INTRODUCTION

Skin has a crucial function as a barrier against external factors. It also maintains the water-electrolyte balance and protects against infections and microorganisms. Wounds and burns damage the integrity of the skin and make it susceptible to harmful factors. The pH of the wounded area changes from acidic to alkaline. Microorganisms invade and proliferate rapidly in the wound site due to the pH change of the wounded site. To prevent these unwanted complications, the necessary treatment should be applied. Wound healing is a regular biological process that includes growth factors, enzymes, and cell types, such as keratinocytes, fibroblasts, and endothelial cells (Gainza et al., 2015). This process also consists of homeostasis, inflammation, cell proliferation, extracellular matrix (ECM) production, and wound closure consecutively (Dreifke, Jayasuriya, Jayasuriya, 2015). It is crucial to determine and use an appropriate formulation in terms of ease of application and patient compliance in wound healing. Hydrogels are frequently used biologically active formulations for the wound healing process as they are safe and easy to apply. With high oxygen permeability and suitable mechanical properties, hydrogels resemble physiological tissue (Rana, Ganarajan, Kothiyal, 2015). Hydrogels in pharmaceutical dosage form are commonly used for drug delivery, for wound healing, and as a wound substitute.

Synthesis and characterization of chitosan-PVA hydrogel containing PEGylated recombinant epidermal growth factor on cell culture for wound healing substitute

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The aim of the current study was to assess the physicochemical characteristics and wound healing activity of chitosan-polyvinyl alcohol (PVA) crosslinked hydrogel containing recombinant human epidermal growth factor (rh-EGF) or recombinant mouse epidermal growth factor (rm-EGF). The hydrogels were prepared and analyses were made of the morphological properties, viscosity, water absorption capacity, mechanical and bio-adhesive properties. The viscosity of the formulations varied between 14,400 - 48,500 cPs, with the greatest viscosity values determined in K2 formulation. F2 formulation showed the highest water absorption capacity. According to the studies of the mechanical properties, H2 formulation (0.153±0.018 N:mm) showed the greatest adhesiveness and E2 (0.245±0.001 mj/cm²) formulation, the highest bio-adhesion values. Hydrogels were cytocompatible considering in vitro cell viability values of over 76% on human keratinocyte cells (HaCaT, CVCL-0038) and of over 84% on human fibroblast cells (NIH 3T3, CRL-1658) used as a model cell line. According to the BrdU cell proliferation results, B1 (197.82±2.48%) formulation showed the greatest NIH 3T3 and C1 (167.43±5.89%) formulation exhibited the highest HaCaT cell proliferation ability. In addition, the scratch closure assay was performed to assess the wound healing efficiency of formulation and the results obtained in the study showed that F2 formulation including PEGylated rh-EGF had a highly effective role.

Keywords: Chitosan. rh-EGF. Hydrogel. Texture analyzer. Cell culture.

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dressing. The combination of natural and synthetic polymers increases the efficiency of the formulation and provides a stable construct (Suri, Schmidt, 2009; Dogan et al., 2009). The hydrogel can be considered a three-dimensional, hydrophilic, crosslinked polymeric network, which can absorb water within its porous structure (Garg, Garg, 2016; Pal, Bantia, Majumdar, 2009). Chitosan is a linear-structured polymer widely used as a natural material for hydrogel production and the cationic property of chitosan increases its interaction and adhesion with the skin (Mohamed, Abu Elella, Sabaa, 2015). It is soluble in dilute solutions of many organic and inorganic acids due to the protonation of its amino groups. It has excellent film-forming ability and can also be used to prepare films, fibers, and nanoparticles (Crosier, Jérôme, 2013). Chitosan stimulates fibroblast proliferation, provides regular collagen deposition, and accelerates the wound healing process by preventing scar formation at the wound site due to the slow degradation into N-acetyl-β-D-glucosamine (Crosier, Jérôme, 2013; Cheung et al., 2015). In addition to repairing tissue, chitosan triggers the activity of leukocytes, fibroblasts, and macrophages due to increasing granulation (Cheung et al., 2015; Raafat et al., 2008). However, chitosan-based hydrogels have poor mechanical properties and swelling capabilities. These deficiencies can be eliminated by using crosslinking methods for hydrogel formulation. Thus, the mechanical strength and swelling ability of hydrogels are increased by crosslinking chitosan with PVA (Cheung et al., 2015; Mohamed, Abu Elella, Sabaa, 2015; Kamoun et al., 2015).

EGF is a macromolecule containing 53 amino acids, which stimulates the proliferation and regeneration of epidermal cells, and plays an essential role in the process of wound healing (Park, Hwang, Yoon, 2017). EGF is a significant stimulator of the migration and proliferation of keratinocytes and fibroblast cells (Park, Hwang, Yoon, 2017; Hardwicke et al., 2008). However, as EGF is a polypeptide structured molecule, it has stability and efficacy problems during the treatment process (Hardwicke et al., 2008). Recent studies have shown that gel formulations including EGF or other macromolecules increased the stability and effectiveness of EGF in the wound healing process (Dogan et al., 2009; Hardwicke et al., 2008). Moreover, the PEGylation of EGF provides crucial changes to its biological activity, stability, and solubility properties. Conjugation with protein-based macromolecules with a PEGylation agent ensures more effective treatment than pure protein-based macromolecules (Ergül, Ergül, Tutar, 2013). Different types of polyethylene glycol (PEG) reagents can be used in PEGylation processes (Gefen et al., 2013). The combination of ethylene oxide units constitutes PEG and it is a suitable conjugation agent, and dissolves in both water and organic solvents due to the hydrophobicity of the ethylene group and hydrophilic properties of the oxygen group (Jevsevar, Kunstelj, Porekar, 2010). Although there are many studies in literature related to the wound healing efficiency of hydrogels including chitosan and PVA, there have been few studies related to increasing the stability and efficacy of the active substances such as growth factors and proteins. Therefore, in this study, r-EGF types were PEGylated to increase the stability and activity of r-EGF. Different hydrogel formulations with or without PEGylated r-EGF were prepared and it was aimed to evaluate their physicochemical properties, wound healing efficiency, cell viability, and cell proliferation activities. In addition, the mechanical properties and in vitro biological activities of hydrogels with or without r-EGF or PEGylated r-EGF were compared and evaluated.

MATERIAL AND METHODS

Material

Medium molecular weight chitosan (MMW, Sigma, code: 448877), high molecular weight chitosan (HMW, Sigma, code: 419419), and PVA (Sigma, code: P1763) used as polymers for hydrogel formulation were purchased from Sigma-Aldrich (USA). rh-EGF (Sigma, code: E9644), methoxy PEG (mPEG) propionaldehyde (MW 5 kD, Sigma, code: JKA 3039), mPEG propionaldehyde (MW 10 kD, Sigma, code: JKA 3033), dialysis sack (MW cut off 12 kD, Sigma, code: D6191) were purchased from Sigma Aldrich (USA). rm-EGF (Gibco, code: P01133) was obtained Gibco (New Zealand). Dialysis cassette (MW cut off 7 kD, Thermo scientific, code: 66710, USA), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT) cell viability kit (Roche,
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code: 11465007001, Switzerland), and bromodeoxyuridine (BrdU) cell proliferation kit (Millipore, code: 2752, Germany) were used in these studies. Unless specified otherwise, sterile bi-distilled water was used to prepare the solution. All materials used in these studies were of analytical grade.

**Synthesis of crosslinked chitosan/PVA hydrogels**

The concentrations of chitosan and PVA were 3% and 2% (w/v), respectively in the hydrogel formulations, which were prepared as previously described in the literature (Agnihotri, Mukherji, Mukherji, 2012). The volumetric ratio of chitosan to PVA was determined to be 4:1 in chitosan-PVA hydrogel formulations (Cevher et al., 2008). Chitosan was dissolved in a 1% (v/v) acetic acid solution by stirring overnight. The PVA solution (2%, w/v) was prepared with bi-distilled water by stirring and heating at 80 °C. When completely dissolved, PEGylated rh-EGF or rm-EGF (10µg/mL) and crosslinking agent, glutaraldehyde (GA), at a concentration of 0.001 g/mL and 0.002 g/mL were added to the PVA solution by stirring at room temperature for 30 minutes. The PVA solution was then added to the chitosan solution and stirred for 4 h. The hydrogels were placed in 50 mL universal bottles and stored at 4 °C. Each formulation was prepared in triplicate. The components of the hydrogel formulations are given in Table I.

### Table I - Viscosity and water absorption capacity results of different chitosan-PVA hydrogel formulations (n=3). Viscosity and water absorption capacity studies were performed on hydrogels that reached room temperature

| Codes | Molecular weight of chitosan | rh-EGF or rm-EGF | Different molecular weight of mPEG propionaldehyde | Glutaraldehyde concentration (g/mL) | Viscosity values (cPs ± S.D.) | Water absorption rate (% ± S.D.) |
|-------|-----------------------------|------------------|--------------------------------------------------|-----------------------------------|-------------------------------|---------------------------------|
| A1    | MMW                         | rh-EGF           | ---                                              | 0.001                             | 17783 ± 490                   | 64.70 ± 0.50                    |
| A2    | MMW                         | rm-EGF           | ---                                              | 0.001                             | 30100 ± 150                   | 56.80 ± 0.70                    |
| B1    | MMW                         | rh-EGF           | 5kD                                              | 0.001                             | 19433 ± 152                   | 73.22 ± 1.21                    |
| B2    | MMW                         | rh-EGF           | 10kD                                             | 0.001                             | 14400 ± 180                   | 78.10 ± 1.12                    |
| C1    | MMW                         | rm-EGF           | 5kD                                              | 0.001                             | 20450 ± 132                   | 76.80 ± 1.71                    |
| C2    | MMW                         | rm-EGF           | 10kD                                             | 0.001                             | 15350 ± 567                   | 78.61 ± 0.32                    |
| D1    | MMW                         | ---              | ---                                              | 0.001                             | 36033 ± 104                   | 68.42 ± 1.30                    |
| D2    | MMW                         | ---              | ---                                              | 0.002                             | 26233 ± 332                   | 68.23 ± 1.10                    |
| E1    | HMW                         | rh-EGF           | ---                                              | 0.001                             | 24250 ± 264                   | 76.50 ± 0.80                    |
| E2    | HMW                         | rm-EGF           | ---                                              | 0.001                             | 42383 ± 644                   | 97.20 ± 1.20                    |
| F1    | HMW                         | rh-EGF           | 5kD                                              | 0.001                             | 19316 ± 189                   | 103.31 ± 1.60                   |
| F2    | HMW                         | rh-EGF           | 10kD                                             | 0.001                             | 45450 ± 250                   | 105.64 ± 0.41                   |
| H1    | HMW                         | rm-EGF           | 5kD                                              | 0.001                             | 23650 ± 250                   | 92.30 ± 0.70                    |
| H2    | HMW                         | rm-EGF           | 5kD                                              | 0.002                             | 35850 ± 755                   | 92.12 ± 1.02                    |
| K1    | MMW                         | ---              | ---                                              | ---                               | 23216 ± 485                   | 63.60 ± 1.01                    |
| K2    | HMW                         | ---              | ---                                              | ---                               | 48500 ± 217                   | 85.60 ± 1.50                    |

All hydrogel formulations contain polyvinil alcohol (PVA, 2%). MMW and HMW indicate medium molecular weight and high molecular weight respectively. rh-EGF: recombinant human epidermal growth factor. rm-EGF: recombinant mouse epidermal growth factor.
Scanning electron microscopy

Samples of different hydrogel formulations were lyophilized with freeze drying (Steris-Lyovac Gt 2E, Germany). The lyophilized hydrogel was placed on metal grids with double-sided adhesive tape, coated with gold to 8 nm in thickness using SCD 005 Sputter coater (Baltec, Liechtenstein) under high vacuum, 0.1 torr at 25 ± 1 °C. The surface morphology of the hydrogel was investigated with scanning electron microscopy (SEM; Carl Zeis-Evo 40, Germany) (Sezer et al., 2007).

Viscosity measurement of hydrogel formulations

Samples of different hydrogels were placed in 50 mL universal bottles for viscosity measurements. The viscosity measurements of the hydrogels were made within 24 h after the hydrogel preparation. The air bubbles were removed from the hydrogels in the ultrasonic bath. The viscosities of hydrogels were measured using a rotational viscometer (Brookfield DV-E, spindle 6, 20 rpm) (Sezer et al., 2008). All formulations were measured in triplicate.

Determination of water absorption capacity of hydrogels

The water absorption capacity of the hydrogels is strongly dependent on the gel ionization and the ionic strength around the solution (Agnihotri, Mukherji, Mukherji, 2012). To determine the water absorption capacity, a modified method of previous researchers was used (Sezer et al., 2008; Cevher et al., 2008). The current studies were performed in pH 7.4 phosphate buffer saline (PBS). A gram of hydrogel was weighed in petri dishes and 1 mL of buffer solution was added to the hydrogels at predetermined time intervals. Tissue paper was used to remove excess water from the hydrogels and the hydrogels were weighed periodically until they reached a constant weight at 25 °C. The water absorption capacity (WAC) of the hydrogels was calculated using Equation (1), where \( W_0 \) was the initial weight, and \( W_t \) was the weight at any time (t). This study was performed in triplicate.

\[
\text{WAC} (\%) = \left( \frac{W_t - W_0}{W_0} \right) \times 100 \quad (1)
\]

PEGylation mechanism of recombinant EGF species

The rh-EGF, rm-EGF (100 µg) and mPEG-propionaldehyde derivatives (MW 5 kD, 1 mg; and MW 10 kD, 2 mg) were dissolved in 10 mL of sodium acetate buffer (50 mM, pH 5.5) with sodium borohydride (2.5 mM) as a reducing agent. The role of sodium borohydride in the reaction was to provide a Schiff base formation between the aldehyde group of mPEG propionaldehyde and the N-terminal amine group of r-EGF types (Lee et al., 2003). The PEGylation reaction was carried out in a 20 mL glass bottle by stirring with a magnetic stirrer. A dialysis sack (MW cut off = 12 kD) was used for the PEGylation of r-EGF types with mPEG-propionaldehyde 10 kD, and a dialysis cassette (MW cut off =7 kD) for the PEGylation of r-EGF types with mPEG-propionaldehyde 5 kD and Tris buffer (Trisma base-HCl, 5 mM, pH 8.0) to terminate the PEGylation reaction and remove unconjugated PEG-propionaldehyde derivatives, native rh-EGF, rm-EGF, and other chemical reagents. The molecular size of the dialysis sack and dialysis cassette in the PEGylation process was determined by the molecular size of r-EGF types and PEG reagents used. Before and after all the dialysis studies, 1 mL of the PEGylated rh-EGF and 1 mL of the PEGylated rm-EGF solutions were analyzed in a Shimadzu (RF-1601 Japan) UV visible spectrophotometer for excitation at 280 nm and for emission at 260 nm (Lee et al., 2003; Alemdaroğlu et al., 2006). The amounts and ratios of PEGylated rh-EGF and rm-EGF were calculated according to the spectral curve equation (2) of r-EGF types. Where \( y \) is the absorbance of PEGylated rh-EGF or rm-EGF, \( x \) is the concentration of PEGylated rh-EGF or rm-EGF.

\[
y = 0,002x + 0,001 \quad (2)
\]

In vitro release studies of r-EGF types from the chitosan-PVA hydrogel

In this study, the release of r-EGF types from the dialysis sack and cassette to the PBS buffer was
calculated depending on the time. The release profiles of rh-EGF and rm-EGF from the hydrogels were assessed using the dialysis method (Alemdaroğlu et al., 2006). A1 formulation containing rh-EGF and an F2 formulation including PEGylated rm-EGF were used in the release study. Initially, 5 mL of chitosan-PVA hydrogels containing rh-EGF (A1) at a concentration of 5 μg/mL were added to the dialysis cassette with a pore size of 7 kD and containing PEGylated rm-EGF (F2) at a concentration of 5 μg/mL were added to the dialysis sack with a pore size of 12 kD. The dialysis cassette and dialysis sack were then immersed in different stirred containers including phosphate buffer saline (80 mL, pH 5.8). The release processes of formulations A1 and F2 were carried out in different containers and under the same conditions. At predetermined time intervals, a 3 mL of sample solution was taken in the recipient compartment and replenished with fresh PBS buffer. Withdrawn samples were analyzed using a Shimadzu (RF-1601 Japan) UV visible spectrophotometer for excitation at 280 nm and for emission at 260 nm. The release ratios of rh-EGF and rm-EGF were assessed according to the spectral curve equation (2).

**Bio-adhesion studies of hydrogels**

TA.XT Plus Texture Analyzer equipped with a 5 kg load cell was used for the bio-adhesion test. Bio-adhesion studies were carried out using the perspex P 0.5 probe (12.5 mm diameter) at room temperature. Fresh chicken back skin was used as model tissue (Ethical approval was given by Marmara University Experimental Animal Implementation and Research Centre with protocol code: 45.2018.mar.). Skin was divided into circular sections 4 cm in diameter and a section of the skin was connected to the lower end of the probe with a thin rubber band. The samples of hydrogels were added to six well-plates. The Texture Exponent software program was used to calculate the results. The area under the curve (AUC) was measured from the force-distance plot as adhesion force (Sezer et al., 2008; Cevher et al., 2008). Each study was performed in triplicate.

**In vitro cytotoxicity studies**

*In vitro* cytotoxicity of the hydrogel formulations was determined using the MTT assay on NIH 3T3 and HaCaT cell lines. The cells were seeded at a density of $5 \times 10^3$ per well in a 96-well plate and incubated overnight (Wolf et al., 2009). The cells were then treated with hydrogels containing rh-EGF or rm-EGF by suspension in Dulbecco’s modified eagle’s medium (DMEM) for 24 h. After the incubation period, 10 μl MTT labeling solution was added to each well. The plate was incubated for 12 h. Later, 100 μl of the sodium dodecyl sulfate (SDS) was added into each well and samples were incubated overnight in the incubator. The samples were checked for complete solubility of the purple formazan crystals and the spectrophotometrical absorbance of the samples was measured using a microplate ELISA reader (Epoch Biotech, USA). The absorbance of the formazan product was measured at a wavelength of 550 nm. The reference wavelength should be 690 nm (Arranja et al., 2014; Şenel, Büyükköroğlu, Yazan, 2015; Wolf et al., 2009).

**BrdU cell proliferation assay**

Non-cytotoxicity of the formulations was verified using the BrdU cell proliferation assay. For this purpose,
the HaCaT and NIH 3T3 cells were seeded in a 96-well plate at a density of 5x10^3 cells per well and incubated overnight for attached cells. The next day the cells were treated with samples for 48 h. Then 4 µl of BrdU stock solution was withdrawn and completed to 2 ml with BrdU dilution solution. A further 20 µl of diluted BrdU solution was added to each well and incubated for 24 h in the incubator. After incubation, cells were fixed to detect the BrdU tag by the anti-BrdU monoclonal antibody. DNA was denatured by adding 200 µl fixation solution to each well and incubated for half an hour without light. The fixation solution was completely removed and the dry plate was placed in a locked bag. Then, BrdU cell proliferation kit procedure was performed respectively. 100 µl of TMB peroxidase substrate was added to each well and incubated for 30 min at room temperature. Finally, 100 µl of the termination solution was added to each well. The absorbances of the samples were read using an ELISA reader set at a dual-wavelength of 450 nm and 550 nm (Wolf et al., 2009).

Wound healing assay (Scratch test)

The researchers focused on the wound healing process according to the rate of scratch closure (Jonkman et al., 2014; Hulkower, Herber, 2011). This assay was carried out with the optimized formulation (F2), including PEGylated rm-EGF at a concentration of 10 µg/ml, as a result of characterization studies. First, HaCaT and NIH 3T3 cells were seeded in six-well plates (TPP, Switzerland) at a density of 5x10^5 cells/well in DMEM. The cells were incubated in an incubator at 37 °C and 5% CO2 until they reached a confluence of approximately 80%. Then scratches were made through each well using the tip of a sterile 200 µL pipette and the medium was exchanged for fresh medium (Demirci et al., 2016; Wolf et al., 2009). Photographs of the cells with scratches at the initial time and after 24 h were taken using an inverted microscope (Olympus CKX 41 Japan) equipped with an Olympus phase contrast slider camera system (IX2 SLP, Japan) and the wound closure rate was measured using image analysis software (ImageJ, NIH, Bethesda, MD). After the scratched cells had been photographed (40x), the cells were kept in the incubator for a further 48 h, then photographs were taken again in the same position.

Statistical analysis

The results obtained from the studies were statistically evaluated using one-way analysis of variance (ANOVA) followed by Newman–Keuls multiple comparisons test. A value of p<0.05 was considered statistically significant. The results were expressed as the mean and ± standard deviation (SD) values of the mean of every three samples.

RESULTS AND DISCUSSION

The morphological analysis of hydrogels

According to the SEM images of the hydrogels, the structure was seen to have a porous surface (Figure 1). Since the mechanical properties of the B1 and F2 formulations were more suitable and reasonable than those of the other formulations, the SEM images of these formulations were used. The porous structure of the hydrogels makes it easier to embed the active substance in the hydrogel (Sezer et al., 2007; Wikanta et al., 2012). Therefore, the SEM images of the hydrogels showed that they had suitable morphological properties.
Characterization of chitosan-PVA hydrogels

An ideal hydrogel used for wound healing should be compatible with the application site and retained for an extended time on the wound site (Berger et al., 2004; Kiyozumi et al., 2007). Therefore, hydrogels should have an appropriate viscosity. In the current study, the molecular weight of chitosan was a crucial factor in the determination of the viscosity of chitosan-PVA hydrogels \( (p<0.05) \). The viscosity values of the hydrogels were determined to be between 14.400 and 48.500 cPs (Table I). In the majority of formulations, the viscosity of hydrogels containing HMW chitosan was higher than that of hydrogels containing MMW chitosan (F2: 45.450±250 cPs; B2:14.400±180 cPs). The results obtained were compatible with the literature since the hydrogels with viscosity less than 10.000 cPs were retained for a relatively short time on the skin (Sezer et al., 2008). It has been stated in literature (Mohamed, Abu Elella, Sabaa, 2015; Sezer et al., 2007) that suitable hydrogels absorb exudates and keep the wound surface moist during treatment. Therefore, the water absorption capacity of the hydrogels is a crucial factor in wound healing. In the current study, the water absorption capacity of the chitosan-PVA hydrogels showed differences according to the molecular weight of chitosan and concentration of GA. The water absorption rate of hydrogels with MMW chitosan were determined as 56.80±0.70\% - 78.61±0.32\% and hydrogels with HMW chitosan were 76.50±0.80\% - 105.64±0.41\% (Table I). In a study by Sezer et al. (2008) hydrogels prepared with HMW or a higher concentration of chitosan were found to have ideal properties in respect of water absorption capacity. The current study results were compatible with the results of Sezer et al. (2008), and F2 was the ideal formulation in terms of water absorption capacity. It could be said that an increase in the molecular weight of chitosan accelerated the water absorption capacity of hydrogels \( (p<0.05) \). However, types of r-EGF and PEGylation agents did not have any significant effect on the water absorption capacity of hydrogels \( (p>0.05) \). Depending on the studied concentration of GA, covalent bond bridges have been reported to form in the hydrogels, creating a stiff structure in the polymer network, which is expected to decrease the water absorption capacity of the hydrogel (Capanema et al., 2018). However, in the current study, the presence and concentration of GA in the formulation as shown in Table I had no significant effect on the water absorption capacity of the hydrogels \( (p>0.05) \).
PEGylation and in vitro release study results of r-EGF types

PEGylation and release study results of rh-EGF and rm-EGF were calculated according to the EGF spectral curve Equation (2). PEGylation results of different samples are shown in Table II. In the dialysis study performed with mPEG 5K propionaldehyde and rh-EGF, the PEGylation rate was calculated as 78.92±0.54%. PEGylation rate of rm-EGF and mPEG 5K propionaldehyde was calculated as 82.44±0.48%. The rate of PEGylation with rh-EGF and mPEG 10K propionaldehyde was calculated as 56.42±0.84% and PEGylation with rm-EGF and mPEG 10K propionaldehyde was calculated as 64.60±0.58% (Table II). According to the results, the PEGylation rate with r-EGF types and mPEG 5kD propionaldehyde was higher than that of PEGylation with mPEG 10kD propionaldehyde. It could be said that the dialysis process with the dialysis cassette (MW cut off =7 kD) was more easily applicable than the dialysis process with the dialysis sack (MW cut off = 12 kD) and the similarity of molecular weight of mPEG 5kD propionaldehyde and r-EGF types were significant factors determining the efficiency of PEGylation.

In the release study, formulations containing PEGylated rh-EGF or rm-EGF were used as they were more stable to denaturation than formulations containing rh-EGF or rm-EGF without PEGylation agent. Alemdaroğlu et al., (2006) studied the release of EGF from the chitosan gel and it was found to be 97.30% after 24 h. In the current study, the release rate of rh-EGF and PEGylated rm-EGF from the chitosan-PVA hydrogel was measured as 98.50% and 96.60% respectively, to PBS for up to 72 h. In the current study, the release rates of r-EGF were similar to the release results of Alemdaroğlu et al., (2006). In addition, it might be concluded from these results that, chitosan-PVA hydrogel formulations released r-EGF types for a longer period of time compared to that of r-EGF containing chitosan gel without PVA and crosslinker in the study by Alemdaroğlu et al., 2006. Moreover, the release results of rh-EGF and PEGylated rm-EGF were compatible with each other. From the results in Figure 3, approximately 50% of rh-EGF and PEGylated rm-EGF was released from the hydrogel formulations within the first 3 h. The present results showed that crosslinked hydrogel samples released r-EGF types in an extended period due to the strong intermolecular bonds.
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**TABLE II - PEGylation rate of different samples.** Dialysis cassette (MWCO 7 kD) was used for samples including mPEG 5K propionaldehyde, rh-EGF, and rm-EGF. Dialysis membrane (MWCO 12 kD) was used for samples including mPEG 10K propionaldehyde, rh-EGF, and rm-EGF

| PEGylation rate (%±S.D.) | Dialysis cassette (MWCO 7 kD) | Dialysis sack (MWCO 12 kD) |
|-------------------------|-------------------------------|-----------------------------|
| mPEG 5K propionaldehyde and rh-EGF | 78.92±0.54 | --- |
| mPEG 5K propionaldehyde and rm-EGF | 82.44±0.48 | --- |
| mPEG 10K propionaldehyde and rh-EGF | --- | 56.42±0.84 |
| mPEG 10K propionaldehyde and rm-EGF | --- | 64.60±0.58 |

**FIGURE 3 - In vitro release profile of rh-EGF(A1) and rm-EGF(F2) from chitosan-PVA hydrogel at pH 5.8 phosphate buffer saline at room temperature (n=3).** rh-EGF: Recombinant human epidermal growth factor. rm-EGF: Recombinant mouse epidermal growth factor.

**Evaluation of texture profile analysis results of hydrogels**

Hardness is known as the force required for the deformation of hydrogels. Low hydrogel hardness value provides the minimum force necessary for the removal of hydrogels from the vessel and reduces the retention time of hydrogel on the application site. Therefore, hydrogel should have a suitable hardness value for the effective repair of the wound (Cevher et al., 2008; Pal, Banthia, Majumdar, 2009). Cevher et al. (2008), and Sezer et al. (2008), showed that a hardness value of less than 0.396 N was found to be acceptable and appropriate for application on to the epidermal barrier. Similar results were obtained in the current study and it could be said that hardness values of the hydrogels were in an applicable range based on the above-mentioned studies. The results of the current study demonstrated that the hardness values of the hydrogels examined increased significantly (B2: 0.034±0.001 N; E2: 0.052±0.001 N) with the addition of HMW chitosan in the hydrogel formulations. Cevher et al. (2008), explained in their studies that the inclusion of 2% polymer possessed appropriate hardness values, and acceptable hardness. Similarly, in the current study, hydrogels which include 2% PVA and 3% chitosan, showed acceptable hardness values. A2 and F2 were
both optimized formulations in terms of adhesiveness, elasticity, and compressibility; the texture profile analysis graphs of A2 and F2 formulations are shown in Figure 4. These figures provide important information about the adhesiveness and hardness values of the hydrogels. Other properties of the formulations, such as elasticity, compressibility, and cohesiveness were calculated from the data obtained. To provide a suitable treatment period, the hydrogel should be easily applied and should be stable during the treatment period (Sezer et al., 2008).

FIGURE 4 - Texture profile analysis graphs of hydrogel formulations (a: A2, b: F2). All of the formulations have texture profile analysis graphs but A2 and F2 formulations were selected because these formulations were optimized in terms of mechanical properties. The first peak of the graphs expresses the hardness value. The area under the curve between 3 and 4 (AUC3-4) is calculated as the adhesion value.

The hydrogel formulations containing HMW chitosan (H2: 0.153±0.018 N.mm.; F2: 0.125±0.006 N.mm.) were approximately two-fold more adhesive ($p<0.05$) than hydrogels containing MMW chitosan (A1: 0.072±0.005 N.mm; B1: 0.067±0.016 N.mm). Similar results were obtained to those of the studies by Cevher et al. (2008), and Sezer et al. (2008). It has been previously said that the molecular weight and concentration of polymers have a crucial role to adhesiveness. Moreover, it was observed that the concentration of GA increased the adhesiveness of the formulations (D1:0.069±0.003 N.mm; D2:0.081±0.002 N.mm; H1:0.118±0.010 N.mm;
H2: 0.153±0.018 N.mm). In comparison to other hydrogel formulations, the formulation (H2) containing HMW chitosan and high concentration GA (0.002 g/mL) exhibited the greatest adhesiveness and that which (C1) contained MMW chitosan and low concentration GA (0.001 g/mL) exhibited the lowest adhesiveness (Table III). The high cohesiveness values provided significant recovery at the application site after hydrogel application. According to the current study results, the cohesiveness values of hydrogels showed a considerable difference (p<0.05), whereas some of the hydrogel formulations did not show a significant difference (p>0.05) depending on the molecular weight of chitosan (B2: 0.864±0.007; E1: 0.851±0.003; D1: 0.843±0.008; H2: 0.852±0.009). The cohesiveness values of hydrogel formulations containing MMW chitosan were higher than those of hydrogels with HMW chitosan. According to the results, the cohesiveness values of the formulations were between C2 (0.873±0.005) and F2 (0.762±0.016), demonstrating that C2 was the most suitable formulation for cohesiveness. The elastic polymer chains form strong adhesive bonds through interaction between the polymer and epithelial barrier (Capanema et al., 2018; Cevher et al., 2008). From the result obtained, the elasticity of the hydrogels was affected according to the molecular weight of chitosan in the hydrogel formulations (p<0.05). Hydrogels containing MMW chitosan were more advantageous than the other formulations in terms of elasticity (Table III). The data obtained supported the view that the elasticity values of the hydrogel formulations were suitable for the wound healing application. The results showed that B2 (0.978±0.003) was the most suitable formulation in terms of elasticity compared to the other formulations.

The hydrogel was taken from the vessel and the low compressibility value ensured easy application and spreading property. According to a study by Cevher et al. (2008), the compressibility of hydrogels increased depending on the increased polymer concentration. In the current study, the compressibility of the hydrogel increased when the molecular weight of chitosan increased (p<0.05). When the compressibility results of Cevher et al. (2008) were compared with the current study results, it could be concluded that the current study formulations were more useful in terms of application because of the lower compressibility values. The compressibility values of the hydrogels ranged between 0.424±0.010 mj and 0.658±0.016 mj. In comparison to other formulations, those containing HMW chitosan (F1: 0.648±0.006 mj; K2: 0.658±0.016 mj) exhibited the greatest compressibility values. From the results, it was observed that both r-EGF types and PEGylation agent types did not significantly affect the compressibility of the hydrogels (A1: 0.431±0.007 mj; A2: 0.452±0.004 mj; B1: 0.464±0.054 mj; B2: 0.433±0.004 mj; p>0.05).

### TABLE III - Mechanical and bioadhesive properties of hydrogel formulations (n=3). The mechanical and bioadhesive characterizations of hydrogels were measured when the hydrogels reached room temperature

| Formulations | Hardness (N) ± S.D | Adhesiveness (N.mm) ± S.D | Elasticity ± S.D | Cohesiveness ± S.D | Compressibility (mj) ± S.D | Work of Bioadhesion (mj/cm²) ± S.D |
|---------------|-------------------|--------------------------|----------------|-------------------|--------------------------|---------------------------------|
| A1            | 0.036±0.001       | 0.072±0.005              | 0.962±0.006    | 0.860±0.003       | 0.431±0.007              | 0.142±0.007                     |
| A2            | 0.038±0.001       | 0.080±0.004              | 0.934±0.001    | 0.847±0.004       | 0.452±0.004              | 0.161±0.006                     |
| B1            | 0.061±0.008       | 0.067±0.016              | 0.956±0.008    | 0.821±0.006       | 0.464±0.054              | 0.130±0.006                     |
| B2            | 0.034±0.001       | 0.074±0.003              | 0.978±0.003    | 0.864±0.007       | 0.433±0.004              | 0.077±0.004                     |
| C1            | 0.057±0.002       | 0.065±0.005              | 0.951±0.004    | 0.828±0.002       | 0.424±0.010              | 0.081±0.004                     |
| C2            | 0.034±0.000       | 0.071±0.002              | 0.974±0.008    | 0.873±0.005       | 0.429±0.004              | 0.144±0.007                     |
| D1            | 0.103±0.005       | 0.069±0.003              | 0.956±0.007    | 0.843±0.008       | 0.463±0.002              | 0.145±0.003                     |
| D2            | 0.037±0.000       | 0.081±0.002              | 0.957±0.008    | 0.863±0.004       | 0.440±0.008              | 0.139±0.012                     |
The bio-adhesion test was performed to measure the adhesiveness of the hydrogels to the wound site. The bio-adhesion test results were shown in Table III. Cevher et al. (2008) stated that the findings of the mucoadhesion test showed similarity with the adhesiveness results obtained from texture profile analysis. Similarly, findings of the bio-adhesion test were compatible and similar to the adhesiveness values of hydrogels in the current study. The hydrogels with HMW chitosan and high concentration GA had higher bio-adhesion than the other formulations (E2: 0.245±0.001 mj/cm²; F2: 0.194±0.010 mj/cm²; B2: 0.077±0.004 mj/cm²; C2: 0.144±.007 mj/cm²). In addition, some of the hydrogels containing MMW chitosan showed appropriate bio-adhesion values (A1: 0.142±0.007 mj/cm²; A2: 0.162±0.006 mj/cm²; D1: 0.145±0.003 mj/cm²). According to the results of the bio-adhesive property, E2 (0.245±0.001 mj/cm²) and K1 (0.062±0.005 mj/cm²) formulations exhibited the highest and lowest bio-adhesion values. Based on these results, the bio-adhesion graph of the E2 formulation is shown in Figure 5. Sezer et al. (2008) showed that the molecular weight of chitosan and the amount of fucoidan influenced the mucoadhesive ability of the formulations. Similarly, the current results showed that the molecular weight of chitosan and the concentration of the GA had a crucial effect on the bio-adhesion strength of the hydrogels (Table III).
Evaluation of in vitro cell cytotoxicity results

Cell viability below 70% is considered to be a cytotoxic effect. No significant alterations of cell viability were observed after application of both SLN and CMS carrier systems according to the study of Wolf et al. (2000). Similarly, the current MTT results showed that the formulations were not cytotoxic on the NIH 3T3 and HaCaT cell lines. In these experiments, the cell viability rate of the positive control group was determined to be 100%. A significant difference was observed between the MTT results of the control group, the samples of hydrogel formulations containing rh-EGF, rm-EGF, and those not containing rh-EGF or rm-EGF ($p < 0.05$). Dogan et al. (2009) stated that EGF has a crucial role in cell viability and the wound healing process. Similarly, the results of the MTT cell viability showed that rh-EGF, rm-EGF and PEGylation agents had a positive effect on cell viability (Figure 6). According to the MTT results, both A1 (94.60±3.03%) and B1 (93.90±2.70%) formulations showed the greatest NIH 3T3 cell viability and K1 (84.20±2.19%) formulation exhibited the lowest NIH 3T3 cell viability compared to the other hydrogel formulations. Moreover, B1 (91.83±2.04%) and D1 (76.20±2.06) formulations had the highest and lowest cell viability activity, respectively. Due to the cytotoxicity of the GA, the formulations might be considered to be cytotoxic. Formulations which were not cytotoxic on HaCaT and NIH 3T3 cells due to the low concentration (0.001-0.002 g/ml) of GA in the formulations were classified confidential in terms of cytotoxicity (Figure 6A and Figure 6B).

FIGURE 5 – The bio-adhesion graph of E2 formulation. The bio-adhesion is shown as the area under the curve between 1 and 2 (AUC1-2). E2 formulation has a higher bio-adhesive value than the other formulations.
Evaluation of in vitro cell proliferation results

The BrdU cell proliferation test results showed that the formulations proliferated both NIH 3T3 and HaCaT cells ($p<0.05$). According to the BrdU cell proliferation results, B1 (197.82±2.48%) formulation showed the greatest NIH 3T3 cell proliferation activity. Moreover, C1 (167.43±5.89%) formulation exhibited the greatest HaCaT cell proliferation ability. It was observed that hydrogel formulations containing rh-EGF, rm-EGF, PEGylated rh-EGF, or PEGylated rm-EGF had a significant effect on cell proliferation (Figure 7A and Figure 7B). It was crucial that the cell proliferation activity of all the hydrogels was higher than that of the control group. According to these results, it could be said that formulations trigger cell proliferation and could be improved for investigations of wound healing studies.

**FIGURE 6** - MTT cell viability results of NIH 3T3 (A) and HaCaT (B) cell lines (n=3). Positive control (non-treated) group includes NIH 3T3 (A) and HaCaT (B) cells respectively. Negative control (non-treated) group does not include cells. Values are the mean of triplicate determination (n=3) ± standard deviation and statistically significant at *$p<0.05$.
Wound closure results of hydrogel

The wound closure assay was performed using HaCaT and NIH 3T3 cell lines. This study aimed to evaluate the effects of polymers and rh-EGF in hydrogel formulations on wound closure and cell migration. Wolf et al. (2000), evaluated the effects of nano-carriers containing TGF-β1 on the migration of HaCaT cells. It was observed that the migration rate in the cells carrying nano-carrier was twice that of the control group (Wolf et al., 2000). Similar results were obtained in the current study. The migration rate of the F2 formulation, containing PEGylated rh-EGF, in NIH 3T3 and HaCaT cells was calculated as approximately 50% more than the migration rate of the control group. The F2 formulation was used in this study because it has previously shown very suitable results in mechanical characterization.

FIGURE 7 - BrdU cell proliferation results of NIH 3T3 (A) and HaCaT (B) cell lines (n=3). Positive control (non-treated) group includes NIH 3T3 (A) and HaCaT (B) cells consecutively. The cell proliferation ratio of the positive control group was accepted as 100%. The negative control (non-treated) group does not include cells. Values are the mean of triplicate determination (n = 3) ± standard deviation and statistically significant at *p<0.05.
and cell viability studies. From the current study results, the wound closure rate of the control, rh-EGF, and optimized F2 formulation showed significant differences (Figure 8 and Figure 9). On NIH 3T3 cell lines, the wound closure rate of the control, rh-EGF, and F2 were 47.60±2.57%, 59.56±3.35%, and 73.07±2.33% respectively, and on HaCaT cell lines, these rates were 49.40±2.35%, 68.03±2.54%, and 78.87±2.63% respectively. Based on these results, it could be said that rh-EGF stimulates HaCaT and NIH 3T3 cell migration and accelerates scratch closure. The F2 formulation containing PEGylated rh-EGF showed a higher wound closure rate than the control and rh-EGF, indicating that PEGylation with rh-EGF had a positive effect on cell proliferation and wound closure.

**FIGURE 8** - Scratch test images of NIH 3T3 cell: A: Control group (t:0 h), B: Control group (t:24 h), C: rh-EGF (t:0 h), D: rh-EGF (t:24 h), E: F2 formulation (t:0 h), F: F2 formulation (t:24 h). F2 was the optimized formulation so was used in the scratch assay. Scratch closure ratio was measured using image analysis software. Values are the mean of triplicate determination (n = 3) ± standard deviation. Statistically significant value at *p < 0.05.
**CONCLUSION**

In this study, chitosan-PVA hydrogel formulations were prepared and evaluations were performed to determine viscosity values, water-absorption capacity, bio-adhesive activity, mechanical properties, cell proliferation, cell viability, and wound healing efficiency. The results of these parameters revealed that F2 was the most suitable formulation for *in vitro* cell proliferation, cell viability, and scratch closure studies. According to the results, r-EGF or PEGylated r-EGF types used in formulations have an important role in terms of *in vitro* cell viability, cell proliferation, and scratch closure activity. In conclusion, chitosan-PVA hydrogels containing PEGylated rh-EGF or rm-EGF can be considered to be effectively promising formulations for wound healing studies. However, further studies are needed to evaluate their usability as a wound healing material.

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REFERENCES

Alemdaroğlu C, Değim Z, Çelebi N, Zor F, Öztürk S, Erdoğan D. An investigation on burn wound healing in rats with chitosan gel formulation containing epidermal growth factor. Burns. 2006;32(3):319-327.

Agnihotri S, Mukherji S, Mukherji S. Antimicrobial chitosan–PVA hydrogel as a nanoreactor and immobilizing matrix for silver nanoparticles. Appl Nanosci. 2012;2:179-188.

Arranja A, Schroder AP, Schmutz M, Waton G, Schosseler F, Mendes E. Cytotoxicity and internalization of Pluronic micelles stabilized by core cross-linking. J Control Rel. 2014;196:87-95.

Berger J, Reist M, Mayer JM, Felt O, Gurny R. Structure and interactions in chitosan hydrogels formed by complexation or aggregation for biomedical applications. Eur J Pharm Biopharm. 2004;57(1):35-52.

Capanema NSV, Mansur AAP, de Jesus AC, Carvalho SM, de Oliveira LC, Mansur HS. Superabsorbent crosslinked carboxymethyl cellulose-PEG hydrogels for potential wound dressing applications. Int J Biol Macromol. 2018;106:1218-1234.

Cevher E, Taha MAM, Orlu M, Araman A. Evaluation of mechanical and mucoadhesive properties of clomiphene citrate gel formulations containing carbomers and their thiolated derivatives. Drug Deliv. 2008;15(1):57-67.

Cheung RCF, Ng TB, Wong JH, Chan WY. Chitosan: an update on potential biomedical and pharmaceutical applications. Mar Drugs. 2015;13(8):5156-5186.

Croisier F, Jérôme C. Chitosan-based biomaterials for tissue engineering. Eur Polym J. 2013;49(4):780-792.

Demirci S, Doğan A, Aydin S, Dülger EÇ, Şahin F. Boron promotes streptozotocin-induced diabetic wound healing: roles in cell proliferation and migration, growth factor expression, and inflammation. Mol Cell Biochem. 2016;417(1-2):119-133.

Dogán S, Demirer S, Kepenekci I, Erkek B, Hasirci N, et al. Epidermal growth factor-containing wound closure enhances wound healing in non-diabetic and diabetic rats. Int Wound J. 2009;6(2):107-115.

Dreifke MB, Jayasuriya AA, Jayasuriya AC. Current wound healing procedures and potential care. Mater Sci Eng C Mater Biol Appl. 2015;48:651-662.

Ergül M, Ergül M, Tutar Y. Important Anti-Cancer Applications of Protein Based Nanoparticles. Curr Proteomics. 2013;10(4):334-340.

Gainza G, Villullas S, Pedraz JL, Hernandez RM, Igartua M. Advances in drug delivery systems (DDSs) to release growth factors for wound healing and skin regeneration. Nanomedicine. 2015;11:1551-1573.

Garg S, Garg A. Hydrogel: Classification, Properties, Preparation and Technical Features. Asian J Biomater Res. 2016;2(6):163-170.

Gefen T, Vaya J, Khatib S, Harkevich N, Artoul F, Heller ED, et al. The impact of PEGylation on protein immunogenicity. Int Immunopharmacol. 2013;15(2):254-259.

Hardwicke J, Schmaljohann D, Boyce D, Thomas D. Epidermal Growth Factor Therapy and Wound Healing - Past, Present and Future Perspectives. Surgeon. 2008;6(3):172-177.

Hulkower KI, Herber RL. Cell migration and invasion assays as tools for drug discovery. Pharmaceutics. 2011;3(1):107-24.

Jevsevar S, Kunstelj M, Porekar VG. PEGylation of therapeutic proteins. Biotechnol J. 2010;5(1):113-128.

Jonkman JEN, Cathcart JA, Xu F, Bartolini ME, Amon JE, Stevens KM, et al. An introduction to the wound healing assay using live-cell microscopy. Cell Adh Migr. 2014;8(5):440-451.

Kamoun EA, Chen X, Mohy Eldin MS, Kenawy ES. Crosslinked poly (vinyl alcohol) hydrogels for wound dressing applications: a review of remarkably blended polymers. Arab J Chem. 2015;8(1):1-14.

Kiyozumi T, Kanatani Y, Ishihara M, Saitoh D, Shimizu J, Yura H, et al. The effect of chitosan hydrogel containing DMEM/F medium on full-thickness skin defects after deep dermal burn. Burns. 2007;33(5):642-648.

Lee H, Jang IH, Ryu SH, Park TG. N-Terminal Site-Specific Mono-PEGylation of Epidermal Growth Factor. Pharm Res. 2003;20(5):818-825.

Mohamed RR, Abu Elella MH, Sabaa MW. Synthesis, characterization and applications of N-quaternized chitosan/ poly (vinyl alcohol) hydrogels. Int J Biol Macromol. 2015;80:149-161.

Pal K, Banthia AK, Majumdar DK. Polymeric Hydrogels: Characterization and Biomedical Applications. Des Monomers Polym. 2009;12(3):197-220.

Park JW, Hwang SW, Yoon IS. Advanced Growth Factor Delivery Systems in Wound Management and Skin Regeneration. Molecules. 2017;22(8):1-20.

Raafat D, Von Bargen K, Haas A, Sahlg HG. Insights into the mode of action of chitosan as an antibacterial compound. Appl Environ Microbiol. 2008;74(12):3764-3773.
Synthesis and characterization of chitosan-PVA hydrogel containing PEGylated recombinant epidermal growth factor on cell culture for wound healing substitute

Rana P, Ganarajan G, Kothiyal P. Review on preparation and properties hydrogel formulation. WJPPS. 2015;4(12):1069-1087.

Sezer AD, Hatipoğlu F, Cevher E, Oğurtan Z, Baş AL, Akbuğa J. Chitosan Film Containing Fucoidan as a Wound Dressing for Dermal Burn Healing: Preparation and In Vitro/In Vivo Evaluation. AAPS PharmSciTech. 2007;8(39):E1-E8.

Sezer AD, Cevher E, Hatipoğlu F, Oğurtan Z, Baş AL, Akbuğa J. Preparation of Fucoidan-Chitosan Hydrogel and Its Application as Burn Healing Accelerator on Rabbits. Biol Pharm Bull. 2008;31(12):2326-2333.

Suri S, Schmidt CE. Photopatterned collagen–hyaluronic acid interpenetrating polymer network hydrogels. Acta Biomater. 2009;5(7):2385-2397.

Şenel B, Büyükköroğlu G, Yazan Y. Solid lipid and chitosan particulate systems for delivery of siRNA. Pharmazie. 2015;70(11):698-705.

Wikanta T, Erizal T, Tjahyono T, Sugiyono T. Synthesis of polyvinyl alcohol-chitosan hydrogel and study of its swelling and antibacterial properties. Squalen. 2012;7(1):1-10.

Wolf NB, Küchler S, Radowski MR, Blaschke T, Kramer KD, Weindi G, et al. Influences of opioids and nanoparticles on in vitro wound healing models. Eur J Pharm Biopharm. 2009;73(1):34-42.

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