and

a temperature of 37℃. The weight loss of the samples was evaluated for two months at 4-day intervals. For this purpose, the samples were removed from PBS at predetermined times, washed with double distilled water, and dried in an oven at 50℃ for 5 hours. Then, they were weighed after they reached the weight stability. Finally, the degradation rate was calculated based on their weight loss using the following equation:

Eq. 2 $Di = \frac{W0-Wt}{W0} \times 100$

where W_0 is the original weight and W_t is the weight of the sample after immersion time. Each degradation test was repeated three times, and their average was considered.

2.2.8. Mechanical properties

Mechanical properties are one of the most important factors in determining the characteristics of a desirable wound dressing. This study conducted this test to evaluate Young's modulus, elongation, and yield stress from the PVA and CN combination samples. Also, the test results were used to determine the effect of the amount of G on the mechanical properties. The samples were prepared according to the ASTM D882 standard with a length, width, and thickness of 110, 20, and 1 mm, respectively. This test was conducted using the GT7010-D2E tensile machine made by GOTECH Company in Taiwan at 50 mm/min speed and a distance of 50 mm between jaws on samples with the weight percentage mentioned in the previous section and three repetitions. Finally, Young's modulus, fracture strength, and elongation parameters were obtained using the test outputs.

2.2.9. Measuring the pH changes

According to the pH changes at the wound site and the use of wound dressing in this area, the pH changes were measured at the degradation time of the samples. For this purpose, strips with approximate dimensions of 2 mm × 20 mm × 10 mm were prepared from the samples and placed in 20 mL of PBS with a pH of 7.4 and a temperature of 37±3℃. The pH variations were measured and evaluated in the solution for two months and at 4-day intervals.

2.2.10. Differential scanning calorimetry (DSC)

The DSC test shows the thermal properties of substances (e.g., the melting and softening points) and information about crystallinity. It also shows the degree of miscibility of two substances because the degree of solubility directly affects the ultimate properties of the polymer composition. DSC test was performed using a DSC device (model: 200 F3) manufactured by NETZSCH, Germany, according to ISO 11357. The device's temperature was set from 25 to 250℃ at a speed of 5℃/min. From each sample, 10 mg was separated and placed inside the device. This test was conducted on the mentioned samples and pure CN for comparison purposes.

2.2.11. In-vitro drug release study

Prototyping was done by solvent casting method. First, four international drug units were dissolved in 10 mL of 0.1% NaCl solution in distilled water at ambient temperature and on a medium-speed stirrer. The effective concentration of the drug was selected by reviewing the articles, and 25 mIU of the drug solution was loaded into the samples after the temperature of

the polymer solution was reduced to ambient temperature on the stirrer. To determine the drug release rate of samples, they were placed in the PBS solution (250 mL). After that, sample-containing solutions were placed in a shaking incubator at 37 °C.

After 0-0.5-1-4-8-24 hours and every day for 14 days, 2 ml of the extract was replaced with 2 ml of fresh PBS buffer. This experiment was repeated three times for each sample. The extract was diluted ten times to measure the absorption rate and determine the drug concentration.

In order to obtain the absorption wavelength of the drug, a wavelength scan was performed using a UV-vis spectroscopic device, and the maximum absorption for the somatropin drug was found to be 276 nm. To measure the release of the drug from the extract, a UV spectroscopy device model WPA made by Biochrom Company of England based in the biochemistry laboratory of Tarbiat Modares University was used. The standard absorption curve must be drawn first to measure the release rate of the drug. The method of drawing the standard curve for somatropin drug is as follows: Concentrations of 1-10-20-40-60 µg/ml of somatropin were prepared in PBS buffer solution. Absorbance was read at 276 nm.

The absorbance at the wavelength obtained for the extracts was read at specified times and calculated using the calibration curve equation of the extracted drug concentration.

In addition, a UV Spectroscopy device was used to measure the drug release rate from the extract (samples containing PBS). This study provided the standard absorption diagram to measure the drug release rate. First, the wavelengths of different drug concentrations were obtained. Next, the equation of the line passing these points was provided. Here, drug concentration was determined by inserting the absorption wavelength. The extracts' absorption at the obtained wavelength was read at specified times. Next, the concentration of the extracted drug was calculated using the calibration curve equation.

2.2.12. Cytotoxicity test

The cytotoxicity test was performed using the MTT method according to ISO10993-5 on the drug-containing samples to measure cell viability near the somatropin drug. In addition, the effectiveness of this drug was examined in increasing the growth of fibroblast cells.

The samples were extracted by sterilizing the C100, C70P30, and C68P30G2 samples with and without drug loading by UV. Next, they were placed in 24-well plates, and each sample was added with 500 µl of PBS. Finally, they were placed in an incubator at 37℃ and 50 rpm for 24 hours, and their extract was used for the cytotoxicity test. In this research, 2 x 10^5 HT 1080 Human Fibroblast cells in a 96-well plate with DMEM High Glucose medium and 10% FBS were placed in an incubator at 37℃ and 5% CO_2. After 24 hours, the extracts were added three times to the cells in each well, but one well was added with no extract (i.e., the control specimen). Next, the plate was placed in the incubator for 24 and 48 hours under the above conditions. At 24 and 48-hour intervals, the medium on the cells was removed completely, followed by adding 100 µL of MTT dye to each well. Eventually, they were placed in an incubator at 37℃ and 5% CO_2 for 3 hours. Then, the plate was removed from the incubator, the MTT was removed, and 100 µL of DMSO was

added to each well. Then, the plate was placed in a dark place for 10 min, and the OD value was read using an ELISA Reader.

Finally, the percentage of viable cells was calculated using the following equation:

Eq.3: Percentage of cell viability:

$$\frac{absorption\ at\ wavelength\ of\ 570\ nm}{absorption\ of\ the\ control\ sample} \times 100$$

In this reaction, the mitochondrial dehydrogenase enzyme of viable cells regenerates the tetrazolium dye in to formazan (which is insoluble) and creates a purple color. The higher OD value read with the ELISA reader, the more cells survive and the less toxic the samples are.

3. Results and Discussion

3.1. Morphological properties

In this study, GN was a non-toxic CN cross-linking agent. The thickness of the films was 75 ± 25 µm, and no inhomogeneity was observed due to dissolution, mixing, and phase separation in the samples with different ratios. In general, the films were quite uniform. The pure PVA film was transparent in terms of optical properties. Also, the pure CN film was yellowish, while the GN-cross-linked CN films were blue. Therefore, the reaction between GN and the CN/PVA mixture, accompanied by a change in optical appearance, was examined qualitatively. The blue color was related to forming new double bonds from cross-linking with GN. The yellow color of pure chitosan may make a nurse or doctor think that the patient has an infection. Therefore, it is better to change the color of chitosan simultaneously with this method.

3.2. Morphological study

Figure 1 shows images of an electron microscope for samples with and without drug loading. Images C1 and C2 contain G as well. A uniform and integrated film was obtained in the CN-PVA samples with different weight percentages. However, there were some particles and bubbles in higher magnifications due to the difference in the reaction speed of CN and PVA with GN as a crosslinker and the tendency of GN to react with the CN amine group. According to these images, an increase in the CN content increases surface roughness, which can effectively create clots and increase the speed of blood coagulation. The roughness of CN molecules disrupts the uniformity of the PVA matrix. In addition, the predominance of the CN percentage reduces the free volume of the matrix and leads to an increase in the density of the system. The images show the large structure of CN crystals. As the percentage of CN increases, the surface of the system becomes rougher, and the crystallinity of the polymer increases. As a result, the matrix has a more contact surface and water absorption. G also combines well with CN at the microstructure level. Adding a plasticizer to CN will change the biopolymer structure and improve the optical properties and transparency of the film, making the system more disordered. G can enter the polysaccharide's internal chain, destroy the intra- and inter-molecular hydrogen bonds, and change the polymer to a plastic mode, which can increase the disorder, thereby leading to transparency in the macroscopic dimension and surface roughness in the microscopic dimension. Drug loading led to many changes in the microstructure of the system. In general, loading made the surface of the sample rougher.

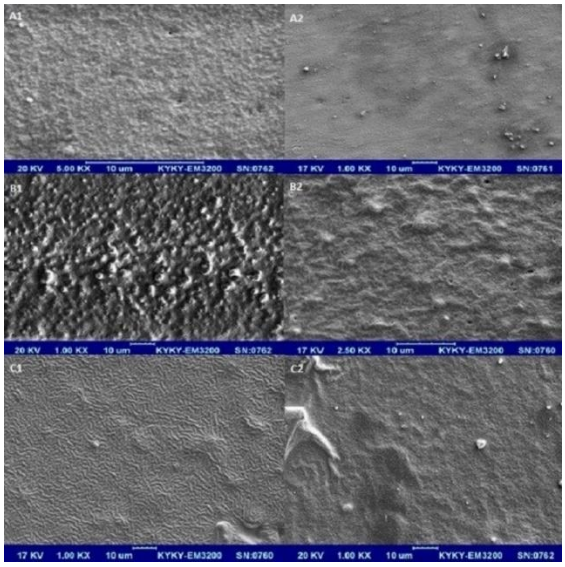

Figure 1. SEM Images: A1) C70P30 with drug, A2) C70P30 without drug, B1) C80P20 with drug, B2) C80P20 without drug, C1) C65P30G5 with drug, C2) C65P30G5 without drug.

3.3. FTIR spectroscopy

Figure 2 shows the ATR spectrum of the samples. The test results were used to ensure the compatibility of the mixture of CN and PVA and to understand the reactions between them. The pure PVA film has an absorption in the peaks of 2940, 1448, 1333, 1248, 1095, and 847 cm^{-1}, which are indexed to V_{α} (CH_2), δ (CH-OH), ω (CH), v (C-O), and v (C-C). According to these figures, pure CN exhibits absorption properties in the peaks around 898 and 1151 cm^{-1}, which belong to the Saccharin structure.

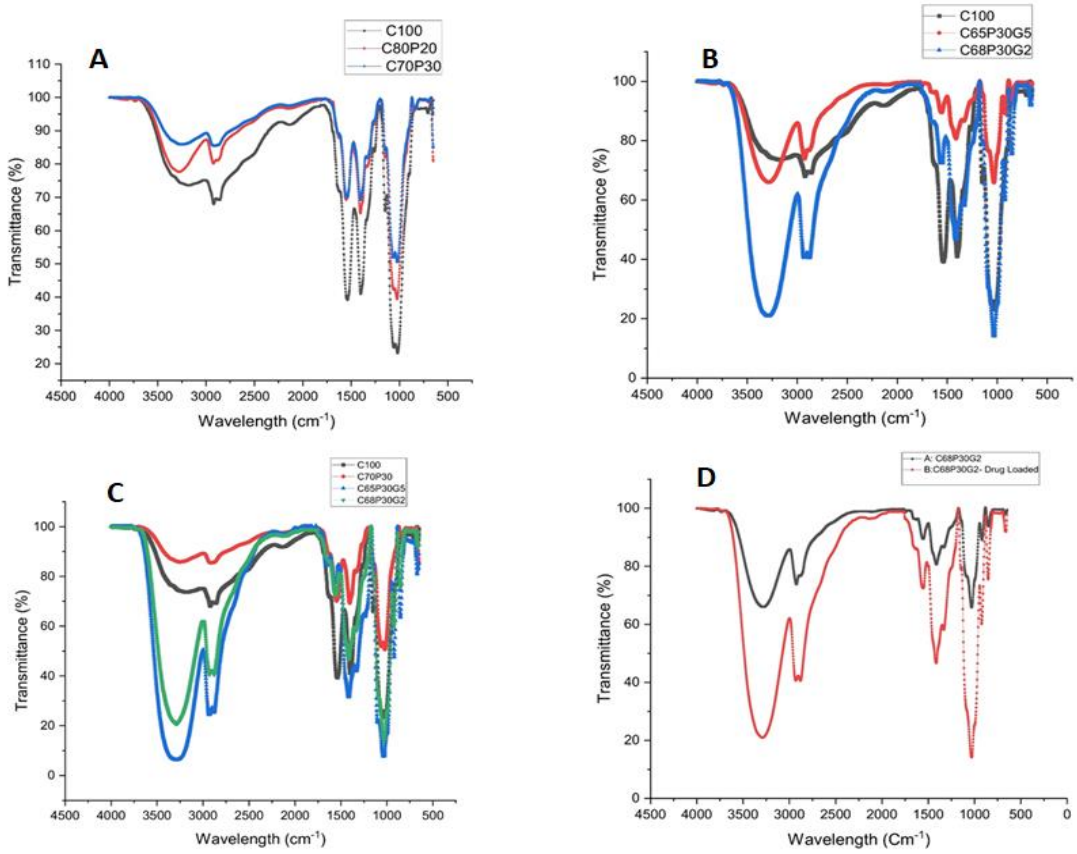

Figure 2. FTIR curves of samples **A)** without G. **B)** with G. **C)** with a constant amount of PVA. **D)** C68P30G2 (with and without drug).

Another amino acid has resonance in the 1255 cm^{-1} peak, which belongs to δ (O-H) bond. Also, the peak at 1383 cm^{-1} is related to the change in the symmetrical form of CH_3. The 3000-3600 cm-1 peaks are related to the bending vibration of the O-H bond of water, which interferes with the tensile bond of N-H in the same range. Water molecules can bond with two polar hydroxyl and amine groups in CN. Water in the IR spectrum affects the stretching bond C-H and amide with primary and secondary structures at 1556 cm^{-1} and 1650 cm^{-1}, respectively [21, 12]. In addition, the cross-linking of CN with GN leads to the opening of the dihydropyran ring and the formation of the tertiary amine structure. Simultaneously, polymerization takes place between GN molecules and the CN amine group. After cross-linking with GN, the amide bond with the second structure type in the 1537 cm^{-1} peak is formed via the reaction of the ester group and the hydroxyl group of GN with the amine group of CN. The 1633 cm^{-1} peak is indexed to the C=O group in the second amide structure. Finally, the vibrations between 1000 cm^{-1} and 1400 cm^{-1} characterize the stretching bond of C-N and C-OH [23].

The spectral separation of the data by the curve algorithm in the 1500-1700 cm^{-1} range, which represents the spectrum of secondary derivatives, was used to estimate the number, position, and relative contribution of independent elements composed of the primary and secondary amides. With the opening of the secondary amide ring, the contribution of the other two components is determined by adding G in the 1559 cm^{-1} and 1597 cm^{-1} peaks [24]. **Table 1** summarizes the functional group of each peak.

Table 1. The wavelength of functional groups**.**

Wavelength (Cm^{-1})	Functional Group
3177-3289	(CH-OH) δ
2909-2931	$V_{\alpha}(CH_2)$
1537-1566	(C-H) Stretching bond Amid
1401-1414	(CH-OH) δ
1240-1259	ω(CH)
1148-1150	Saccharine in Chitosan ‹C-N ‹C-OH
1021-1099	C-N ‹C-OH‹ C-O
845-853	Saccharide, V (C–C)
1331	C-N ‹C-OH

As can be seen from Figure 2-A, the peaks in the 2500-3000 cm^{-1} range and 1500 cm^{-1} range are related to the functional groups δ (CH-OH) and saccharin in CN.

The intensity of this peak decreases following a decrease in the CN percentage. After the minimum peak at 1000 cm^{-1}, a small maximum peak occurs with the addition of PVA to the composition, corresponding to the v(C-O) functional group. Fig. 2-B illustrates that adding G has made the absorption peak in the 3000-3500 cm^{-1} range, which is related to water molecules [25]. To understand the binding type of Somatropin, we evaluated the FTIR curves of the C65P30G5 sample with and without the drug loading. According to the articles, the peak in the 1656 cm^{-1} range defines the structure of the alpha helix in somatropin. Fig. 2-D shows an absorption peak at 1659.63 cm^{-1} in the drug-loaded sample, representing the somatropin drug in the composition.

3.4. Water absorption rate

According to **Figure 3**, most samples had higher water absorption than pure CN, indicating their high hydrophilicity. Samples with a PVA percentage could absorb more water.

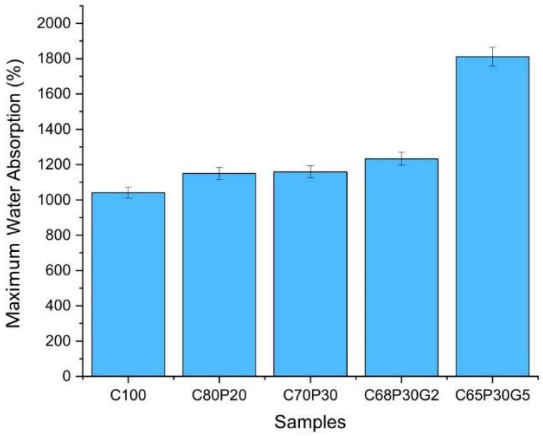

Figure 3. Maximum water absorption of samples.

Their PVA content strongly influences the water absorption rate of the samples. The explanation is that PVA is a hydrophilic and water-soluble polymer, and its combination with CN increases water absorption by adding the hydrophilic group (-OH) to the composition. Moreover, PVA is physically trapped in the CN chains, forming a hydrogel network [26, 27]. The addition of G in the composition also plays an effective role in increasing samples' water absorption rate. The samples had the highest water absorption rate during the first 30 min, but their weight reached a constant value after 4 hours. This feature helps the dressing be used in wounds with high exudates. Also, water absorption will help release the drug at the desired site, which was one of our main goals. Chitosan is a highly biocompatible polymer, and being hydrophobic is one of the major challenges in its use. This problem should be solved using different methods, including the method used in this research.

3.5. Degradation in the in-vitro medium

The degradation rate of the samples was evaluated by recording their weights in certain intervals (**Fig. 4**). The degradation rate was higher than pure CN in all samples. In G-free samples, the weight loss of the samples increased following an increase in PVA and a decrease in the CN content due to the hydrophilicity of PVA and the tendency of the GN crosslinker to bond with the CN amine group. The addition of G led to an increase in the weight loss of the samples because G, as a plasticizer, reduces the intermolecular van der Waals bonds. In this respect, plasticizers are not permanently trapped in the polymer chain and are released in the medium in the presence of water and other factors.

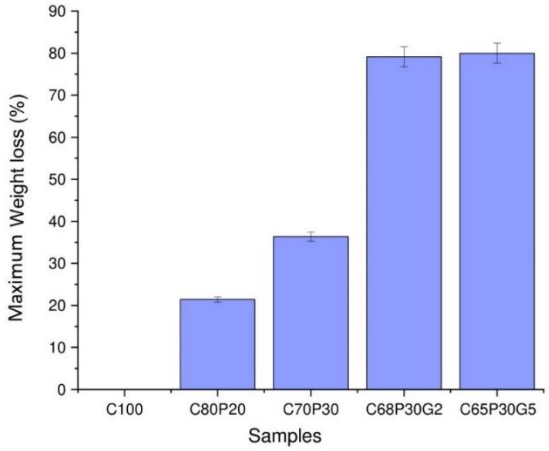

Figure 4. Maximum weight loss of samples.

In general, the degradation rate of the system should correspond to or be close to the tissue formation rate. In the case of wounds, the tissue normally starts to grow again after five days, and it usually takes three weeks for the vessels to regenerate and for the damaged tissue to be replaced [22, 28]. According to the test results, C68P30G2 and C65P30G5 samples had almost the same degradation rate and lost about 80% of their weight in the eighth week of the test. During this period, the tissue reached an optimal level of growth. Therefore, the

degradation rate of these two samples is more consistent with the rate of tissue formation. Biodegradability is one of the important features of new-generation wound dressings. Releasing the drug trapped in it, helping to form new tissue, and separating it easily from the wound site are some of its desirable benefits. One of our goals in this project is to add the biodegradability feature to chitosan, which has successfully used this method.

3.6. Mechanical properties

Mechanical properties of different ratios of the CN-PVA mixture with and without G were also examined. The elongation percentage, strength, and elastic modulus are reported in **Figures 5-6** and **Table 2.**

Table 2. Elastic modulus of samples**.**

Samples	Elastic Modulus (MPa)
C68P30G2	235.7
C70P30	6050.7
C80P20	5790.5
C100	5077.2
C65P30G5	102.6

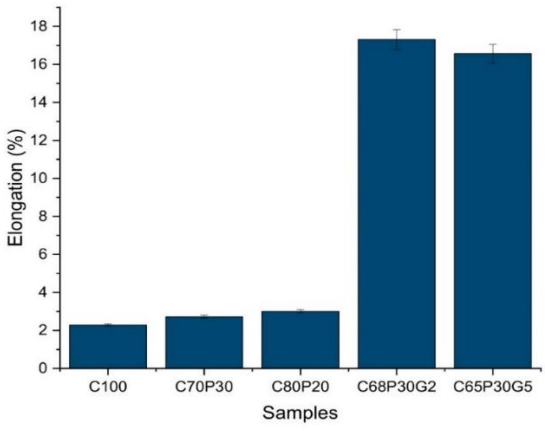

Figure 5. Elongation of samples.

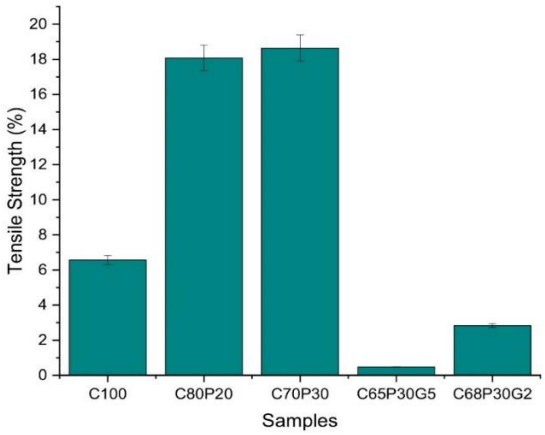

Figure 6. Tensile strength of samples.

3.6.1. Analysis of elongation

Elongation is the ratio of the increase in length of the sample in the rupture mode to its initial length. This ratio is expressed as a percentage of the initial length of the sample. As can be seen, the elongation percentage increased in the G-free samples compared to pure CN (C100) due to adding PVA, a water-soluble synthetic polymer with good film-forming and emulsification capabilities [29]. The addition of G to the composition leads to a significant increase in the elongation percentage. G reduces the van der Waals bonds between polymer chains and the stiffness of the bond network. Making the structure disorderly increases the range of motion of polymer chains and facilitates their motion. G acts as a moisturizer and lubricant with three hydroxyl groups and improves the elongation process [14]. However, analyzing the G-containing samples showed that the samples with 5% G had less strength and elongation than those containing 2% G. This result suggests the necessity of selecting an optimal amount of this plasticizer to add to the composition to achieve the appropriate properties. The explanation is

that G makes the structure disorderly and prevents the sample from increasing in length by creating a space between the polymer chains by increasing its amount in the composition more than a certain level. As **Figure 5** shows, the tensile strength decreased in the sample with five wt.% G, and this sample ruptured before the expected elongation occurred. Flexibility is one of the important characteristics of a desirable wound dressing, which pure chitosan does not have, and it must be improved by using other methods and materials.

3.6.2. Analysis of strength changes

The strength increased in the G-free samples following an increase in the PVA amount and a decrease in the CN amount. The CN's unique structure and cation-like nature have caused it to create a homogeneous composition when dissolved in an aqueous medium with anionic derivatives such as PVA. As a positively charged polysaccharide, CN is adsorbed to the negatively charged hydroxyl group of PVA. Therefore, it enhances the strength and elongation of the plastic film, which is formed by intermolecular hydrogen bonds [30]. The results of this study support this evidence. The addition of G to the composition decreased the strength of the samples compared to the control and G-free samples, which can be due to the loss of van der Waals bonds between molecules.

Table 2 presents the calculated elastic modulus of the samples. In G-free samples, the elastic modulus has decreased due to the increase of CN. A lower modulus value means less stiffness or more elastic deformation at a certain applied stress. After adding G as a plasticizer, Young's modulus decreases, and as expected, the sample tolerates more elastic deformation. In an ideal wound dressing, the developed system should have a high elongation percentage, good strength, and elastic modulus close to the skin tissue. The strength of skin depends on age, anatomical area, and direction. Therefore, researchers have reported different results on the mechanical properties of skin. The reported average strength is 27.9 MPa, and the average elastic modulus is 98.97 MPa [31].

Considering the conditions of the samples and comparing the figures in this research, sample C68P30G2 was considered the optimal mode of material composition because it had the appropriate elongation percentage, an elastic modulus close to the skin tissue, and acceptable strength. However, given the changes in the mechanical properties of the skin according to gender, age, and different anatomical regions of the body, it was better to select the ratio of the constituent materials based on the clinical characteristics of patients when using this type of wound dressing.

3.7. Measuring the pH changes

Figure 7 shows the pH changes during the degradation of samples. As can be seen, the pH changes were in the range of 3%-5%. The pH at the wound site is in the range of 7.15-8.9. Whether a wound is chronic or acute, its healing speed decreases when placed in the alkaline range. The most appropriate wound healing speed occurs at neutral pH. As the wound progresses toward healing, the pH becomes neutral and acidic. It was observed that the pH changes of the samples tend toward acidity. The amount of acetic acid in the G-free samples

increases, and the medium is expected to become more acidic following an increase in CN. However, as shown by the degradation test, an increase in the PVA led to an increase in the weight loss of the sample. As a result, a larger sample material enters the medium, making it more acidic. Because of adding G, the pH changes are reduced and become closer to neutral pH, which can provide ideal conditions for wound healing. Both C68P30G2 and C65P30G5 samples produce almost equal pH changes within the standard range and can thus be useful for wound healing [32].

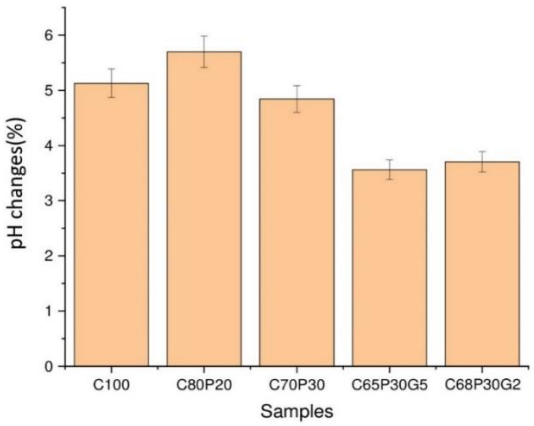

Figure 7. pH changes of samples.

3.8. Differential scanning calorimetry (DSC)

The DSC test shows the thermal properties of materials, such as melting point, softening point, and crystallinity information. It also shows the miscibility of two substances. An important factor in developing new materials by mixing polymers is the degree of solubility in the composition, which directly affects the final properties of the polymer composition. Also, this test was conducted to evaluate the crystallization rate, the flexibility of the developed system, and its temperature stability [21, 33].

Figures 8 and **9** display an endothermic peak related to the melting process of the substance. **Table 3** shows the calculated surface along the melting curve. According to the results, as the percentage of PVA and G in the composition increases, the area of the melting curve shrinks.

Table 3. The area under the T_m peak curve in different samples**.**

Samples	T_m peak curve
C68P30G2	54538/35
C70P30	10013/30
C80P20	36772/31
C100	6929/43
C65P30G5	92178/28

In **Figure 8-A**, a peak at a temperature of about 90℃ is related to the T_g of the composition. The depression after that peak is related to the formation of the crystal structure and the Tc peak. The next endothermic peak is related to the melting of the composition, as can be seen in **Figures 8-B** and **9**; after adding PVA to the composition, the T_g peak can still be seen in the temperature range of 90℃. In the C80P20 sample, the Tc peak is more distinct than the C100 and C70P30 samples at 130℃. The Tm peak is also narrower in this sample. The shape of the peak can indicate the type of thermal event that has occurred. The crystal structure change is usually seen as a sharp peak, and thermal decomposition as a broad peak [34, 35]. As can be seen from SEM images, the C80P20 sample is more crystalline than the C70P30 sample, and this ratio can be considered an optimum point for the formation of crystal structure. The Tg peak has almost disappeared with the addition of G to the composition. This result is because of the possibility of changing the T_g of polymers by adding a small amount of additive.

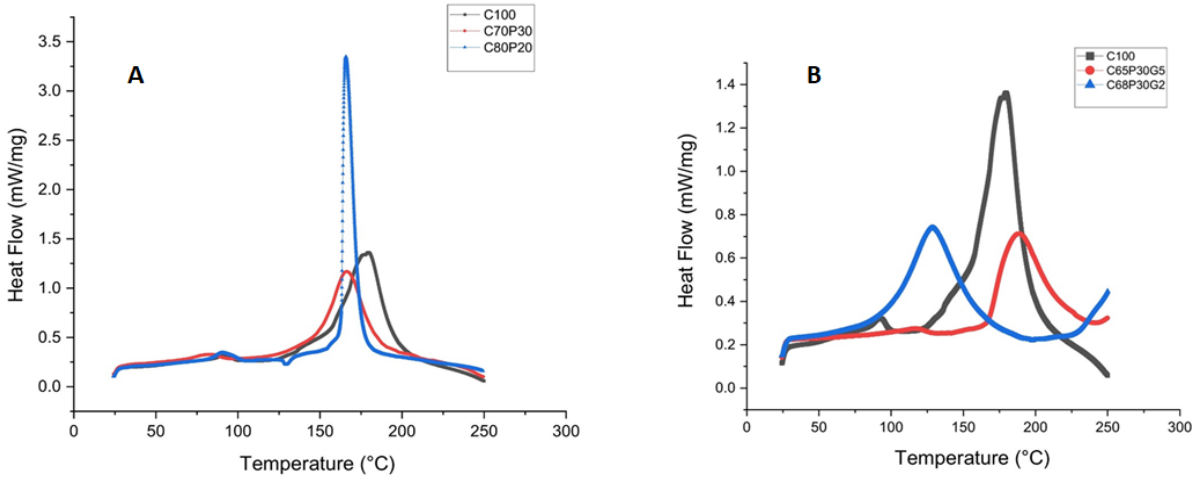

Figure 8. DSC curves of samples **A)** without G. **B)** with G.

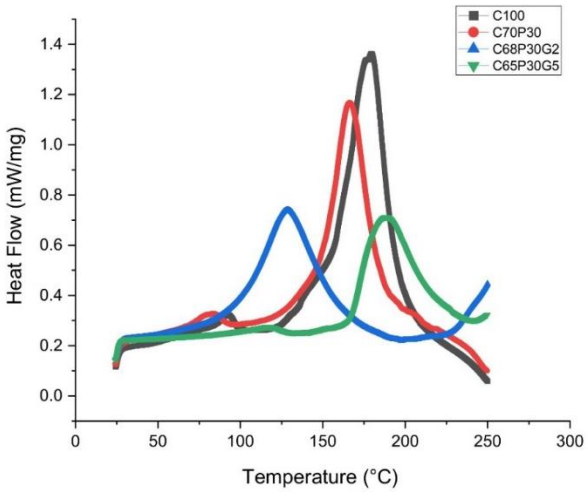

Figure 9. DSC curves of samples with the constant amount of PVA.

In this study, the addition of different concentrations of G caused the T_g peak to disappear following the increase in the mobility of the polymer chains and the greater irregularity of the structure, a phenomenon referred to as "plasticization". The explanation for this result is that G enters the polysaccharide chain (CN) and destroys its intra- and inter-molecular hydrogen bonds. The softening effect of G was also proven in the tensile test.

3.9. Drug release

The release profile of Somatropin was calculated by drawing its standard curve and calculating its release concentration using the formula obtained from **Figure 10.** This figure depicts the standard curve of Somatropin at a wavelength of 276 nm. Accordingly, its concentration can be calculated based on the absorption rate. In the equation below, X and Y

represent the amounts of absorption and concentration, respectively.

Eq. 3: Y=0.0106X+0.0061

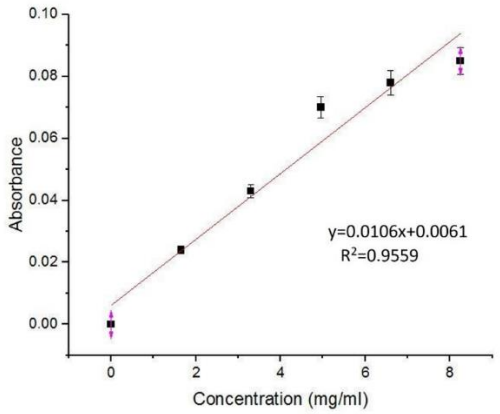

Figure 10. Standard curve of Somatropin.

Figure 11 presents the drug release profile over 130 hours. Since somatropin may cause sensitization in direct contact with skin, it is necessary to release a controlled concentration at the wound site.

The oral LD 50 value of this drug tested on rats is 242 mg/kg. However, ten µg/m^3 may be a sensitizer in contact with the skin. The concentration loaded in each sample is 0.4 IU/ml. For somatropin, each IU is equal to 0.33 g.

The effective dose of this drug in injection for treating wounds and burns is 0.6 IU/Kg/day [16, 36]. According to the drug release graph, the highest release rate is related to the C68P30G2 sample, and the release rate is generally higher in the samples containing G. However, the release rate decreases following an increase in the PVA. In drug diffusion through the polymer matrix method, the concentration of the released drug is measured at different time points. An initial burst release is generally caused by the release of drug molecules entrapped close to the surface. This is followed by a sustained release rate owing to the release of the drug entrapped in the scaffold. When the drug concentration decreases, the release rate indicates a reduction. The polymer gels entrap the drug in the cross-linked matrix as the solution temperature warms to body temperature. Diffusion of the drug from the solid gel allows for sustained-release formulations. In this research, the release profile is investigated during 140 h. Comparing drug release's R2 value (0.9559) with the mathematical models shows that the drug release data fit well in the first-order kinetic model. It is necessary to release somatropin with controlled concentration at a specific time. Because the first 4-6 days after wound formation is a golden time for the healing process, we investigated the release profile to ensure sufficient drug in the wound site. To reach this goal, encapsulation of the drug in biodegradable polymers is a very appropriate method as we use it.

3.10. Cytotoxicity test

Figure 12 depicts the viability percentage of the Human Fibroblast HT 1080 cell near the somatropin-containing samples and the control sample extract.

According to the cytotoxicity test, the cell growth and survival percentages in the drug-containing samples were higher than in the drug-free extracts. In fact, the cytotoxicity test also evaluates somatropin's activity level. More than 50% of cells survived in all samples. According to this test, not only did somatropin cause no toxicity, but it also contributed to the growth of fibroblast cells.

Overall, it can be concluded that adding PVA to the composition decreased the viability percentage of cells, and adding G increased this percentage slightly. Somatropin is a growth hormone and should directly affect the growth and survival of cells. This process is completely evident in the charts. Therefore, it can be concluded that this medicine had the desired effect on this wound dressing, and our dose had no toxicity.

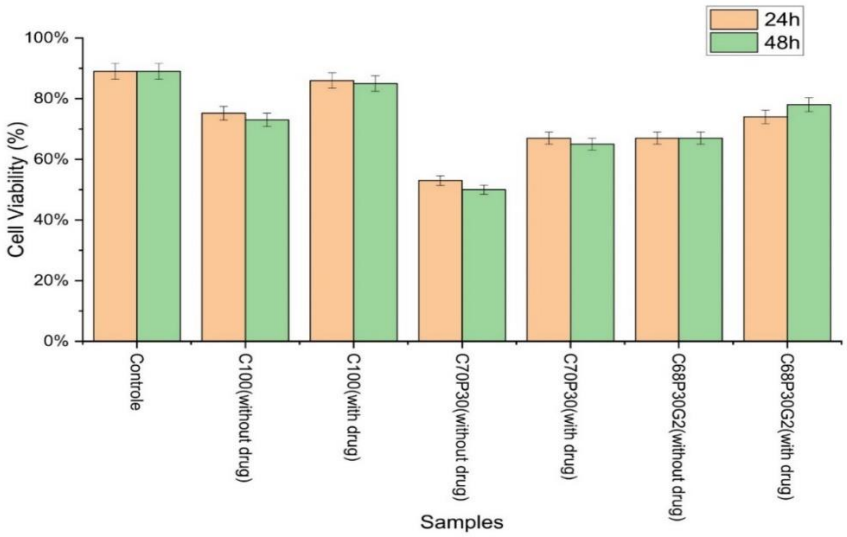

Figure 12. Cell viability after 24 and 48 hours.

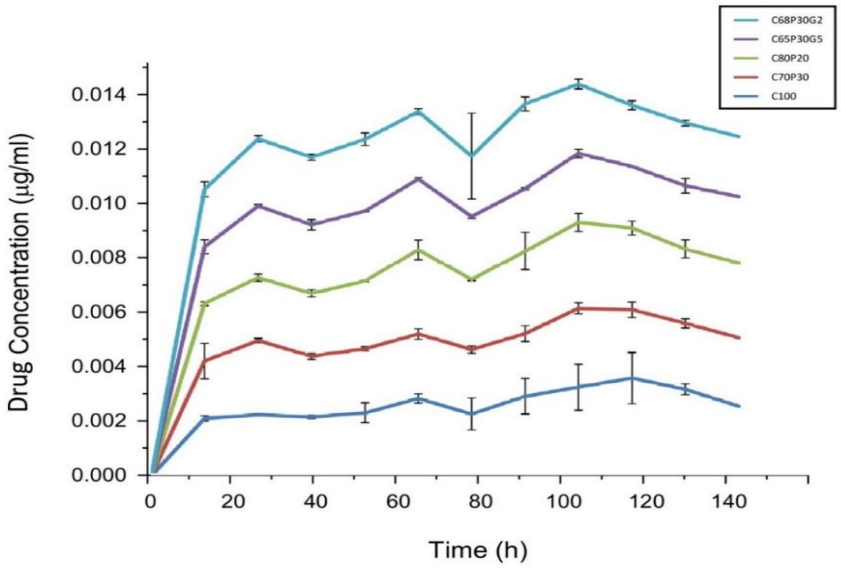

Figure 11. In vitro drug release profile.

4. Conclusion

According to the elongation, strength, and elastic modulus parameters extracted from the mechanical test results and compared with the reported mechanical properties of skin, the value of G leads to an increase in the elongation of the samples, as expected. Therefore, the C68P30G2 sample was selected as the optimal mode. The DSC test also showed that adding G to the composition produces softening. The two samples with G had almost the same degradation rate, which is acceptable considering the wound healing time and the need to change the wound dressing to prevent the accumulation of microorganisms. The pH value at the wound site is usually 7.15-8.9, and the wound healing speed will slow down if it becomes alkaline. Adding G lowered the pH changes and made it closer to the neutral mode. According to the results of the drug release test, the samples containing G had better performance, and the release rate was higher in the first four days of wound healing when there was the greatest need for GH. PVA and G both effectively increase the liquid absorption rate and are thus useful for wounds with a high exudate. The MTT test showed that somatropin increased the viability percentage of cells. In general, this system keeps the wound site moist, plays an effective role in absorbing exudates, and increases the viability of cells by releasing somatropin. GN gave the system a greenish color, which is psychologically relaxing. Moreover, its transparency makes facilitates observing the wound site under the wound dressing.

Conflict of interest

The authors declare to have no conflict of interest.

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