Thiol-Maleimide 반응을 이용한 Poly(ethylene glycol)-케라틴 하이드로젤 연구

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Poly(ethylene glycol)-Keratin Hydrogels Prepared *via* Thiol-Maleimide Reaction

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Abstract: The mechanical properties of hydrogels have a profound effect on cellular responses in tissue engineering applications. In this study, poly(ethylene glycol)-keratin (PEG-KRTN) hydrogels with tunable mechanical properties were prepared by varying molar mass of the maleimide functionalized PEG in the thiol-maleimide chemistry. Reduced keratins were reacted with PEG-maleimides having 2000 Da and 6000 Da molar masses. Viscoelastic and physiochemical properties and cytocompatibility of these hydrogels were tested. Storage modulus values were obtained as 2613 ± 254 Pa and 1313 ± 345 Pa for PEG2000-KRTN and PEG6000-KRTN hydrogels, respectively. Strain sweep data indicate that the linear viscoelastic region (LVER) of the PEG6000-KRTN hydrogel spans up to 40% strain value, whereas it is limited to 10% critical strain for the PEG2000-KRTN hydrogel. PEG6000-KRTN hydrogel presented higher swelling ratios and porosity. CCK-8 test showed that both hydrogels promoted the proliferation of L929 mouse fibroblast cells and, hence, can be applied in soft tissue engineering.

Keywords: keratin, poly(ethylene glycol), michael addition reaction, hydrogel, rheology.

Introduction

Hydrogels are exceptional scaffolds in tissue engineering applications as they are structurally similar to native tissues, and their biochemical and structural properties can be modulated to mimic extracellular matrix to maintain homeostasis.[1,2] Incorporation of the cell adhesion ligands or degradable sequences into the hydrogel matrices has been widely exploited to impart bioactivity to the hydrogel matrices.[3-6] Hydrogel mechanical properties, on the other hand, also influence the morphology, proliferation, migration, signaling, and differentiation of the cells.[7-10]

The mechanical properties of hydrogels can be tuned *via* chemical crosslinking methods. However, the crosslinking agents and byproducts generated can be toxic.[11] Thus, biocompatible

reagents and addition reactions should be used in the crosslinking reactions to overcome potential toxicity problems. The Michael-type addition reactions require a nucleophile as an electron donor and an electron-deficient carbon-carbon double bond present in functional groups such as acrylates, maleimides, and vinyl sulfones as an electron acceptor.[12,13] Maleimides are the most efficient Michael-type acceptors used in the thiol-engaged Michael addition reactions, which ensure rapid crosslinking at physiological conditions.[14-16] Maleimide functionalized poly(ethylene glycol) (PEG) has been frequently preferred in hydrogel formulations due to its biocompatibility, hydrophilicity, and biologically inertness.[15,17] Especially, the hydrogels crosslinked by PEG-maleimides were proposed in cell encapsulation applications where in situ gelation is required.[15,18,19]

Keratin, a fibrous protein extracted from natural sources such as hair, nails, and wool with inherent biocompatibility and biodegradability, is a sustainable hydrogel-forming biopolymer that can serve as a tissue engineering scaffold and drug delivery platform.[20,21] Mechanical and chemical stabilities of the

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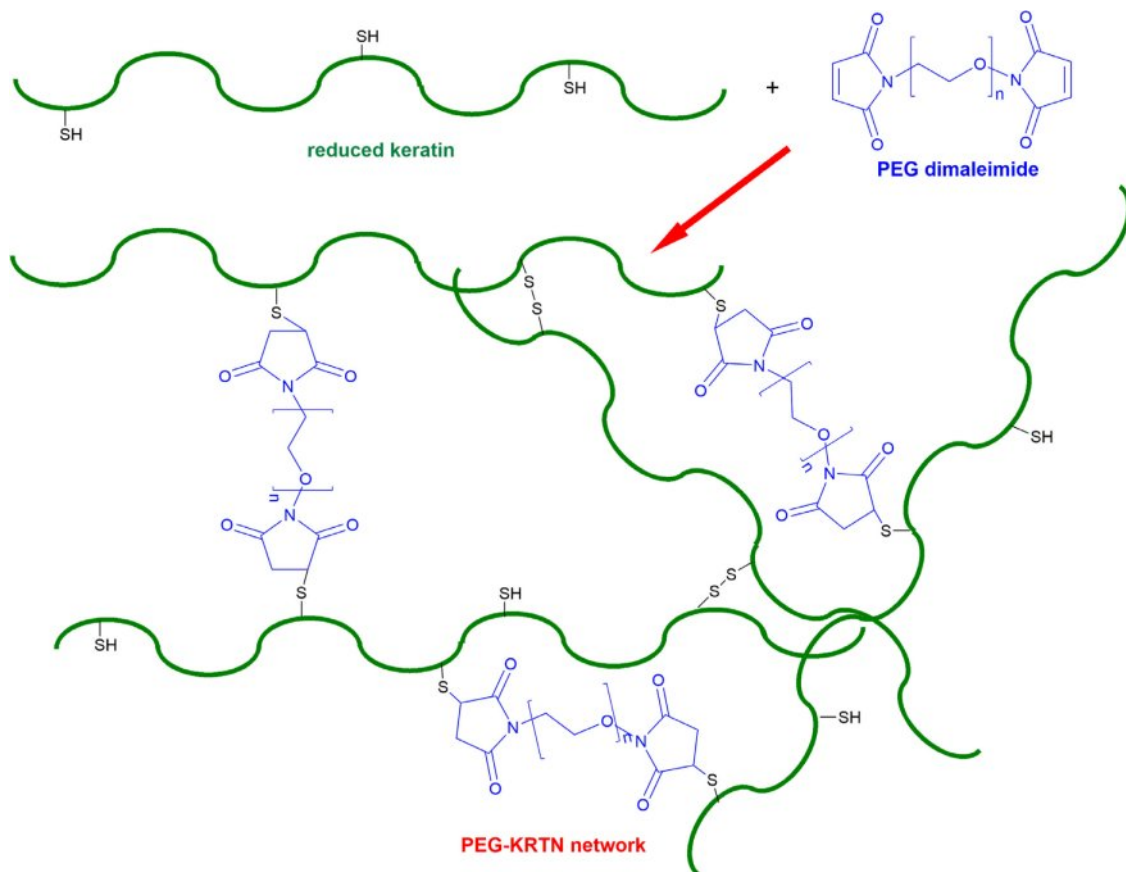
Scheme 1. The network structure of PEG-KRTN prepared *via* thiol-maleimide reaction and disulfide bond formation.

keratin are mainly governed by the disulfide linkages in its structure. However, the reduction of keratins enhances their solubility in water by generating free thiols, which can be exploited in various functionalization and crosslinking processes.[22,23] Additionally, the presence of bioactive domains such as LDV (Leu-Asp-Val), EDS (Glu-Asp-Ser), and RGD (Arg- Gly-Asp) in the keratin proteins promotes the cell attachment characteristics of the keratinous hydrogel matrices.[24,25]

In this study, chemically crosslinked PEG-keratin hydrogels were prepared *via* a thiol-maleimide reaction (Scheme 1). Reduced keratins were used as a Michael-type donor source and reacted with homobifunctional PEG-maleimides (6000 Da or 2000 Da) to form biocompatible hydrogels with tunable viscoelastic and physicochemical properties.

Experimental

Materials. Karacabey Merino sheep wool was kindly provided by the Sheep Breeding Research Institute (Balıkesir, Turkey). Chloroform, methanol, sodium sulfide nonahydrate, DL-

dithiothreitol (DTT), 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB = Ellman's reagent), and cellulose membrane dialysis tubing (MWCO ~14000) were obtained from Sigma-Aldrich. Polyethylene glycol dimaleimides (RAPP Polymere, PEG-$(C_2H_4$-mal$)_2$, M_n = 2000 Da and 6000 Da) were used as crosslinking agents. Cell culture supplements were purchased from Biological Industries. CCK-8 assay (Cayman) was used in the cell proliferation studies.

Extraction and Reduction of the Keratin Proteins. Merino sheep wools were cleaned and defatted using previously reported methods.[26,27] Soluble keratin proteins were obtained by treating 1 g cleaned and defatted wool with 50 mL 125 mM sodium sulfide solution at 40 ℃ by shaking at 150 rpm for 4 h. The keratin extraction solution was dialyzed against deionized water using pretreated dialysis membrane tubing. The dialysis medium was changed thrice a day, and the dialysis process was carried out for 3 days. Then, the keratin solution was lyophilized and stored at -20 ℃. Keratin proteins are exposed to the second reduction process to increase their free thiol content.[28] 500 mg lyophilized keratin was dissolved in 10 mL deionized water at pH 7.4 and room temperature. 200 mg DTT was added to the keratin solu-

tion to cleave the disulfide bonds, and the solution was stirred at room temperature overnight. Next, the reduced keratins were purified using a centrifugal filter (Amicon Ultra-15 with Ultracel membrane, 3 kDa MWCO). Keratin proteins were isolated by freeze-drying and stored at -20 ℃. DTNB assay was used to determine the free thiol groups of the keratin proteins.[29] UV absorbance values at 412 nm were measured on a Shimadzu UV-2450 model UV-Vis spectrophotometer. Free thiol content was determined using the extinction coefficient of 14150 $M^{-1}cm^{-1}$ at 412 nm.[30]

Preparation and Characterization of the PEG-keratin Hydrogels. Chemically crosslinked PEG-keratin hydrogels were prepared by mixing the reduced keratin and PEG-$(C_2H_4\text{-mal})_2$ solutions prepared in degassed PBS (10 mM NaH_2PO_4 and 150 mM NaCl) at pH 7.4 or in degassed deionized water at neutral pH. Immediately formed viscous solutions were incubated at 37 ℃ overnight to promote gelation. In the hydrogel formulations, the concentration of functionalized PEGs was fixed as 5 w%. The keratin amount used was determined according to the thiol-to-maleimide molar ratio of ~1 : 0.58 ± 0.04.

Oscillatory rheology tests of the hydrogels were conducted by following the protocol given elsewhere.[31] A Thermo Fisher Scientific HAAKE MARS model rheometer with a 3.5 cm diameter was used in the experiments.

The swelling behavior of the hydrogels was investigated by soaking the lyophilized hydrogels in excess PBS buffer at room temperature and comparing the dry weight (W_d) and the swollen weights (W_s) of the hydrogels measured at specified periods. The following equation was used to get the swelling ratio (SR) values:[32]

$$SR = \frac{W_s - W_d}{W_d} \qquad (1)$$

The pore structure of the hydrogels was determined using an FEI Quanta 250 FEG model scanning electron microscopy (SEM). The hydrogels prepared in deionized water were frozen in liquid nitrogen and freeze-dried at -80 ℃. A cross-sectionally fractured portion was observed after the sample was coated with gold. CCK-8 assay was used to investigate the cytocompatibility of the PEG-keratin hydrogels. CCK-8 analysis and cultivation of the L929 mouse fibroblasts were performed according to the previously used protocols.[26,27] For the CCK-8 assay, absorbance measurements at 450 nm (A_{450}) were taken with a Varioskan Flash model microplate reader. The relative cell proliferation rate (RCPR) values were determined using the following formula:[27]

$$RCPR = \frac{A_{450} \text{ of the hydrogel}}{A_{450} \text{ of TCPS at the end of Day 1}} \qquad (2)$$

Results and Discussion

Keratin proteins were solubilized using sodium sulfide. The free thiol amount obtained in the sulfitolysis reaction is relatively low (~0.07 mmol/g keratin) due to the reformation of disulfide bonds during dialysis. For this reason, the keratin proteins were subjected to the second reduction process with DTT and purified very quickly using a centrifugal filter. Upon DTT reduction, the free thiol amount increased to 0.62 mmol/g keratin. Similar to our study, sodium sulfide was used to extract keratin proteins from human hair, and the free thiol amount of the keratin proteins was determined to be less than 0.01 mmol/g keratin. After the DTT reduction process, the free thiol content of the human hair keratins was reported as 0.55 mmol/g keratin.[28]

PEG-keratin hydrogels were prepared at a fixed functionalized PEG concentration of 5 w% and ~1 : 0.58 ± 0.04 thiol to maleimide ratio. At these conditions, the hydrogels with 12.5 w% and 10 w% total polymer concentration (keratin + functionalized PEG) were formulated for 2000 Da or 6000 Da PEG-$(C_2H_4\text{-mal})_2$ components and the resultant hydrogels were denoted as PEG2000-KRTN and PEG6000-KRTN, respectively. After mixing reduced keratin with 2000 Da or 6000 Da PEG-$(C_2H_4\text{-mal})_2$ at pH 7.4 and incubating at 37 ℃, self-standing hydrogels were obtained. As thiols were used in excess amounts, the network formation is mainly due to the Michael addition reaction and the formation of disulfide bonds upon oxidation (Scheme 1).

The viscoelastic behavior of the PEG-keratin hydrogels was investigated using frequency sweep, strain sweep, and cyclic strain time sweep experiments. First, the frequency sweep tests were done from 0.1 Hz to 15 Hz at a constant 0.2% strain, and the resultant curves are shown in Figure 1. Storage modulus (G') values of the PEG-keratin hydrogels were higher than those of the loss modulus (G'') values, confirming gel-like structures composed of the crosslinked viscoelastic network.[33]

Plateau values of storage moduli were determined as 2613 ± 254 and 1313 ± 345 Pa for PEG2000-KRTN and PEG6000-KRTN hydrogels, respectively. The higher stiffness of the PEG2000-KRTN hydrogel can be attributed to its higher number of crosslinking points, as the amount of keratin is higher in the PEG2000-KRTN hydrogel formulation. It is also possible that shorter and more mobile PEG2000-dimaleimide chains
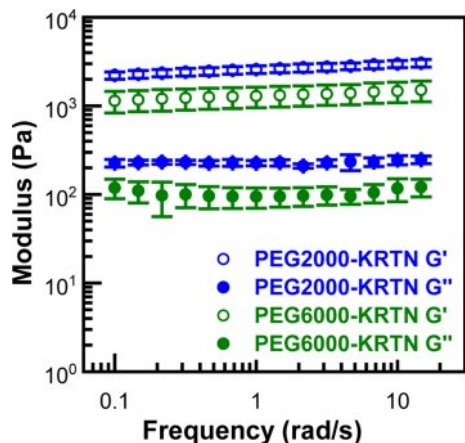
Figure 1. Frequency sweep plots of the hydrogels.

can react with the thiol groups more readily, contributing to the crosslinking density. G' values of the PEG-keratin hydrogels obtained in this study are much higher than those of the tetrakis (hydroxymethyl) phosphonium chloride (THPC) crosslinked keratose hydrogels (between ~0.06 and 0.8 kPa) prepared at 7.5 w% biopolymer concentration.[27]

Linear viscoelastic regions (LVER) of the hydrogels were determined using strain sweep data given in Figure 2. LVER of the PEG2000-KRTN hydrogel was observed up to 10% strain. However, PEG6000-KRTN hydrogel exhibited a broader range of LVER with a 40% critical strain value. After these critical strain values, G' values decreased, and G'' values started to increase, indicating an increase in the viscous behavior and destruction of the network structure of the hydrogels.[34] The higher critical strain of the PEG6000-KRTN hydrogel can be attributed to its lower crosslinking density.[35]

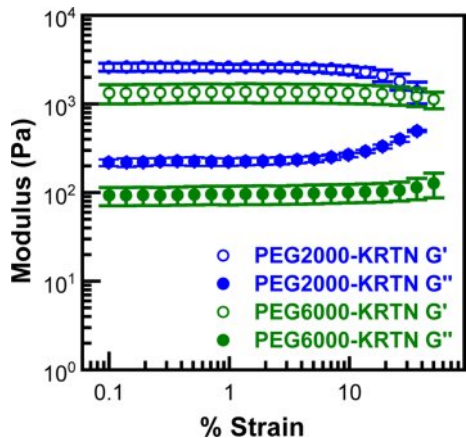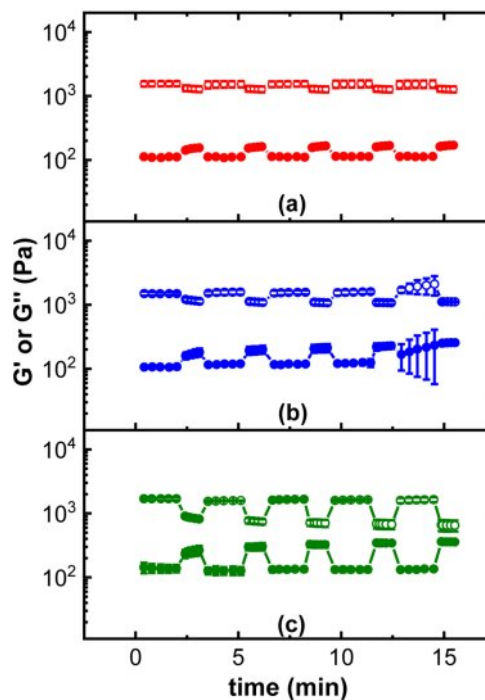Cyclic time sweep experiments were performed using 40%,

Figure 3. Cyclic strain time sweep plots of the PEG6000-KRTN hydrogel exposed to 0.2% low strain followed by (a) 40%; (b) 60%; (c) 100% high strain. G' = open circles, G'' = closed circles.

60% and 100% strains outside the LVER region of the hydrogels. The PEG2000-KRTN hydrogel structure failed upon exposing high strain values. PEG6000-KRTN hydrogel, on the other hand, restored its structure and did not show elastic to viscous transition up to 100% strain, confirming its more elastic nature (Figure 3).

The swelling kinetic curves of the PEG-hydrogels are shown in Figure 4. The average swelling ratios of the PEG2000-KRTN and PEG6000-KRTN hydrogels were determined as 13.7 ± 1.0
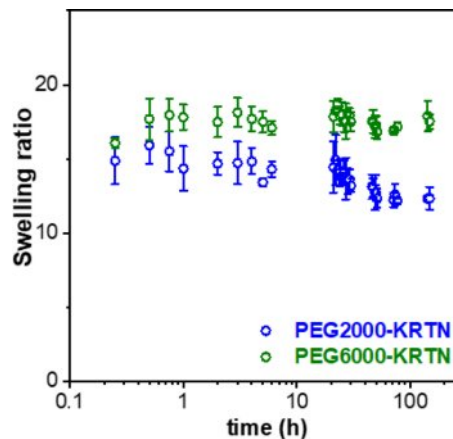

Figure 2. Strain sweep plots of the hydrogels.

Figure 4. Swelling behaviors of the freeze-dried PEG-KRTN hydrogels.

and 17.6 ± 0.5, respectively. The lower swelling ratio of PEG2000-KRTN hydrogel can be explained by its stiffness, which restricts water penetration into the network structure.[36,37] Both hydrogels maintained their structures for up to 6 days. After six days, the samples started to dissolve in the PBS buffer.

Figure 5 shows SEM images of freeze-dried hydrogels. A highly irregular pore structure with open and closed pores was obtained for both hydrogels. PEG6000-KRTN hydrogel
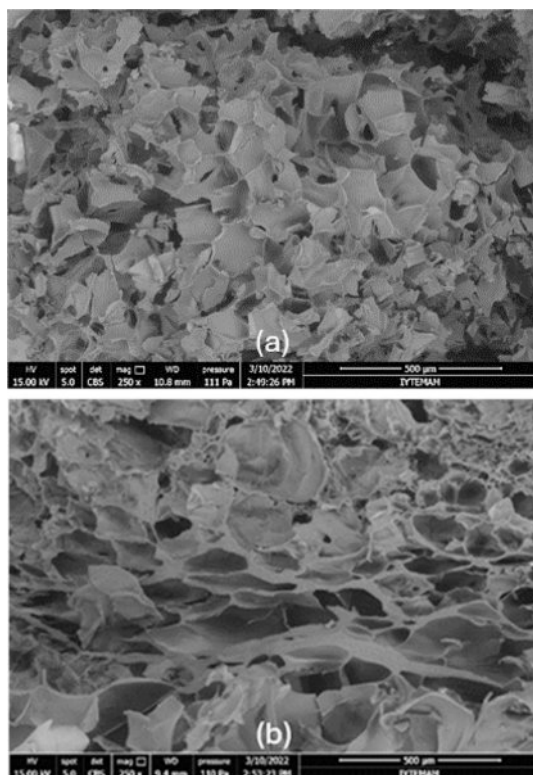

Figure 5. SEM images of the freeze-dried: (a) PEG2000-KRTN; (b) PEG6000-KRTN hydrogels. Scale bars: 500 μm.
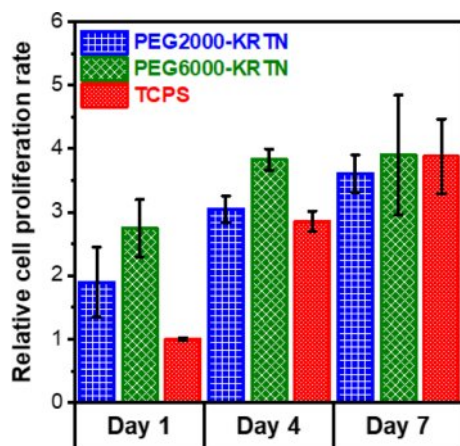

Figure 6. Cell proliferation results of the PEG-KRTN hydrogels.

exhibited a more porous structure, which can be attributed to the higher molar mass of PEG and its lower number of cross-linking points. Additionally, fibrillar structures around the pores and pore walls of the PEG6000-KRTN hydrogel were observed.

Cell proliferation tests were performed to examine the interactions between the hydrogels and the L929 mouse fibroblast cell line using CCK-8 assay. Relative cell proliferation rate results are given in Figure 6. For day 1 and day 4, both hydrogels promoted cell proliferation at a rate higher than tissue culture polystyrene (TCPS). However, THPC crosslinked keratin hydrogels prepared by our group exhibited lower cell proliferation than TCPS.[27] The superior biocompatibility of the PEG-keratin hydrogels can be attributed to their higher stiffness and larger pore sizes. Between the fourth and seventh days, slower cell proliferation was obtained, suggesting the saturation of the seeded surfaces by the cells.

Conclusions

PEG-keratin hydrogels were successfully prepared by applying simple thiol-maleimide chemistry. The initial solubilization of keratin proteins using sodium sulfide generated a relatively small number of thiols, but the subsequent reduction with DTT significantly increased the thiol content of the keratin. Using different molar masses of PEG dimaleimides (2000 Da and 6000 Da) in the crosslinking reaction profoundly affected the mechanical and physicochemical properties of the resultant hydrogels. The PEG2000-KRTN hydrogels presented higher stiffness due to increased crosslinking points, whereas the PEG6000-KRTN hydrogels exhibited higher elasticity, attributed to the longer PEG chains and lower crosslinking density. Swelling experiments showed that the PEG6000-KRTN hydrogel had a higher swelling ratio, correlating with its more porous structure, as revealed by its SEM image. PEG-keratin hydrogels promoted a higher cell proliferation rate than the TCPS surfaces, confirming their biocompatibility. The results indicate that these hydrogels with tunable properties can find applications in soft tissue engineering.

Conflict of Interest: The authors declare that there is no conflict of interest.

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