XANTHAN-GELATIN AND XANTHAN-GELATIN-KERATIN WOUND DRESSINGS FOR LOCAL DELIVERY OF VITAMIN C

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ABSTRACT

XANTHAN-GELATIN AND XANTHAN-GELATIN-KERATIN WOUND DRESSINGS FOR LOCAL DELIVERY OF VITAMIN C

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In recent years, functional dressings that can protect the wound area from dehydration, support healing (interactive), and protect the wound against infections have gained importance instead of dry and passive dressings. In this study, it was aimed to develop temporary xanthan gelatin (XGH) and keratin xanthan gelatin hydrogels (KXGHs) that have high exudate absorption capacity, oxygen permeability, and applicability as a wound dressing for medical applications. Firstly, xanthan gelatin hydrogels were produced using different glycerol concentrations. Then, different keratin concentrations were added into xanthan/gelatin hydrogels to determine optimum keratin concentrations. The produced XGH and KXGHs were immersed into different Vitamin C (VC) solutions for loading. Physical properties, mechanical strength, biodegradability, FTIR, DSC, TGA analysis, and biocompatibility of XGH, KXGH, and VC-loaded KXGH (VC-KXGHs) were analyzed. The addition of keratin to XGH improved the viability of L929 fibroblast cells, increased the protein release from the hydrogels. The addition of VC and keratin didn’t affect the water vapor transmission rate and oxygen permeability

significantly. However, VC immersed hydrogels showed a higher water uptake percentage and higher weight loss at the end of the 5th day. VC was released in 100h. VC loaded hydrogels increased collagen synthesis by the fibroblast cells. Also, microbial penetration test proved that XGH, and KXGH, can be a good barrier against bacteria. Therefore, VC-XGH and VC-KXGH can be suggested to hold potential as candidate wound dressing material for skin wounds.

Keywords: Xanthan, Gelatin, Keratin, Glycerol, Wound dressing

ÖZ

LOKAL VİTAMİN C SALIMI İÇİN KSANTAN-JELATİN VE KSANTAN-JELATİN-KERATİN YARA ÖRTÜLERİ

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Son yıllarda kuru ve pasif pansumanların yerine, yara bölgesini dehidrasyondan koruyabilen, iyileşmeyi destekleyen (interaktif) ve yarayı enfeksiyonlara karşı koruyabilen fonksiyonel pansumanlar önem kazanmıştır. Bu çalışmada, tıbbi uygulamalar için yara örtüsü olarak yüksek eksüda emme kapasitesi, oksijen geçirgenliği ve uygulanabilirliği olan geçici ksantan / jelatin (XGH) ve keratin / ksantan / jelatin hidrojellerin (KXGHs) geliştirilmesi amaçlanmıştır. İlk olarak, ksantan / jelatin hidrojeller, farklı gliserol konsantrasyonları kullanılarak üretildi. Daha sonra, optimum keratin konsantrasyonlarını belirlemek için ksantan / jelatin hidrojellerine farklı keratin konsantrasyonları ilave edildi. Üretilen XGH ve KXGH'ler yükleme için farklı Vitamin C (VC) solüsyonlarına daldırıldı. XGH, KXGH ve VC yüklü KXGH'nin (Vc-KXGH) fiziksel özellikleri, mekanik mukavemeti, FTIR, DSC, TGA, biyobozunurluk ve biyouyumluluk özellikleri test edildi. XGH'ye keratin ilavesinin, L929 fibroblast hücrelerinin canlılığını iyileştirdiği, hidrojellerden protein salınımını arttırdığı gözlendi. VC ve keratin ilavesi, su buharı geçirgenliği oranını ve oksijen geçirgenliğini önemli ölçüde etkilemedi. Bununla birlikte, VC solüsyonlarına daldırılmış hidrojeller, 5. günün

sonunda daha yüksek su alım yüzdesi gösterdi ve daha yüksek oranda ağırlık kaybına neden oldu. VC 100 saat içinde ortama salındı. VC yüklü hidrojeller, fibroblast hücrelerinde kolajen sentezini arttırdı. Mikrobiyal penetrasyon testi, XGH ve KXGH'lerin bakterilere karşı iyi bir bariyer olabileceğini kanıtladı. Bu nedenle, VC-XGH ve VC-KXGH, cilt yaraları için yara pansuman malzemesi olarak kullanılabilecek potansiyeli barındırdığı önerilebilir.

Anahtar Kelimeler: Ksantan, Jelatin, Keratin, Gliserol, Yara örtüsü

Dedicated to my mother, who has always supported me more than anyone else during the hard and exhausting times

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LIST OF ABBREVIATIONS

ABBREVIATIONS

AG: Alginate

ANOVA: Analysis of Variance

ASTM: American Society for Testing and Materials

AV: Aloe vera

BC: Bacterial cellulose

CS: Chitosan

CSH: Corn starch

DMEM: Dulbecco's Modified Eagle's Medium

DO: Dissolved oxygen

DS: Degree of substitution

DSC: Differential scanning calorimetry

DTA-TGA Differential Thermal Analysis-Thermogravimetric Analysis

ED: Enzymatic degradation

EDS: Equilibrium degree of swelling

EDTA: Ethylene diamine tetra acetic acid

EPP: Extract of propolis

FBS: Fetal bovine serum

FDA: Food and Drug Administration

FTIR: Fourier transform infrared spectrophotometry

G: Gelatin

GLY: Glycerol

GRAS: Generally recognized as safe

HA: Hydroxyapatite

HA-g-Pu: Hyaluronic acid grafted pullulan

K: Keratin

KXGHs: Keratin beared xanthan gelatin hydrogels

P: Porosity

PBS: Phosphate buffered saline

PUUF: Polyurethane-urea foam

PVA: Poly (vinyl alcohol)

SD: Standard deviation

SSD: Silver sulphadiazine

SEM: Scanning electron microscope

SF: Silk fibroin

VC-KXGHs: VC containing keratin xanthan gelatin hydrogels

WVTR: Water vapor transmission rate

X: Xanthan

XGHs: Xanthan gelatin hydrogels

VC: Vitamin C

CHAPTER 1

INTRODUCTION

1.1 Skin

Skin is the largest organ that covers the whole surface of the human body with mucous, which constitutes %15 of the total weight of the body. The skin, with its accessory structures such as hair, nail, glands, and nerves, comprises the integumentary system, which supports body protection (Kolarsick A. J. P., BS, Kolarsick M., MSN, ARNP-C, Goodwin C., APRN-BC, 2006). The main function of the skin is not only the protection of the body but also the obstruction of water loss and supplying thermoregulation (Kanitakis, 2002). Skin also has a storage role for the body: The deepest layer of skin stores water, fat and metabolic products and also produces hormones. The skin includes mainly three layers: epidermis, dermis, and subcutaneous tissue. Skin can vary in terms of color texture and thickness (*How Does Skin Work?* 2019)

The epidermis includes the stratum corneum is the outermost layer, which consists of dead keratinocytes (also known as corneocytes) and intercellular lipids. The layer hinders the entry of the water and water-soluble substances. Besides its barrier function, many holes such as hair follicles and sweat ducks are present (Kabashima et al., 2019). The dermis is located under the epidermis layer as an integrated system of fibrous, filamentous, and amorphous connective tissue. As a response to any stimuli, lymphocytes, leukocytes, and macrophages can enter the dermis. The skin takes its pliability, tensile strength, and elasticity from the dermis. Furthermore, the dermis has the role of protecting the skin from injury (Fitzpatrick et al., 2008). Subcutaneous tissue, also known as the hypodermis, is the innermost layer of the

epidermis. It comprises not only connective tissue with blood vessels but also adipocytes which are in groups in lobules. Depending on the body part, the number of adipocytes can vary. However, their size is determined by the nutrition state of the body. Gender is another factor that affects the number of adipocytes in the body. Adipocytes are needed as an energy source for the thermoregulation of the body.

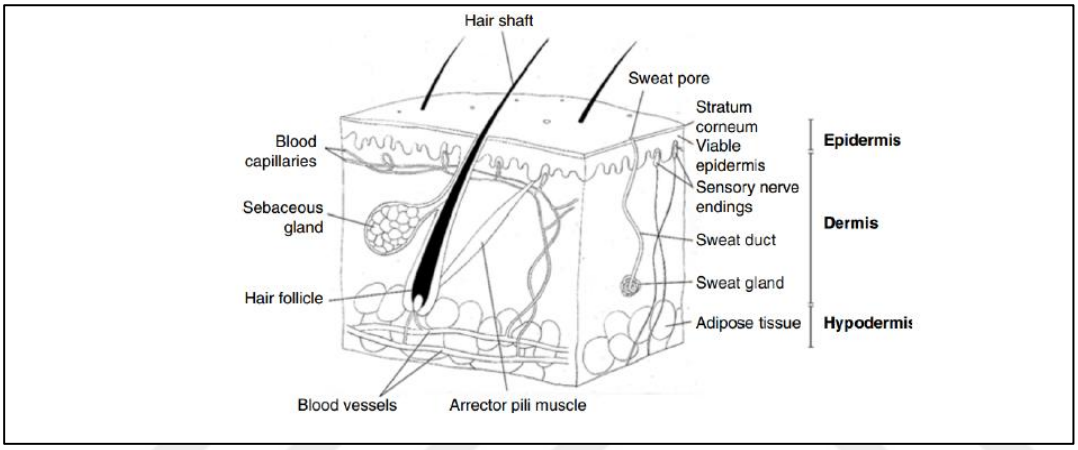

Figure 1. 1. Layer of skin (Ng & Lau, 2015)

The main function of the skin is to protect the body and its physiological environment from external factors. The stratum corneum acts as a physical barrier. It prevents the entry of exogenous material also hinders water loss. Alongside being a physical barrier, the skin has a chemical barrier function which is called ‘acid mantle’. Due to the acidic environment of the skin whose pH values are between pH 4- pH 6, skin can prevent the growth of bacteria that lives in an alkali environment. Furthermore, this acidic environment provides aggregateness of the stratum because many enzymes have an important role in terms of homeostasis of stratum corneum lipid as shown in Fig 1.1 (Ng & Lau, 2015). Many cells such as Langerhan cells, dendritic cells, and macrophages, which are responsible for defending mechanisms of the immune system can be found in the skin (Zaba et al., 2009). They are part of the

primary immune response by activating naïve T cells. For thermoregulation, thermoreceptors detect the cold or the heat. Once the heat is detected, the signal is transmitted to the hypothalamus which controls the thermoregulation mechanism of the body. Additional insulation is supplied by body hairs on the skin surface, which can be maximized by the erection of the hairs. Perspiration through sweat pores also reduces the body temperature. Blood vessels can dilute or contract depending on the heat or the cold, which is part of thermoregulation. Sensory nerve endings in the dermis can detect the pain, which is crucial for the survival skills of the human. Thanks to the dermis, the body can synthesize vitamin D. After synthesizing the precursor vitamin D in the stratum spinosum, it is converted to vitamin D by keratinocytes (Bikle, 2011). Many minerals and organic wastes as dissolved are removed through sweat. In the case of physical shock, the subcutaneous tissue protects the inner organs (Ng & Lau, 2015).

1.1.1 Types of Wounds

A wound is explained as an injury or deterioration of the function and structure of the skin (Velnar et al., 2009). Wounds can be categorized depending on the number of damaged skin layers. If a wound occurs in the epidermal layer alone it is called superficial wounds. The wounds located in the epidermal and deeper layers that contain damaged hair follicles, blood vessels, and sweat glands, are known as partial-thickness wounds; and wounds located in the depth of the subcutaneous layer are named full-thickness wounds (Joshua Boateng & Catanzano, 2015).

The wounds can be characterized into two categories: Acute and Chronic wounds. Acute wounds can be sourced by surgeries or trauma such as burns (chemical, thermal and electrical), animal bites, and a splash of an object. These kinds of wounds can completely heal with four reparative phases (Gurtner et al., 2008). However, if the healing process is longer than four weeks, an acute wound becomes a chronic wound (Yazdanpanah et al., 2015). This could be due to the destruction of

the extracellular matrix, hypoxia, reactive oxygen species production, and bacterial invasion (Azzimonti et al., 2016).

Chronic wounds need a frequent change of the dressing and cause physical limitations which result in a decrease in the life quality of the patients (Fife et al., 2012). Chronic wounds have other obstacles such as a large volume of exudate; for venous leg ulcers, it is reported that the volume of the exudate can be 12 $L.m^{-2}$ (Cutting, 2003). Chronic wounds are prone to be stuck in the inflammatory phase of the healing process (Falanga, 2000). For healing of the wound, debridement (dead wound tissue) should be removed because it can retard the healing by preventing keratinocyte migration over the wound bed. To remove debridements, surgical, autolytic, enzymatic, biological, and mechanical methods can be used. Although surgical debridement removal is efficient and rapid, it can cause damage to the viable tissue. When the wound is moist, endogenous enzymes degrade the debridement from the wound bed, which is called the autolytic method. This method is more selective and less painful (Steed, 2004).

Acute wounds can be categorized into accidental wounds and surgical wounds. They can be caused by trauma, such as burns, lacerations, and abrasions (Pitts et al., 2008; Reichman & Greenberg, 2009). While surgical wounds need minimal intervention for quick healing, other injuries such as burn and gunshot may require surgical debridement and antimicrobial therapy. Acute wounds show normal wound physiology, mostly, they progress through the normal phases of wound healing (Okur et al., 2020). A wound can be identified as infected when it shows the symptoms of infection such as oedema, heat, purulent exudate, and pain. To inhibit infection of wounds, disinfectants can be used to remove bacteria, soil, and debris due to mechanical cleansing (Edlich et al., 2010). Previous research about acute wounds claimed that the presence of certain bacteria in a wound may signify an infection (Robson, 1997).

There are certain characteristic differences between acute and chronic wounds. While acute wounds have low levels of bacteria, cytokines, proteases, and reactive

oxygen species, chronic wounds have not only high levels of bacteria, cytokines, proteases, but also reactive oxygen species. Also, acute wound fibroblasts show high mitogenic activity with an intact matrix. However, fibroblasts of chronic wounds cause low mitogenic activity with a dysfunctional degraded matrix in the body (Morton & Phillips, 2016).

1.1.2 Repair Mechanisms of Skin

The repair mechanism of the skin comprises four different overlapping processes that are hemostasis, inflammation, proliferation, and remodeling. In the hemostasis stage, fibrin and platelet plugs stimulate the coagulation. Platelets have a role in the production of chemotactic factors and transforming growth factor-beta. These chemotactic and growth factors stimulate macrophages, neutrophils, smooth muscle cells, fibroblasts, and endothelial cells which are needed for inflammatory and proliferative phases (Robert S. Kirsner et al., 1993). Fibrin acts as a chemo-attractive matrix not only for macrophages but also for fibroblasts (Budzynski & Shainoff, 1986). When the neutrophils adhere to the endothelium, the inflammatory phase starts (Wilgus et al., 2013). Neutrophils can migrate into the extracellular space due to the activity of elastases and collagenases (Wilgus et al., 2013). Pathogenic organisms degrade wound debris and can cause stimulation of granulation tissue formation and angiogenesis (Shah et al., 2012). The proliferation phase includes fibroplasia, granulation, epithelization, and angiogenesis. This phase starts within 24 hours of the wound infection (R. S. Kirsner & Eaglstein, 1993). Recruited endothelial cells proliferate in response to vascular endothelial growth factors that also stimulate smooth muscle cell migration (Qu et al., 2010). After injury formation, the proliferation of fibroblasts, regulated by the fibroblast growth factor, is very important for dermal matrix formation (Bainbridge, 2013). Fibroblasts produce proteins such as collagen, elastin, extracellular matrix protein (Bainbridge, 2013). Collagen secretion can start after the 48 and 74 hours of the injury occurrence however, its maximum secretion can be seen 5 to 7 days post-injury. Extracellular

matrix components such as glycosaminoglycans, proteoglycans supply strength, and support collagen (R. S. Kirsner & Eaglstein, 1993). The remodeling process can last from weeks to years. The contraction of the wound can start by day 5 due to phenotypic changes of fibroblasts to actin-laden myofibroblasts (J. Boateng & Catanzano, 2015). Collagens may provide %20 of tensile strength after 3 weeks, and 80% of its strength at month 12 (Morton & Phillips, 2016).

For acute wound treatment, the treatment can vary depending on whether the wound is closed or open. While closed wounds don't need cleansing, dirty open wounds such as bite, or cut wounds should be cleansed. For wound cleansing, drinkable tap water can be applied by using lukewarm water. Moreover, disinfectants shouldn't be used for acute wound cleansing (Ubbink et al., 2015). The treatment of chronic wounds can be more complex than acute wounds. For chronic wounds including diabetic foot ulcers, venous leg ulcers, and pressure ulcers, debridement, control, and moisture balance are very important. There are different treatment methods for the treatment of ulcer wounds, for example, for diabetic foot ulcers, infections are treated. The use of shoes can redistribute plantar pressure and also orthotics/special devices can be used (Nicks et al., 2010). For unhealing chronic wounds, arterial revascularization surgery, venous vascular surgery, and blood glucose control are used for arterial insufficiency ulcers, venous insufficiency ulcers, and diabetic foot ulcers, respectively (Steed et al., 2006).

Wound dressings can be seen as a part of wound care of chronic wounds. Regular changes in dressing help the healing process by hindering infection. It was claimed that hydrogel dressings can be more functional in the healing process of diabetic foot ulcers compared to basic wound contact dressings (Forster & Pagnamenta, 2015; Saleh & Sönnergren, 2016).

1.2 Wound Dressings

Throughout the history of humanity, various strategies have been developed to solve problems encountered in stopping bleeding, accelerating healing in acute wounds, and caring for chronic wounds. Particularly in clinical situations where the wound healing process does not proceed normally and complete functional regeneration of functional skin tissue cannot be achieved, it has very serious consequences, including death when wound care is inadequate. The wound care process significantly reduces the quality of life of the patients and causes socioeconomic problems due to health expenditures and labor losses(Sen et al., 2009).

Wound dressings are produced to protect the wound against any external environment. An ideal dressing should be able to absorb excess exudate formed on the wound surface, control moisture in the wound area, allow gas passage, protect the wound from microorganisms, provide desired mechanical and physical conditions, be non-toxic and allergic, if necessary, be biodegradable, support cell growth and help the healing of tissue. It should be easily changed without damaging the wound, it should be easy to apply (Andreu et al., 2015). Additionally, it should have a long shelf life and be economical (Andreu et al., 2015; V. J. Jones, 2006; MacEwan et al., 2017; Sood et al., 2014). Therefore, it is very important to evaluate the wound's condition, needs, costs, and choose the right dressing. Although a wide variety of dressings are used in the clinic, there is still no dressing that contains all the features that an ideal dressing should have, and there is a need to develop new-generation dressings suitable for different kinds of wounds.

A broad array of commercialized wound dressings are used in clinical use for different kinds of wounds originated by traumatic, pathological, or surgical events. Aquaflo ™, Tegaderm ™, Mepiform®, BIOPAD®, Cutifilm, AQUACEL® are some of the dressings frequently used in the clinic. Such dressings contain natural or synthetic polymers such as polyvinyl alcohol, polyvinylpyrrolidone, polyacrylamides, alginate, chitosan, bacterial cellulose, carboxymethyl cellulose, gelatin, dextran, polyurethane, and polyglutamic acid (Gupta et al., 2019). The

dressings used in the clinic are selected based on the wound type, characteristics, and the stage of wound healing, and they are replaced with different dressings according to the need in the same wound treatment process (Dabiri et al., 2016). Clinical and experimental studies conducted in the literature involve dressings that are generally synthetic and natural polymer-based. They are categorized as passive, interactive, and bioactive dressings.

1.2.1 **Passive Barrier Type Dressings**

Passive barrier type dressings are the first type of dressing used in the clinic to preserve the wound area from external factors and help the healing process (Moura et al., 2013). Traditional methods such as gauze are generally used. Passive barrier dressings are frequently preferred today because they are inexpensive and provide protection. The use of traditional dressings for wounds following the normal healing process may be useful, but the use of such dressings in the treatment of chronic wounds cannot prevent infections in the wound area (Han, 2016). Besides, their inability to release therapeutic agents to the wound area and not being suitable for all kinds of wounds limit the use of these dressings. Passive dressings are used directly in uninfected wounds with a dry or small amount of wound exudate, but only as secondary dressings in chronic injuries. One of the biggest problems encountered in the use of such dressings is that traditional dressings adhere to the wound and give rise to trauma during the removal of the wound surface (White et al., 2012).

1.2.2 **Interactive Wound Dressings**

In recent years, it has become important to develop functional dressings that can protect the wound area from dehydration, support healing (interactive), and protect the wound against infections instead of dry and passive dressings ((Dhivya et al., 2015; Dreifke et al., 2015)). Apart from covering and protecting the wound area completely, an ideal dressing should also keep the wound area moist and help wound

healing (Andreu et al., 2015; Vowden & Vowden, 2017) Therefore, interactive dressing has been frequently studied in recent years in forms such as hydrocolloid, hydrogel, semipermeable film, and foam as shown in Table 1.1 since it interacts with wound bed components (Dealey et al., 2012; Dhivya et al., 2015; Mustafa Mir et al., 2018).

1.2.2.1 Foam Dressings

Wound dressings in the form of foam, which have been used since 1979, are mostly preferred in the clinic because of their high liquid absorption and permeability properties against moisture vapor, they provide thermal insulation in the wound area, do not stick to the wound and can be applied easily (Dhivyaa et al., 2015; Mir et al., 2018). However, this type of dressing has disadvantages such as frequent replacement, the need for a second protective layer in practice, and its use for dry wounds without exudate (Dhivyaa et al., 2015; Ilenghoven et al., 2017). Effectively, they are preferred for the prevention of pressure ulcers. On the other hand, there is no strong evidence that declares foam dressing is more efficient than the other dressing types (Davies, 2016; Dumville et al., 2013a).

Table 1. 1. List of interactive wound dressing types and commercial wound dressing products

Type of dressing	**Advantages**	**Disadvantages**	**Commercial Products**	**Reference**
Semi-Permeable Films	Moist environment Semi-permeable to gas and water The inhibiting entry of bacteria No need for a secondary dressing Water repellent Elastic and durable	The adhesive feature can cause damage during the removal Being non-absorbent Insufficient for removing exudate	Tegaderm Blister Poly skin II Silon-TSR	Arroyo et al., 2015
Hydrocolloid Dressings	Moist environment Exudate absorbing Nonpermeable to gas and water	Not suitable for infected and heavily exudating wounds Odour formation at the wound site	Iodosorb (Cadexomer) Debrisan (Dextranomer)	Martin et al., 2010; Skórkowska-Telichowska et al., 2013; Brölmann et al., 2013; Chaby et al., 2007

Table 1. 1. (cont) List of interactive wound dressing types and commercial wound dressing products

Foam Dressings	Moist environment High absorption Easy to remove The ability of loading with agent/ antimicrobial	Not proper for low exudate wounds Need often change for heavy exudate wound Maceration formation	Flexzan Biopatch Crafoams Biatain Cutinova Reston Lyofoam Ivalon	Santamaria et al., 2015; Song et al., 2017; Powers et al., 2016
Composite Dressings	Moist environment Non-adhesive Easy to remove	Adhesive borders are not suitable for every type of skin Not sufficient to create a humid environment	Gentell Comfortell DermaDress Island Dressing	Joshi et al., 2019 Baranoski, 2008 *WOUND & SKIN CARE: Choosing a Wound Dressing, Part 1 | Article | NursingCenter*, n.d.

Table 1. 1. (cont) List of interactive wound dressing types and commercial wound dressing products

Hydrogel Dressing	Easy to remove (non-adherent) Injectable In situ crosslinking Responding to stimuli Moist retention	Mechanical features can decline in the swollen state Maceration can be observed Need for a secondary dressing	Cultinova Gel Biolex TegaGel, Carrasyn	Gupta et al., 2016 Powers et al., 2016; Tyeb et al., 2018; Cirillo et al., 2017

Generally, foam dressings are produced using highly absorbent polyurethane. However, the other kind of foam can be hydrofiber, which becomes a gel when contacts in a moist environment (Wasiak et al., 2013). Non-adhesive foam dressings are applied with gauze and tape. Some foam dressings include antibacterial agents such as silver and silver sulphadiazine (SSD) (Wasiak et al., 2013). Foam dressings containing the antibacterial agent are commercially available. Commercial foam dressings are IodoFoam (iodine), Acticoat (Ag), Algidex (Ag, alginate, maltodextrin), PolyMem silver (Ag), Optifoam (Ag), HydraFoam (Ag), Kendall AMD antimicrobial foam (PHMB) (Chaganti et al., 2019).

In a study polyurethane (PU) foam wound dressings which were covered with poly diallyl-dimethylammonium chloride, were produced by Tran et al.,(2015) to prevent bacterial growth especially, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii* for both acute and chronic wounds. The results showed that this kind of foam could prevent the migration of these three types of bacteria in wound dressing and biofilm formation. Namviriyachote et al. (2019) produced polyurethane foam dressings using natural polyols to enhance the properties of the

foam and the release of microbial agents such as silver and asiaticoside. After the coaction of polyols and diisocyanate, hydroxypropyl methylcellulose, chitosan, and sodium alginate were combined with polyols and polypropylene glycol. The content of the natural polyols didn't affect significantly the properties of foam in terms of the pore size, water sorption-desorption, and compression strength, however, the main impression was observed in the release of both active components. In natural polyols formulations, foam sheets including alginate released the highest silver and asiaticoside. Studies on fibroblasts cells also confirmed that the foam was not toxic. In an *in vivo* study (pig), foam dressing composed of 6% alginate, 1 mg/cm^2 silver, and 5% asiaticoside enhanced wound healing in terms of percentage of the wound closure without any dermatologic reactions (Namviriyachote, Lipipun, Akkhawattanangkul, Charoonrut, & Ritthidej, 2019). In another study, kaolin incorporated PU foam was produced by a one-pot synthesis method (Lundin et al., 2017). Kaolin was scattered throughout the PU matrix, however, higher loadings caused the minor aggregation. Kaolin-PU composites released ciprofloxacin over 2 h, and the initial release rates enhanced with the high amount of kaolin content. Kaolin (5 and 10wt%)/ PU foams increased the hemostatic capability of the dressings. Kaolin loading foams showed the hemostatic property at low loading levels (5 wt%). Besides, kaolin didn't affect water absorption.

1.2.2.2 Film Dressings

Film dressings, which include adherent and transparent polymer and permit O_2 and CO_2 exchange, are very elastic and flexible. This transparent feature allows the observation of a wound without removing the dressing (Felgueiras & Amorim, 2017). Due to the transparency of the film dressings, wound closure is expectative without removal of the dressing. These kinds of dressings are preferred for superficial wound, epithelializing wound and shallow wound that has low exudates, the commercially available film dressings for mentioned wounds are Opsite™, Tegaderm™, Biooclusive™ respectively. Commercial film dressings can be

different in terms of their vapor permeability, adhesive characteristics (Dhivya et al., 2015). However, film dressings have some disadvantages such as limited ability to absorb a high amount of wound exudates, which can cause edema because of excessive fluids. This situation can damage skin cells and rise bacterial infection possibility (Miranda & Srinivasan, 2016). They are preferred as primary dressings in lacerations minor burns and as a postoperative layer for sutured wounds (Dabiri et al., 2016).

Hyaluronic acid grafted pullulan polymers (HA-g-Pu) containing various hyaluronic acid (HA) moieties degrees of substitution (DS) were produced and analyzed by FTIR, H-NMR, and differential scanning calorimetry measurement (DSC) (H. Li et al., 2018). HA-g-Pu polymers showed better degradation both in vitro and in vivo studies, based on different DS. The HA-g-Pu films were composed of small pores whose diameter is between 0 -100 μm that were detected by scanning electron microscopy (SEM). HA-g-Pu films had a higher swelling ratio compare to the pullulan/or HA films. HA-g-Pu films had higher absorption and stronger protection of the wound bed from the accruement of exudate. Furthermore, the biocompatibility of HA-g-Pu polymers was tested by cytotoxicity, skin irritation, hemolysis test, and cell proliferation. The HA-g-Pu films stimulated wound healing. Furthermore, the HA-g-Pu polymers showed coagulation and rapid hemostasis property that helps heal wounds. Other studies have been working on the antibacterial properties of HA-based films. For this purpose, a cornstarch/HA dressing, which is incorporated with ethanolic extract of propolis (EEP), due to a casting method, was produced (Eskandarinia et al., 2019). The results showed that the cornstarch/HA films with EEP can help the healing process with the prevention of bacteria on the wound surface. Similarly, HA/sodium alginate (SA) films, which were crosslinked with Ca^{2+}, Zn^{2+}, and Cu^{2+} cations, were produced to maintain homeostasis for wounds (Abou-Okeil et al., 2018). To enhance the antimicrobial activity, HA/SA films were loaded with silver nanoparticles and sulfadiazine. *In vivo* results, a higher reduction in the size of wound on treated rats was observed with a decreased amount of

produced inflammatory mediators and oxidative stress markers such as nitric oxide and malondialdehyde, respectively.

1.2.2.3 Hydrocolloid Dressings

Hydrocolloid dressing comprises hydrophilic gel that can be made of gelatin, pectin, and sodium carboxymethylcellulose on a film layer (Kane & Krasner, 1997). Alongside sheet form, hydrocolloids can be produced as paste or granules for the closure of the deep wound (Kane & Krasner, 1997). Hydrocolloid dressings can form gels upon exposure to exudate and they can absorb a high amount of exudate due to hydrophilic gel formation (Lanel et al., 1997). Hydrocolloids bring about autolytic debridement by creating a moist environment for the skin (A. M. Jones & San Miguel, 2006). Generally, amorphous hydrocolloids are chosen to treat pressure ulcers. For pressure ulcers, the use of hydrocolloid is more beneficial compared to gauze dressing in terms of peddling of the pressure ulcer dimension, absorption capacity, and the pain through dressing changes (Alm et al., 1989; Heyneman et al., 2008). However, hydrocolloid usage is not recommended on infected wounds. Additionally, the continuous use of hydrocolloids can cause hyper granulation (Swanson et al., 2014). Hydrocolloids can be applied for the treatment of chronic venous ulcers, burns, partial-thickness wounds (Dumville et al., 2013b).

Sodium carboxymethylcellulose hydrocolloid fiber material based on hydro-fiber dressings, produced by using Hydrofiber® technology, is used in the clinic for medium-heavy exudate wounds due to their ability to absorb exudate directly into their fibers and cause no tissue destruction when they are removed from the wound. Composite Hydrofiber dressings (AQUACEL®) containing silver, alginate, and maltodextrin are also preferred in the clinic because of their antimicrobial properties. Although there are many commercial examples like Duoderm® (ConvaTec), NuDerm® (Johnson & Johnson Medical), Comfeel® (Coloplast Sween, Inc), Hydrocol® (Dow Hickam), hydrofiber dressings have limited clinical use due to their negative properties, such as being non-sticky, needing a secondary dressing to

be fixed on the wound and not being suitable for third-degree burns and severe bleeding wounds (Dhivyaa et al., 2015).

Different research groups are working on hydrocolloid dressings. Thu et al. (2012) produced alginate hydrocolloid film wound dressing that includes an upper layer embrued with the drug (ibuprofen) and non-drug containing the lower layer and film properties such as tensile strength, stiffness, swelling, water vapor permeability, and SEM analysis were examined. The results showed that the bilayer structure had stronger mechanical and rheological features compared to the single layer. This kind of structure caused a higher healing rate *in vivo* (albino rats), compared to the control (Thu et al., 2012a). In another study, hydrocolloid wound dressings composed of different hydrophilic polymers were compared in terms of swelling, bioadhesion, and mechanical strength (Jin et al., 2016). Hydrocolloid dressings were produced by a hot melting method using blends of the styrene-isoprene-styrene copolymer and polyisobutylene (PIB) with six different polymers such as sodium alginate, sodium carboxymethyl cellulose hydroxypropyl methylcellulose, polyvinyl alcohol, poly (vinyl pyrrolidone), and poloxamer (Jin et al., 2016). Karayehesive® is a hydrocolloid dressing that was analyzed for surgical wounds. It was observed that Karayahesive® made the healing process more comfortable for patients. Moreover, due to its cost-effectiveness, it is considered a better option than gauze dressing for neurosurgical wounds (Fujimoto et al., 2008). Hydrocolloid films composed of alginate were investigated as a slow-release wound healing vehicle (Thu et al., 2012b). In another study, hydrocolloids consisted of an upper layer that included ibuprofen as a drug and a lower layer, which had a release rate-controlling membrane. Features of the hydrocolloids such as solvent loss, morphology, rheology, mechanical properties, in vitro drug release were analyzed. The bilayer structure of hydrocolloid films showed a low moisture vapor transmission rate and higher healing rate in vitro with faster granulation compared to the control group. Hydrocolloid dressing has adhesion loss problems which decrease therapeutic efficiency. For this reason, the adhesion loss mechanism of hydrocolloids was studied due to sodium carboxymethyl cellulose-filled hydrocolloid dressings

exposing to the physiological environment (D. Kong et al., 2020). The group studied the surface energy of the dressing and tried to understand and associate the adhesion mechanism and contact angle tests. The effused CMC caused a significant increase in surface energy and adhesion loss. This study helped to understand the reason for the loss of adhesion property in hydrocolloid wound dressings and can assist to produce hydrocolloid dressings that might have moisture control and sustained self-adhesiveness.

1.2.2.4 Hydrogel Dressings

Hydrogel dressings are one of the most promising materials in wound care due to their properties such as absorbing large amounts of wound exudate, keeping the wound moist, completely closing the sensitive wound tissue, reducing the pain by cooling, the potential of active intervention during the wound healing period (Koehler et al., 2018). Hydrogel dressings can control scar formation and allow cell proliferation for recovery (Kamoun et al., 2017). Moreover, the inclusion of antimicrobial agents, wound healing bioactive agents, or cells into the hydrogel structure increases the potential of hydrogel dressings in clinical use. They can be easily applied to the wound by injection (Gupta et al., 2019).

As an antibacterial agent, octenidine incorporation into bacterial cellulose hydrogel resulted in the inhibitory effect against *S. aureus* (Moritz et al., 2014). Wound dressing containing octenidine was stable, releasing the biologically active agent for 6 months. It was shown that thickness, water content, and the surface area to volume ratio affected the release property. Moreover, bacterial cellulose hydrogel including tetracycline hydrochloride showed the antimicrobial effect on *E. coli, S. aureus*, and *C. albican* (Shao et al., 2016). The antimicrobial activity of bacterial cellulose hydrogel, which is containing zinc oxide nanoparticles has bees testes in burn BALBc mice model and the result proved that the hydrogels had antimicrobial activity against *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus*,

and *Citrobacter freundii* bacteria with healing activity (Khalid et al., 2017). The agents which are used in wound dressing were shown in Table 1.2.

Table 1. 2 List of agents that are used for wound dressing types

Form of dressing	**Agent**	**Activity of agent**	**Application**	**References**
Hydrogel	Octenidine	Anti-microbial	Drug Delivery	Moritz et al., 2014
Hydrogel	Tetracycline hydrochloride	Bactericidal	Wound Healing	H. Chen et al., 2017
Hydrogel	Tigecycline	Antibacterial	Treating infected chronic wounds	Nimal et al., 2016
Hydrogel	Vancomycin, Gentamicin	Antibacterial	Treating burn wounds	Nuutila et al., 2020
Film	Ceptazidime	Anti-microbial	Wound healing	Jana et al., 2016
Film	Vancomycin	Anti-microbial	Wound healing	M. Li et al., 2019
Composite	Zinc oxide (ZnO) nanoparticles	Anti-microbial	Wound healing for burn patients	Khalid et al., 2017

1.2.2.5 Composite Dressings

Composite dressings are produced by the association of distinct components into a single dressing that brings in features such as bacterial barrier, absorption, being semi-adherent, or non-adherent (Joshi et al., 2019). They can be used as both primary and secondary dressing for wound closure (S. Sharma et al., 2014). Generally, composites have three layers. The outer layer of the dressing has a role in the frustration of the infection. While the middle layer, which includes absorptive materials, keeps the wound moist and helps autolytic debridement, the bottom layer that is made of non-adhesive material prevents the wound from sticking to the wound. Since each layer has a different function, composite dressings can create a proper environment for healing the wound (G. Schoukens, 2009). Composite dressings are classified as an island and layered composite dressing. While island dressings include an absorbent layer located between the semi-permeable backing sheets, layered composite composed of multiple layers that are stacked over each other (Joshi et al., 2019). Sodium alginate/poly(vinyl alcohol)/nano ZnO composite nanofibrous dressings were produced by the electrospinning technique (Shalumon et al., 2011). The antibacterial effect of the composite dressing on *S. aureus* and *E.coli* was observed at high ZnO (0.5–5%) content. L929 cells proliferated on 1% ZnO containing dressing showing the cytocompatibility of the dressing. Besides, for wound healing applications, chitin/nano silver composite scaffolds showed a bactericidal effect for both *S. aureus* and *E. coli* (Madhumathi et al., 2010). The hemostatic activity of the chitin scaffold was improved by silver, which resulted in better blood clotting activity. However, this composite dressing showed a toxic effect on fibroblasts *in vitro*. Davis & Balasubramanian et. al (2016) produced Azadirachta indica (as an antibiotic agent) containing the chitosan matrix system by using solvent casting. This hybrid composite had an inhibitory effect against gram-positive *S.aureus* and gram-negative *K. pneumoniae* (Davis & Balasubramanian, 2016). In another study, nano-Ag/ZnO-loaded chitosan composite dressing was produced by lyophilization technique, and Ag/ZnO nanocomposites were loaded into chitosan

sponge (Lu et al., 2017). The features such as porosity, swelling, blood clotting, and drug-resistant pathogenic bacteria were studied. The novel composite dressing had high porosity and swelling accompanied by improved blood clotting and antibacterial feature. Moreover, in vivo studies (mice) showed that the chitosan-Ag/ZnO composite dressing not only assisted the wound healing but also triggered re-epithelialization and collagen deposition.

1.2.3 Bioactive Dressings

Another type of dressing used in modern dressings is bioactive dressings that have wound healing activity and/or can act as a support for the release of bioactive compounds such as antimicrobial agents, growth factors. Bioactive dressings can be composed of materials that have an endogenous activity such as hydrocolloids, alginates, collagens, and chitin as shown in Table 1.3 (Schoukens, 2009).

Alginate® (Smith & Nephew), Kaltostat® (ConvaTec), Tega gen® HG or HI (3M Health Care), Comfeel SeaSorb® (Coloplast AS), AlgiDERM (Bard), Algosteril® (Johnson & Johnson). Furthermore, Comfeel Contour® (Colo -plast AS), Granufl ex® (C o nvaTe c), Tega s orb® (3M Health Care), and Duoderm® (C onvaTec) are commercial alginate hydrocolloid dressings.

Important features of these types of dressings are that they can absorb wound exudate, create a moist environment for the wound, and prevent scar formation (Schoukens, 2019). Moreover, a significant feature of bioactive dressings is that they neutralize the wound alkalinity and enable the skin to regain its natural pH (Schoukens, 2019). This neutralization step needs an acidic carboxylic group or the production of acidic compounds having to the hydrolysis of the dressing (Schoukens, 2019). Some commercial bioactive dressings can be loaded with bioactive materials such as calcium, zinc, and silver (Rajendran, 2018). In literature, bioactive compounds-loaded chitosan film was investigated as a wound dressing material to be used for diabetic patients (Colobatiu et al., 2019). Both antioxidant effect and

effect on cell viability of the films were studied *in vivo* in a streptozotocin-induced diabetic rat model. Results of this study showed that chitosan films had a proliferative effect with antioxidant property and biocompatibility. Moreover, chitosan film enhanced the wound healing process. In another study, bioactive wound dressing including chitosan and *Leptospermum scoparium* honey, known as Manuka Honey was investigated (Rathinamoorthy & Sasikala, 2019). The novel wound dressing containing honey as a drug was produced with the solvent-casting method. The wound healing ability of dressing was tested in three different wound types, which are excision, incision, and burn wound model. After application, the contraction percentage was determined with histopathological examinations. Complete healing was recorded on the 18^{th} and 21^{st} for excision /incision and burn wounds, respectively. The wound contraction percentage of the chitosan honey dressing in the excision wound model was better than the commercial dressing. Moreover, for the incision wound model, the tensile strength of healing skin to which the developed dressing was applied was higher than the commercial dressing.

1.3 Polymers Used in Hydrogel Wound Dressings

Dressing materials can be a natural and synthetic polymer or their combination as film, sponges, and hydrogels (Moura et al., 2013). The natural polymer has aroused interest in biomedical applications thanks to its biocompatible and environmentally friendly features (Bai et al., 2017). Polysaccharides are natural polymers that include monosaccharides and their derivatives (Jain et al., 2007). Because of the chemical similarities of polysaccharides to heparin, they have low cost and hemocompatibility (Malafaya et al., 2007). Dressings that are made up of natural polymer contain metals, antibiotics, proteins to accelerate the healing process. While there are limited studies on chitin, pullulan, and starch as wound dressing material, many studies have focused on bacterial cellulose and chitosan (Naseri-Nosar & Ziora, 2018). The reason for this can be that chitosan has antimicrobial activity and proliferative effects on fibroblast cells (Paul & Sharma, 2004). Also, proteins are natural polymer. Many collagen dressings are combined with gels, polymers. The collagen dressings that may include alginate and cellulose can increase absorbency, flexibility, and ability to create a moist environment (Brett, 2008).

Table 1. 3 Types of bioactive dressings (Gustaaf Schoukens, 2009)

	Advantage	**Commercial Product Examples**	**References**
Alginates	• Biocompatibility • Non-toxicity • Moist environment • Reduced pain feel • Highly absorbent • Need a secondary dressing • Acts as a hemostat	Algisite® (Smith&Nephew), Kaltostat® (ConvaTec), Tegagen® HG or HI (3M Health Care), Comfeel SeaSorb® (Coloplast AS) AlgiDERM (Bard)	Gustaaf Schoukens, 2009; Vowden & Vowden, 2017
Hydrocolloids	• Exchange of water vapor • Moist environment • No need for frequently dressing change	Comfeel Contour® (Colo - plast AS), Granufl ex® (C o nvaTe c), Tegasorb® (3M Health Care) Duoderm® (ConvaTec)	Gustaaf Schoukens, 2009; Schaffner, 1997

Table 1. 3 (cont) Types of bioactive dressings (Gustaaf Schoukens, 2009)

Hydrogel	• Moist wound environment • Suitable for wounds with minimal to moderate exudate • Exchange of gaseous CO_2, O_2, and H_2O	Granugel® (ConvaTec), Intrasite Gel® (Smith & Nephew), Nu-Gel® (Johnson & Johnson).	Rajendran, 2009; Vowden & Vowden, 2017; Koehler et al., 2018
Collagen	• Low inflammation • Biocompatible • Hemostasis • Provides strength/integrity	BioBrane® (Smith&Nephew) OrCell® (Ortec International Inc.)	Chattopadhyay & Raines, 2014; Gustaaf Schoukens, 2009; Supp & Boyce, 2005; Ehrenreich& Ruszczak, 2006; Lockhart et al., 2001
Honey Dressing	• Rapid healing • Neutralize pH wound • Antimicrobial property	Apinate (Derma Sciences)	Gethin et al., 2008

Table 1.3 (cont.) Types of bioactive dressings (Gustaaf Schoukens, 2009)

	Advantage	**Commercial Product Examples**	**References**
Chitin/Chitosan Derivatives	• High protein content • Low immunogenic • High nitrogen percent (chelating agent) • Biocompatibility, • Biodegradability • Non-toxicity	Beschitin® W	Muzzarelli, 1977; Azuma et al., 2015

Some studies combine polysaccharides with proteins to produce wound dressing. For instance, psyllium seed husk polysaccharide and human hair keratins hydrogels were produced by sodium tri-metaphosphate crosslinking, then morin was loaded onto the hydrogels (Ponrasu et al., 2018). Independently of the presence of morin, the hydrogels showed high (%80) porosity with good swelling capacity. In vivo diabetic wound healing studies showed that psyllium seed husk/keratin/morin hydrogel reduced the time of reepithelization and increased the wound contraction rate by increasing collagen synthesis in diabetic rats. In another research, silk fibroin and konjac glucomannan sponges were crosslinked to produce protein/polysaccharide sponges that have regulatable mechanical features for dressing applications (Feng et al., 2019). The pore structure of sponges depended on the blend ratio of silk fibroin and konjac glucomannan. An increase in konjac glucomannan improved water-absorption, water-retention abilities with compression strength. Furthermore, the

sponges showed a similar compressive modulus with the skin tissue. Also, sponges had great biocompatibility.

The most preferred polymers for hydrogel wound dressings are presented in Table 1.4.

Table 1. 4 List of natural and synthetic polymers used in hydrogel wound dressings

	Properties	**Commercial Product Examples**	**References**
Poly(vinyl alcohol)	• Biocompatibility • Non-toxic • Exudate absorbance	Not available	Kenawy et al., 2014; Kamoun et al., 2015; Kamoun et al., 2015
Chitosan	• Hemostasis • Bacteriostasis • Biocompatibility • Biodegradability	Tegasorb®, Tegaderm®, KytoCel® (MasterCare Medical GmbH)	Hu et al., 2018
Gelatin	• Low antigenicity	Gelfoam Surgifoam	Mishra et al., 2011
Alginate	• Enhanced wound healing	AlgiSite AlgiDerm Sorbsan Pharma-Algi®, Pharmaplast	Thomas et al., 2000 Abbasipour et al., 2014 Varaprasad et al., 2020

Table 1. 4 (cont) List of natural and synthetic polymers used in hydrogel wound dressings

Hyaluronic Acid	• Role in angiogenesis	Bionect® Hyalofill® Hyalomatrix®	Ho et al., 2016 Vasir et al., 2003 Colletta et al., 2003 Longinotti, 2014
Dextran	• Accelerated endothelial cells recruitment • Soft structure • Non-toxic • Biodegradability • Biocompatibility	Not available	G. et al., 2011 Van Tomme & Hennink, 2007
Polyethylene glycol	• Nontoxic • Hydrophilic • Biocompatible • Not immunogenic	Not available	Yesilirmak et al., 2015 Mariam Mir et al., 2018
Bacterial Cellulose	• High water content • Biocompatibility • Flexible • High Strength	Not available	Czaja et al., 2006 Lin et al., 2013 French, 2014

1.3.1 Synthetic Polymers

1.3.1.1 Polyurethane

Polyurethanes, which are comprised of repetitive urethane groups formed by conjugation between diol and diisocyanate groups due to polymerization, are synthetic polymers, as shown in Fig 1.2 (Wright et al., 1998). The reason for the use of polyurethane in medical applications is its strength and biocompatibility. Polyurethanes that are used in biomedical are non-toxic and elastomeric accompanied by good toughness and abrasion resistance. In wound healing, they assist epithelization. Furthermore, they have both barrier property and oxygen permeability (Francesko et al., 2015).

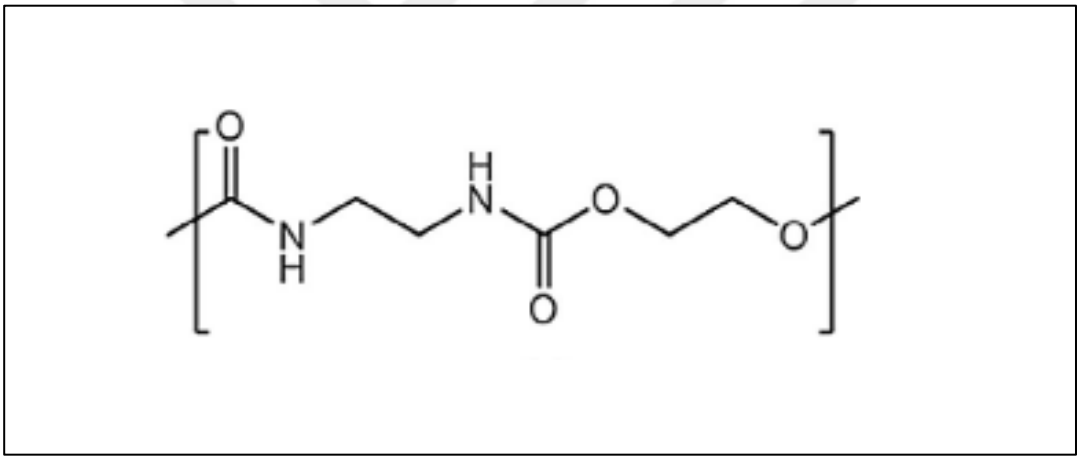

Figure 1. 2 Chemical structure of polyurethane (Kamoun et al., 2017)

In a previous study, polyurethane (PU) foam wound dressing was produced with the salt leaching/solvent casting method and was combined with propolis which is an anti-bacterial agent (Khodabakhshi et al., 2019). The PU foam wound dressing showed significant improvement in cytocompatibility and *in vivo* wound healing. In another research, polyurethane-hyaluronic acid nanofibrous wound dressing was produced and analyzed with different amounts of ethanolic extract of propolis (Eskandarinia et al., 2020). The result showed that wound dressing had higher antibacterial activity against *S. aureus* and *E. coli* with accelerated healing of the

Wistar rats' skin excisions. Park and Nho (2003) used a new method to prepare PVA-PVP-glycerin-chitosan hydrogel membranes coated with polyurethane to be used in wound dressing applications that involved γ-irradiation or F-T cycles crosslinking and then irradiation. Membranes that were produced with the F-T cycle followed with irradiation had a higher cross-linked structure as regard to the irradiation process only. Polyurethane wound dressings are commercially available as films (Blisterfilm (The Kendall Co), Omniderm (Omikron Scientific Ltd), Proclude (ConvaTec)), as foams (Flexzan ® (Dow Hickam), Hydrasorb ® (Tyco/Kendall Co), Lyofoam ® (ConvaTec)) and as hydrocolloids(Duoderm ® (ConvaTec), NuDerm ® (Johnson & Johnson Medical), Comfeel (Coloplast Sween, Inc)). (Homaeigohar & Boccaccini, 2020).

1.3.1.2 Poly (Vinyl Alcohol)

Poly (vinyl alcohol) (PVA) is the oldest synthetic polymer that is preferred for wound dressings, drug delivery systems, and wound management (J. K. Li et al., 1998). It has a structure that is made up of a pendant hydroxyl group as shown in Fig 1.3. Its structure is formed by the polymerization of vinyl acetate to polyvinyl acetate, which is pursued by hydrolysis of polyvinyl acetate (Hassan & Peppas, 2000). It has good biocompatibility, biodegradability, hydrophilicity, and mechanical strength because of the existence of cross-linking hydrogen bonds (Syed et al., 2018). On the other hand, PVA hydrogel has some disadvantages such as inadequate elasticity, stiff membrane (Kamoun et al., 2017).

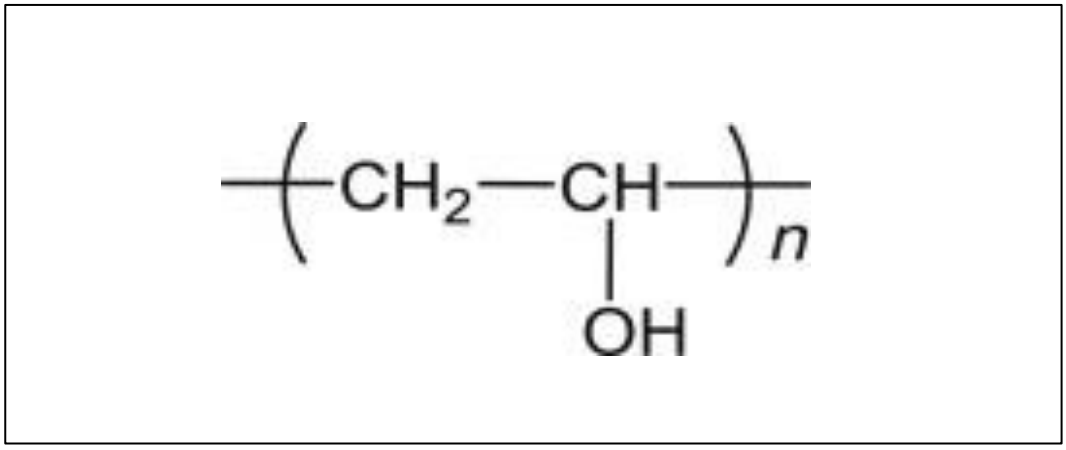

Figure 1. 3 Chemical structure of poly (vinyl alcohol, PVA) (Brady et al., 2017)

In a previous study, PVA-chitosan dressing membranes are produced by gamma radiation (El Salmawi, 2007). It showed that the increase of PVA concentration improves mechanical properties and microbe penetration. However, high chitosan concentration increases antimicrobial activity and swelling capacity. There are studies about PVA-including wound dressings in the literature (Kamoun et al., 2017). For example, PVA-glucan membranes were produced by the physical blending method with drying at 110°C (Huang & Yang, 2008). The results of this study showed that the wound area was recovered fast thanks to glucan release which has anti-inflammatory properties. Glucan release can be caused by the absence of the covalent bond between PVA and glucan. Furthermore, PVA-bentonite, PVA-Ag, and PVA-clove extract membranes were produced via the F-T cycles method (Gonzalez et al., 2012). In the presence of bentonite and silver nanoparticles, the hydrogels showed enhanced water-vapor transmission rate, antibacterial activity against *E. coli*, and swelling capacity.

1.3.1.3 Polyethylene Glycol (PEG)

Polyethylene glycol (PEG) formed by repeating units of ethylene glycol is a water-soluble synthetic polymer as illustrated in Fig 1.4 (Milton Harris & Chess, 2003). Its biological features such as nontoxicity, biocompatibility/biodegradability, transparency make it proper for the use of several medical applications (Syed et al., 2018).

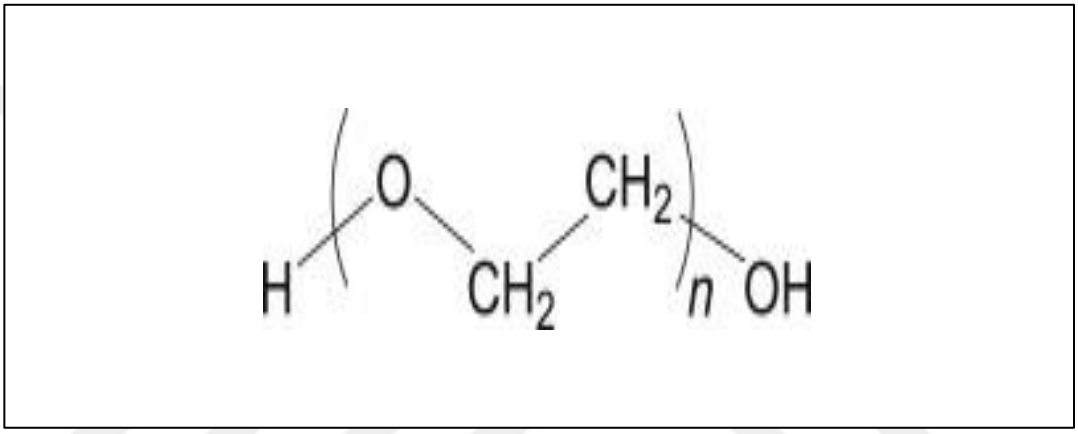

Figure 1. 4 Chemical structure of polyethylene glycol (PEG) (Brady et al., 2017)

Also, the Food and Drug Administration has approved the use of polyethylene glycols in biomedical applications (Syed et al., 2018). In literature, the PVA-PEG-$CaCl_2$ hydrogel is produced by irradiation crosslinking (Dutta, 2012). The use of $CaCl_2$ solution as a plasticizer and a gelling material to increase the synergistic effect of wound dressing and PEG is used to increase thermal stability. Results showed that this kind of dressing doesn't have an inhibitory effect on cell proliferation, and PEG helps wound healing rate. Moreover, the bioactive gold nanoparticles loaded PEG/PCL copolymer matrix was produced for wound healing (F. Wang et al., 2018). Au-PEG/PCL nanocomposites showed inhibitory effects, which makes them a possible treatment to inhibit bacterial infection. This kind of antibacterial nanocomposite dressing can be useful to assist wound healing due to its therapeutic effect. In another study that used PEG, they synthesized asiaticoside loaded

polyvinyl alcohol/polyethylene glycol hydrogels via the freeze-thaw method for drug delivery (Ahmed et al., 2018). Results claimed that there was no microbial growth on hydrogels and produced wound dressings that were swellable, elastic, and safe.

1.3.2 Natural Polymers

1.3.2.1 Protein

1.3.2.1.1 Collagen

Collagen is the protein that is procured by fibroblast in the human body. It composes of repeating amino acid sequences of Gly-X-Y (X and Y are mainly proline and hydroxyproline amino acids) as shown in Fig 1.5 (Gaspar-Pintiliescu et al., 2019).

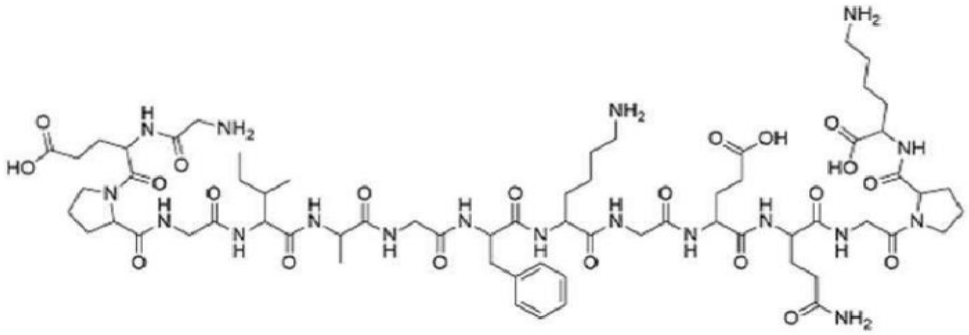

Figure 1. 5 Chemical structure of collagen (Houacine et al., 2019)

Collagen participates in wound healing phases and it helps new tissue formation by activating cellular migration. Collagen-based wound dressings stimulate immune cells such as macrophages (El Masry et al., 2019). They can be easily applied and removed. Their source can be bovine, porcine, and avian (Fleck & Simman, 2010). Due to its hemostatic property, biocompatibility, and stimulating effect on cellular growth and attachment, collagen is used in wound dressings (Gaspar-Pintiliescu et

al., 2019). Collagen fibers, which are found in the collagen-based dressings, can be degraded by metalloproteinases in the wound exudates, which makes it proper for wound healing (Brett, 2008). Collagen, when it is used with thymol, shows antibacterial features except for *P. aeruginosa* by inhibiting biofilm formation (Michalska-Sionkowska et al., 2017). Furthermore, it helps the healing of burn wounds (Oryan et al., 2018). Collagen is commercially available: Integra ®, Matriderm ®, Orcel ®, Apligraf ® (Gottleib & Furman, 2004; Min et al., 2014; Still et al., 2003; Zaulyanov & Kirsner, 2007). These commercial products have some advantages and disadvantages. As an example, although Integra is strong and flexible, it can't be used for infected wounds (Gottleib & Furman, 2004). Also, Matriderms is flexible but highly cost (Min et al., 2014). Finally, Orcel is resorbable, however, it can be used for infected wounds and it is highly cost (Still et al., 2003).

Collagen wound dressing can be formed in the type of sponge, aerogel, and film. Collagen sponges can be manufactured by using the freeze-drying method and these kinds of wound dressing can show antibacterial activity against *S. Aureus and P.aeruginosa* and stimulate wound healing due to the increase of hydroxyproline content (M. S. Kumar et al., 2010). Collagen aerogels which are produced by UV-light crosslinking followed by salt leaching and freeze-drying showed that antimicrobial activity against *B. subtilis* and *E.coli* and stimulation of wound healing (Govindarajan et al., 2017). Furthermore, collagen films such as Thymol, are produced by the casting method, which has antioxidant activity and antibacterial properties against *S. aureus, B. subtilis, E. coli,* and *P. aeruginosa* (Riella et al., 2012). Collagen dressings which are loaded with a polyphenolic extract from *Artemisia absinthium* stimulated not only proliferation but also attachment of fibroblasts and keratinocytes, also it triggers the synthesis of extracellular matrix component (Gaspar-Pintiliescu et al., 2018). *Hamamelis virginiana* polyphenols containing collagen wound dressings caused inhibitory effects on chronic wound enzymes like myeloperoxidase and collagenase (Antonio et al., 2011). Although collagen is biocompatible, it may cause some antigenic/immunogenic reactions in the body because of the presence of N-and C-terminal telopeptides (Holmes et al.,

2017). Previous studies showed that collagen-included wound dressing may have disadvantages such as requiring secondary dressing, low elasticity, and not being proper for infected wounds (Donaghue et al., 1998; Kalin et al., 2015; Still et al., 2003). Collagen scaffolds for skin substitutes can not be proper for infected wounds and can be highly costly (Gottleib et al., 2004; Min et al., 2014).

1.3.2.1.2 Gelatin

Gelatin is a collagen-derived protein. Like collagen, it includes repeating amino acid sequences of Gly-X-Y, which X and Y represent mostly proline and hydroxyproline as presented in Fig 1.6 (Gaspar-Pintiliescu et al., 2019).

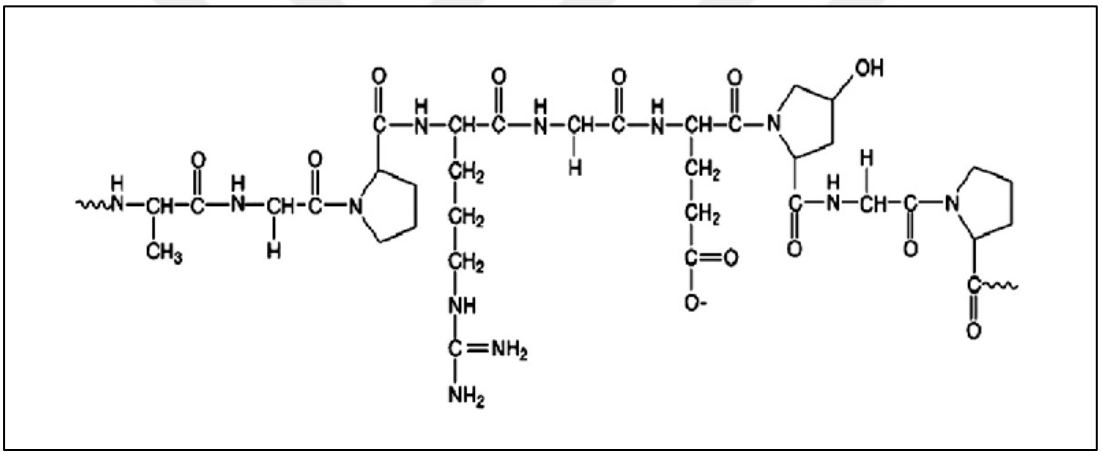

Figure 1. 6 Chemical structure of gelatin (Kommareddy et al., 2007)

It is not only inexpensive but also less antigenic than collagen because of denaturation (Malafaya et al., 2007; Mogoşanu & Grumezescu, 2014). Thanks to its biocompatibility and biodegradability, it is accepted as Generally Recognized As Safe (GRAS) by the Food and Drug Administration (FDA) (Elzoghby, 2013). Gelatin can easily form hydrogel and film. The arginine-glycine-aspartic motif found in gelatin promotes cell adhesion (Eisenbud et al., 2003). Moreover, the structure of the gelatin includes flexible amino acids that have free functional groups that permit chemical conjugation or modification to occur (Eisenbud et al., 2003)003). Hydrogels of gelatin are widely used due to their advantages. Firstly, gelatin can

absorb a large volume of water or can help with wound hydration. Also, it can cool the wound area, which decreases the pain feel with the feature of permeability to water vapor and oxygen (Eisenbud et al., 2003). For unusual shape wounds, it can easily be applied because of the gel-like structure (Eisenbud et al., 2003). To enhance the mechanical properties of gelatin with the reduction of the solubility or degradation rate, gelatin is crosslinked. The most commonly used cross-linker is aldehydes such as glutaraldehyde, which is toxic. Alternatively to the aldehydes, less toxic cross-linkers such as genipin, lactose, and citric acid have gained importance (Etxabide et al., 2018; Garcia-Orue et al., 2019; Lien et al., 2008). Silk fibroin/gelatin electrospun nanofibrous dressing incorporated with astragaloside IV was produced for deep partial-thickness burn wound due to electrospinning nanotechnology (Shan et al., 2015). *In vitro* studies, the AS-loaded silk fibroin/gelatin nanofibrous dressing showed that stimulation of cell adhesion and proliferation improved wound healing and wound closure. Besides, increased angiogenesis and collagen organizations were observed. Bioactive gelatin-oxidized starch nanofibers including Lawsonia Inermis (known as henna), which was produced for the treatment of second-burn wounds, improved fibroblast attachment, proliferation, and collagen secretion, and also showed the antibacterial property. *In vivo* studies on BALB/c mice showed that the wound was closed with a low inflammatory response, but without detrimental suppurative effect in the burn wound (Hadisi et al., 2018). Microparticles that were composed of gelatin and collagen were improved by a water-in-oil emulsion/cross-linking (Jiménez et al., 2015). Then, these nanoparticles were combined with collagen suspension. Scaffolds obtained with lyophilization were loaded with C. officinalis extract and analyzed *in vitro*. Results showed scaffolds produced by microparticles had resistance to enzymatic degradation and low cytotoxicity.

There are commercially available gelatin sponge wound dressings such as Gelfoam, Surgifoam (Sabel & Stummer, 2005; Schonauer et al., 2004). Although they are both hemostatic and produced for bleeding wounds, while Gelfoam is nonelastic, Surgifom has a substrate that is convenient for bacterial growth (Sabel & Stummer, 2005; Schonauer et al., 2004).

1.3.2.1.3 Keratin

Keratin is the protein that comprises cysteine-rich amino acids and disulfide bridges as illustrated in Fig 1.7. The disulfide bridges with fibrous structures give mechanical strength (Hill et al., 2010). Keratin, which can be extracted from human hair, is preferred for biomedical application due to its autologous transplantation potential, biodegradability, and biocompatibility (Shavandi et al., 2017).

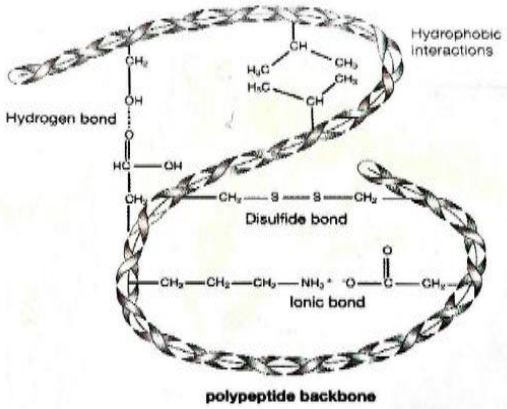

Figure 1. 7 Chemical structure of keratin (Feroz et al., 2020)

In literature, the structure of keratin was discussed as alpha and beta keratins (Spearman, 1967). While alpha keratin is the main component of the hair, nail, and horns, beta-keratin is the primary component for feather and reptilian claws (Fraser et al., 1972; Schweizer et al., 2006). In alpha keratins, the polypeptide changes arrange as alpha helices whereas, in beta keratins, keratins are in plate beta-sheets. Two polypeptides, which are twisted, form a coiled-coiled structure (L. N. Jones et al., 1997). The keratin is composed of different amino acids that act as a backbone including inter and intramolecular bonding. Disulfide, hydrogen, and ionic bonds make the keratin structure stable and strong (M. Park et al., 2013; Poole et al., 2011). Keratin is the structural protein of many animal species in the structure of hair, wool, feathers, nails, and horns. It is also abundant in the outer layer of human skin, hair, and nails. Due to its easy availability, biocompatibility, and lack of keratinase enzymes in mammals, slow degradation, the use of keratin has been increased as a

biomaterial (Sharma et al., 2017). Keratin-based biomaterials have been used in applications such as wound healing (Kelly, 2016) and bone (Tachibana et al., 2005), hemostat (Aboushwareb et al., 2009), and peripheral nerve repair (Apel et al., 2008; Sierpinski et al., 2008). Replicate ™, Keragel®, KeragelT®, and Keramatrix® are keratin-based dressings used in the clinic. Also, keratin-based hydrogels are more successful in controlling blood loss than commercial hemostatic agents (Aboushwareb et al., 2009). Keratin isolation from human hair is a better option in terms of producing biomaterials since it occurs in very light conditions compared to isolation from animal tissues (Lee et al., 2014). Also, due to its biocompatibility, biodegradability, and interaction with cells, keratin isolated from human hair is used in biomedical applications (Lee et al., 2014).

A novel antibacterial carboxymethyl cellulose-human hair keratin wound dressing was produced for topical clindamycin delivery (Sadeghi et al., 2020). The results showed that the increase of keratin content not only decreased the water vapor transmission rate but enhanced the water stability of the wound dressing. *In vitro* studies, hydrogels that contain higher keratin had a slower release of clindamycin. Also, hydrogel showed antibacterial features against *S. aureus* bacteria. In another study, three different nonwoven keratin-containing dressings were produced and characterized by SEM and FTIR, these were developed from chicken feather keratin, keratin-sodium alginate, and keratin-chitosan (Shanmugasundaram et al., 2018). Keratin-sodium alginate and keratin-chitosan dressings had an antibacterial effect against *Staphylococcus aureus* (gram-positive) and *Klebsiella pneumonia* and *Escherichia coli* (gram-negative). *In vivo* studies on (Albino Wistar rats) showed complete healing 15, 17, 21, and 23 days for keratin-chitosan, keratin-sodium alginate, feather keratin, and control respectively.

1.3.2.2 Polysaccharides

1.3.2.2.1 Alginate

Alginate is formed by the linear binary polymer of (1-4) linked beta-D-mannuronic acid (M block) and alpha-L-guluronic acid residues (G block). It includes three fractions, while two of them are composed of a homopolymer of guluronic and mannuronic acids, the third fraction includes equal portions of both guluronic and mannuronic acid as shown in Fig 1.8 (Steinbüchel & Rhee, 2005).

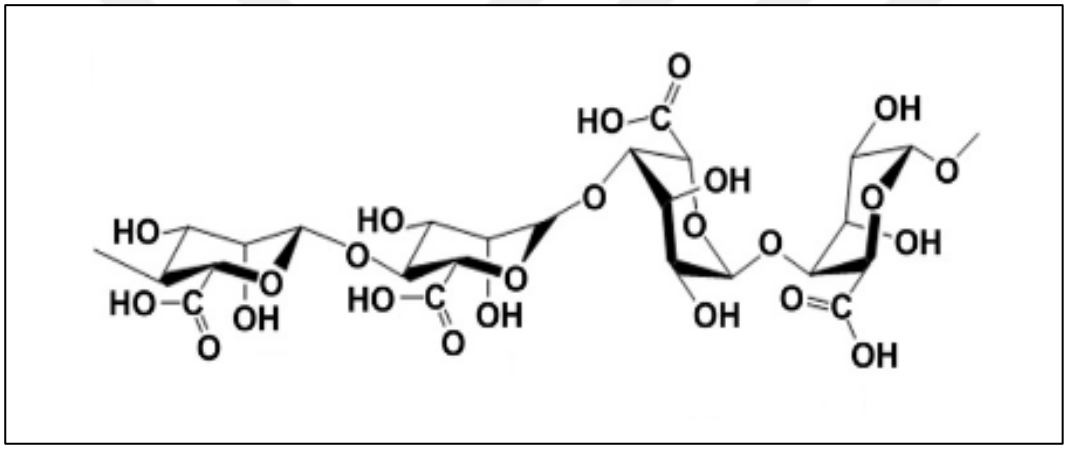

Figure 1. 8 Chemical structure of alginate (Pawar & Edgar, 2012)

The M/G ratio may affect the properties of alginate (Varaprasad et al., 2016). While alginate containing high M blocks can be useful for wound healing applications, because of its cytokine production (Del Gaudio et al., 2020). The quality of alginate can vary based on algal species. Furthermore, alginate is a hydrophilic polymer that is non-toxic, biocompatible, biodegradable, and biostable (K. Y. Lee & Mooney, 2012). Depending on the research, water-soluble alginate such as sodium alginate has important rheological properties, such as gelling, viscosifying, and stabilization (Torres et al., 2007). Due to electrostatic, ionic interaction, and redox reactions, alginate can bind to metal ions. As an example, alginate produces an egg-box-like structure with Ca^{+2} interaction (Goh et al., 2012). Because alginate can adjust not

only the structure of biomaterials but also antibiotics delivery from the wound dressing, it can be preferred for biomedical products, containing skin wound dressings to stimulate healing (Varaprasad et al., 2020). Wichai et al (2019) improved the wound dressings that are made of bacterial cellulose (BC), alginate (AG), chitosan (CS), and copper (II) sulfate. They claimed that BC/CS composite had lower cell viability compared to BC/AG and CS/AG. Moreover, alginate increased the cell viability of both BC, CS composites. BC/AG/CS-Cu (0.1) composite had better antibacterial activity compared to commercial products such as Acticoat®, As-kina®, and BluRibbon®. Chen et al. (2019) produced iodine/carboxymethyl chitosan/sodium alginate film including hydroxylate lecithin due to the microwave drying method. Whereas lecithins were used to increase the stability of iodine, iodine was used as an antibiotic. The film's wound repairing was observed due to a rat model for infected wounds. Kaygusuz et al.(2017) crosslinked alginate by cerium ion, in the place of calcium ion, which gave the dressing antibacterial properties (Kaygusuz et al., 2017).

There are commercially available alginate wound dressings such as Giderm (Bard); Algosteril (Johnson & Johnson Medical); Algisite (Smith & Nephew); Curasorb (The Kendall Company); Algisorb (Calgon-Vestal, St Louis, MO); Kaltostat (ConvaTec). However, their gel form may smell bad or because of their appearance, they can lead to the misunderstanding that the wound is purulent. They can need a secondary dressing (Leveriza-Oh et al., 2012).

1.3.2.2.2 Chitosan

Chitosan is a chitin derivative (β-(l-4)-2-amino-2-deoxy-D-glucopyranose). It is a polysaccharide with N-acetyl-glucosamine and N-glucosamine unit as shown in Fig 1.9. The degree of deacetylation (DDA), which is the number of N-glucosamine units, affects its physicochemical properties, biodegradability, and immunological activity (Tolaimate et al., 2000).

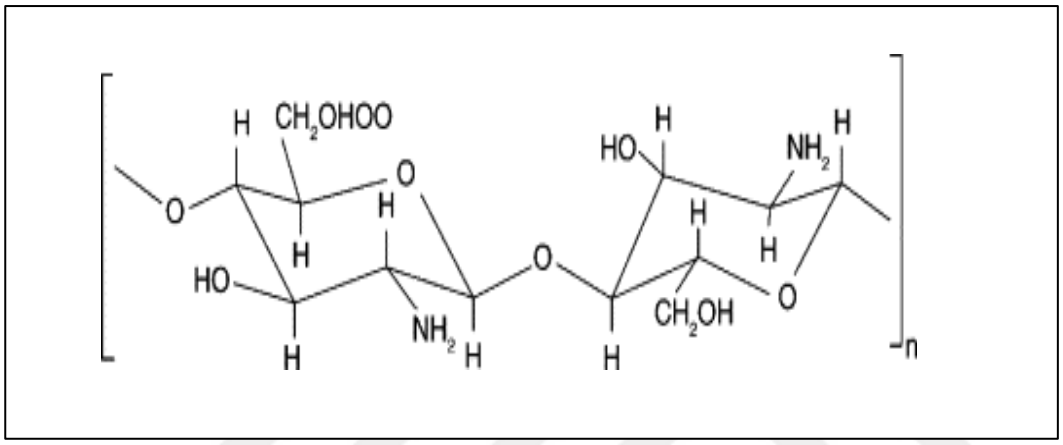

Figure 1. 9 Chemical structure of chitosan (Krasaekoopt & Bhandari, 2012)

Chitosan is not only biocompatible, hemocompatible, and biodegradable but also antimicrobial, which makes it proper for wound dressing applications (Pillai et al., 2009; Ueno et al., 2001). It can be produced as a sponge, hydrogel, etc. For wound dressing, hydrogel form is more suitable since it can protect the wound and facilitate the healing of the wound by creating a moist environment (Wang et al., 2012). Wang et al. (2012) reported that chitosan-gelatin-honey and gelatin hydrogels can be applied for burn wounds. They studied the effect of chitosan-gelatin (20wt%) blend hydrogels with varying honey content (less than %20) on burn wounds. It was observed that chitosan and honey had a synergistic effect due to their antimicrobial activity. Hydrogel composed of chitosan (0.5 wt%), honey (20 wt%), and gelatin (20 wt%) was the optimum choice in terms of the moist environment with antimicrobial activity. In another study chitosan, a gentamicin-loaded liposome immobilized nanofiber mesh was produced with antibacterial activity (Monteiro et al., 2015). Results showed that the mesh had antibacterial activity against *Escherichia coli,*

Pseudomonas aeruginosa, and *Staphylococcus aureus.* Furthermore, a minocycline-loaded PVA and chitosan wound dressing was produced with lyophilization (Sung et al., 2010). Chitosan decreased strength and thermal stability and it enhanced the water vapor transmission rate, porosity, and elasticity of the blend hydrogel. The minocycline-loaded dressings were more flexible and elastic because of the weak interaction between PVA and chitosan. They caused faster healing in rat dorsum comparing to the control which was sterile gauze thanks to antifungal activity.

There are commercially available chitosan wound dressings: Tegasorb ®, Tegaderm®, HemCon Bandage™, Chitodine®, Trauma DEX(Jayakumar et al., 2011).

1.3.2.2.3 Starch

Starch is made up of anhydroglucose units, which contain two subunits: amylose and amylopectin as seen in Fig 1.10 (Castro et al., 2005; Suortti et al., 1998). Although it is biocompatible, biodegradable, and non-toxic, its poor mechanical features limit the application of starch in wound dressing. This problem can be solved either by combining it with the other polymers or chemical modification of the starch to advance its features for biomedical applications (Pal et al., 2006). As an example, Aquaform® hydrogel dressing, composed of modified starch and glycerol, reduces the pain feel and inflammation. It helps complete the healing of the wound by preventing infection. Askina® Gel (B. Braun Medical Ltd.) and Flexigran (A1 Pharmaceuticals) are other commercially available starch-based hydrogels (Ellis Horwood Ltd, 1989).

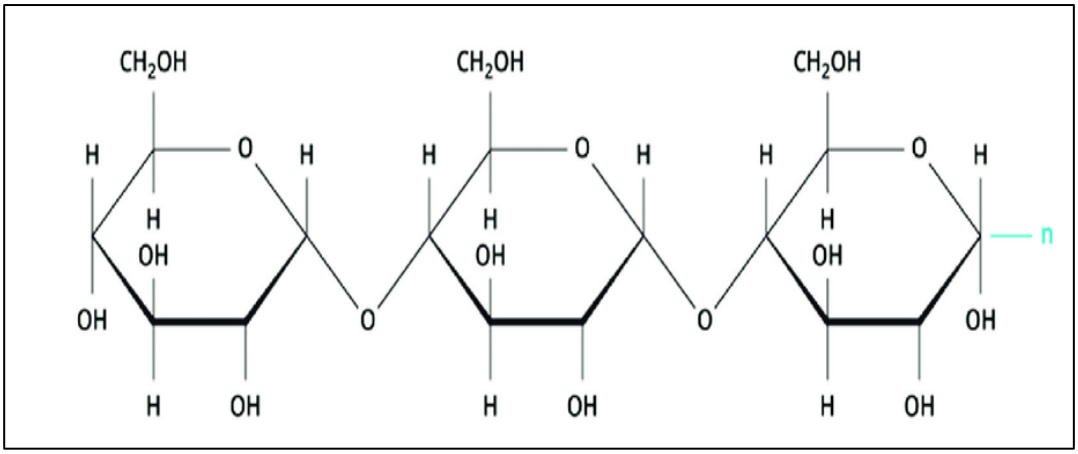

Figure 1. 10 Chemical structure of starch (Othman et al., 2018)

Starch has been studied for biomedical applications (Torres et al., 2013). For example, silk fibroin (SF)/poly (vinyl alcohol) (PVA) /aloe vera (AV) nanofibrous dressing which contains starch nanoparticles was produced to carry Vitamin E (Kheradvar et al., 2018). Vitamin E encapsulated starch nanoparticles were combined with SF/PVA/AV fibers due to the electrospinning method. *In vitro* studies showed that the release of Vitamin E was controlled by Fickian diffusion and faster in dressings including more nanoparticles. The addition of aloe vera and vitamin E increased the proliferation and attachment of fibroblast with collagen secretion. Novel Ag nanoparticles, which were synthesized by *Diospyros lotus* fruit extract, were combined with starch and PVA to create hydrogel membranes for wound dressing applications (Batool et al., 2019). The increase of nanoparticle concentration raised swelling capability and the hydrogel containing Ag nanoparticles had the highest tensile strength. On the other hand, the presence of nanoparticles resulted in lower water evaporation from the membrane. In antibacterial tests, Ag nanoparticles gave comparable results with ciprofloxacin. Furthermore, cornstarch/hyaluronic acid/ ethanolic extract of propolis (CS/HA/EEP) was produced due to the solvent-casting method for biomedical applications (Eskandarinia et al., 2019). The dressing containing 0.5%EEP had the highest antibacterial activity against *Staphylococcus aureus, Escherichia coli,* and *Staphylococcus epidermidis* compared to the CS, CS/HA, and CS/HA/0.25%EE

films. Moreover, it is not observed the cytotoxic effect of the film on the L929 cells. *In vivo* studies showed that film enhanced wound healing at Wistar rats' skin.

1.3.2.2.4 Xanthan

Xanthan is a polysaccharide that has a branched structure and, the backbone and side chains containing a trisaccharide comprise of D-mannose (b-1,4), D-glucuronic acid(b-1,2), and D-mannose as shown in Fig1.11 (Jansson et al., 1975). Xanthan is produced due to gram-negative bacteria fermentation. There are many different strains such as *X. arboricola, X. axonopodis* for xanthan production (Petri, 2015); (Janse, 2005). *X. campestris* is the most preferred strain for the industrial manufacture of xanthan gum (Palaniraj & Jayaraman, 2011). Xanthan is a water-soluble hydrocolloid. The hydration time of the xanthan can be reduced by mixing. Generally, the hydration time is based on ionic strength. When the temperature rises, xanthan becomes a disordered state due to conformational transition. Xanthan has viscoelastic properties. As an example, it can form a weak gel or have to suspend properties in liquids. The behavior of xanthan doesn't vary between pH 3 and 10. It is claimed that xanthan is more stable and resistant to enzymes (Wüstenberk, 2014). In 1969 xanthan is approved by the FDA for use as food products. In 1974, Europe has let the use of xanthan as a food additive (Imeson, 2010). Also, it is biocompatible, high viscous at low concentration, and thermally stable against hydrolysis (Stokke & Christensen, 1996). Xanthan has a high number of hydroxyl groups and free carboxyl groups which can be used for chemical functionalization to arrange its physicochemical and biological properties (A. Kumar et al., 2018).

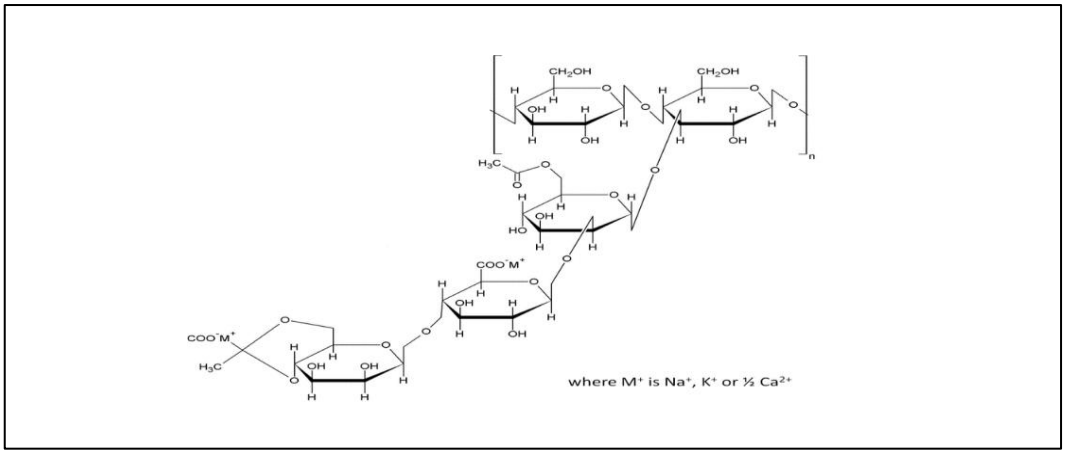

Figure 1. 11 Chemical structure of xanthan gum (Quinten et al., 2011)

Due to the natural properties of xanthan, it is used for biomedical studies (Petri, 2015). As an example, Xanthan-PVA-zinc oxide nanoparticles were produced by using eco-friendly 60Co γ-ray irradiation (Raafat et al., 2018). The presence of zinc oxide not only formed a porous structure but also controlled fluid uptake, water uptake, and water vapor transmission. The wound dressing showed antibacterial properties against *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans* with good biocompatibility. The stable membrane of xanthan-chitosan complexes was produced (Veiga & Moraes, 2012). The membranes were analyzed in terms of morphology, thickness, maximum uptake capacity, and mechanical resistance. An increase in polysaccharide (chitosan to xanthan ratio) content resulted in high water absorption and lower tensile strength at break.

1.4 Crosslinkers Used in Hydrogel Wound Dressings

1.4.1 Glycerol

Glycerol is trivalent alcohol that is present in human tissue as the skeleton of the triglycerides as shown in Fig 1.12 (Bunge, 1971; Dulhunty et al., 1973). Glycerol, also called glycerin, is trihydroxy alcohol, which is highly viscous, low toxic, and odorless. In physical, it is a colorless, hygroscopic, and sweet taste liquid. While the boiling point is 290 ºC, the melting point is 13 ºC (Speight, 2002). High viscosity and boiling point are caused by the intermolecular hydrogen bonding of glycerol. It is capable of holding moisture from the air under contact with the air (Miner & Dalton NN, 1990). It can completely be dissolved in both water and alcohol due to hydroxyl groups, however, it is insoluble in hydrocarbon (Pagliaro & Rossi, 2008).

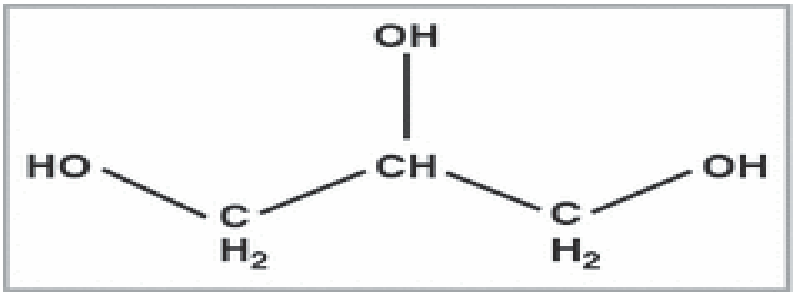

Figure 1. 12 Chemical structure of glycerol (glycerin) (Fluhr et al., 2008)

It is a good solvent for iodine, bromine, phenol because of hydroxyl groups. Under normal storage conditions, it is stable, however, when it contacts an oxidized agent such as potassium chloride it can be explosive (Miner & Dalton NN, 1990). The primary and secondary alcohol groups make glycerol reactive molecules. It can take part in reactions to form ether, ester, amine, and aldehyde (Tan et al., 2013). In medical applications, glycerol is used for the treatment of brain edema, throat

lozenges (Gregory, 2018). Glycerol-added alginate films were produced by crosslinking different calcium chloride concentrations. Glycerol with calcium chloride showed a synergistic effect on mechanical properties and alginate films. Ca crosslink caused an increase in tensile strength but a decrease in fracture strain. Glycerol increased the fracture strain. When Ca concentrations were equal to or higher than 1%, glycerol content, it decreased the tensile strength. A high amount of either glycerol or Ca beyond the limit causes the film to become weaker (Giz et al., 2020).

1.5 Vitamin C

Vitamin C is composed of enediol structure conjugated with the carbonyl group of the lactone ring as presented in Fig 1.13. The acidic feature of vitamin C is sourced by the two enolic hydrogen atoms which also supply the electrons for its antioxidant property. Oxygen can transform ascorbic acid into dehydroascorbic acid (Barba et al., 2014)

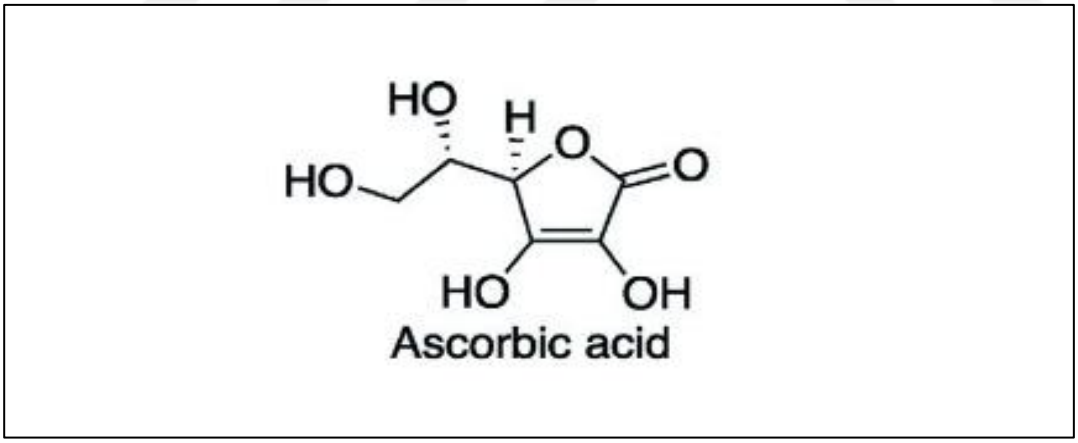

Figure 1. 13 Chemical structure of vitamin C (ascorbic acid)(Avendaño & Menéndez, 2008)

The wound healing process needs collagen and crosslinked fibers to bring damaged tissue in tensile strength. Studies showed that maximum tensile strength was achieved in guinea pigs after vitamin C applications (Bourne, 1946).

Skin includes a higher amount of vitamin C compared to other body tissues. Mostly, vitamin C can be found in intracellular compartments mostly in the millimolar range (McArdle et al., 2002; Rhie et al., 2001; Shindo et al., 1994). In the dermal layer of the skin, vitamin C moves into cells from a blood vessel.

Vitamin C was chosen because of its convenient properties for skin healing. Firstly, vitamin C shows cofactor properties for proline and lysine, which are responsible for not only stabilizing collagen structure but also activating collagen gene expression (Pullar et al., 2017). Moreover, due to its antioxidant feature, vitamin C can neutralize and remove oxidants that are sourced by environmental pollutants and UV radiation (Evans & Lawrenson, 2017). The previous study claimed that vitamin C has a positive effect on the keratinocytes to enhance epidermal barrier function. As an example, vitamin C improves the differentiation of rat epidermal keratinocytes cells (Pasonen-Seppänen et al., 2001). Besides, vitamin C triggers the production of barrier lipids depending on this information, vitamin C can be beneficial for the formation of stratum corneum and inhibiting water loss of skin (Boyce et al., 2002; Savini et al., 2002). Also, VC has a role not only in the formation of cartilage but also in the endothelium of blood vessels. In the lack of ascorbic acid intake, many tissues can be damaged (Wahli et al., 2003).

Furthermore, depending on studies, the application of 500 mg to 1.0 g/day of vitamin C was suggested to expedite the healing process, especially for post-operative patients as vitamin C was utilized for collagen synthesis at wound area (Hellman & Burns, 1958). Vitamin C with micronutrients improves both skin barries' function and protective activities of immune cells (Maggini et al., 2007). Because vitamin C has antioxidative, photoprotective, antiaging, and anti-pigmentary effects, it has been studied for medical applications (Al-Niaimi & Zhen Chiang, 2017). As an example, vancomycin-loaded polycaprolactone nanoparticles covered with polyelectrolyte-Vitamin C in the polyvinyl alcohol-alginate gel were studied (George et al., 2017). D-PCL nanoparticles were formed by the emulsion solvent evaporation method. However, this reduced the antibacterial activity against *S.aureus*. PAH–vitamin C

coating caused stronger adsorption on D-PCL. This kind of formulation showed better anti-staphylococcal action, compared to the available antibiotics with good permeation. The better anti-staphylococcal action can be correlated to polyelectrolyte. Also, the cytotoxicity test on L929 proved that the gel was biocompatible even at high concentrations. Our body needs vitamin C to have healthy physiological functions. In the body, vitamin C is used for the synthesis and metabolism of tyrosine, proline, catecholamine, hydroxylation of glycine, lysine carnitine, tryptophan, and folic acid. Moreover, because it is an antioxidant, it has the role of protecting the body from free radicals, pollutants, and toxins (Chambial et al., 2013).

1.6 Aim of The Study

In recent years, a wide variety of dressings have been improved to assist wound healing and care. In selecting the appropriate dressing, the wound type, healing process, and cost are taken into consideration and different dressings are used at different stages of the healing process. Despite the variety of dressings used in the clinic, there is still no dressing that is suitable for each type of wound and studies have been conducted to develop new dressings.

In this study, our goal was to design and characterize different xanthan and protein (gelatin and/or keratin) based composite hydrogels as cost-effective, multifunctional, temporary wound dressing materials for the first time in the literature. After characterization studies, the most convenient hydrogel composition, in terms of biocompatibility, degradation, and oxygen permeability, and water vapor transmission, was combined with vitamin C (VC). The methods used for the production of a hydrogel wound dressing that will provide local VC delivery were also easily applicable. Gelatin was chosen because of its low immunogenic properties and good biocompatibility. Hydrogels are preferable owing to their cooling effect of the wound area, which decreases the pain feel with its permeability to water vapor and oxygen. Xanthan was studied due to its biocompatible, high viscous structure at low concentration, and thermal stability, biocompatibility. The study contributes to the literature in terms of understanding the vitamin C and keratin presence in xanthan gelatin hydrogels. Besides, although keratin is a well-studied polymer, its effect on xanthan gelatin hydrogels has not been investigated before. The change in mechanical and chemical properties of xanthan gelatin hydrogels formed by glycerol cross-linking because of vitamin C and different concentrations of keratin will contribute to the literature as well as contribute to the production of bioactivate dressings for future investigations. XGHs and KXGHs wound dressings are designed to deliver VC locally. The presence of vitamin C neutralizes the basic environment of wounds, which helps the healing process. Also, an acidic feature of vitamin C can inhibit the bacterial infection of a wound. Furthermore, other delivery

methods have some disadvantages. As an example, oral delivery of bioactive agents can reduce the solubility, stability, and absorption of the agent because of the harsh environment.

The thesis comprises three parts. The first part was aimed to produce xanthan gelatin hydrogels with glycerol crosslinking. The second part of the thesis involves the investigation of the effect of keratin on the xanthan gelatin hydrogels and finding the optimum keratin concentration. The third part focused on the addition of vitamin C to the keratin xanthan gelatin hydrogels, and examination of the effect of vitamin C addition on the properties of the wound dressing. The main steps are shown in Fig 1.14.

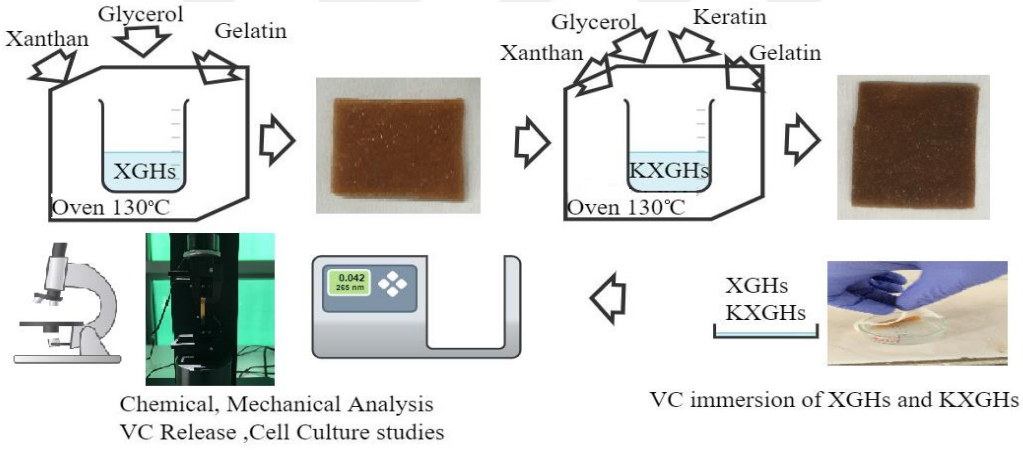

Figure 1. 14 The experimental design of the thesis.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

For hydrogel production; xanthan, gelatin and glycerol were obtained from Sigma USA. Other commercially available products to isolate keratin were supplied by Sigma USA.

For in vitro studies; DMEM low glucose (1 g/l) with L-glutamine, heat-inactivated fetal bovine serum (FBS), penicillin/streptomycin DMEM high glucose (4.5 g/l) with L-glutamine, and trypsin EDTA were bought from Biochrom (Germany). Dimethyl sulfoxide (DMSO) (molecular biology grade) and Trypan blue solution (0.4%) were purchased from AppliChem (Germany) and Sigma-Aldrich (Germany), respectively. Alamar Blue was taken from Invitrogen. Sodium azide, bicinchoninic acid (BCA) reagent, and Nutrient broth were purchased from Sigma (Germany) and (Merck, Germany), respectively.

2.2 Methods

2.2.1 Isolation and Characterization of Keratin

Keratin was isolated from human hair samples using the modified protocol of a previous study (Shui-qing, Lin, Haixia, & Gang, 2017). Firstly, natural hair samples taken from hairdressers and barbers, which have not been applied such as dye, perma, highlighting were left in 70% ethyl alcohol solution and then washed with distilled water. Washed hair samples were delipidated in chloroform/methanol (2: 1) solution for 24 hours. Afterward, chloroform and methanol solutions were removed and the samples were dried in a drying oven at 70° C for 2 hours. The dried hair samples that were cut into small sizes were weighed in a certain amount and left in a water bath at 55°C for 4 hours in the extraction solution containing 8 M urea, 0.10 M sodium dodecyl sulfate (SDS), and 0.50 M sodium sulfite (Na_2SO_3). The solution obtained at the end of the isolation process was transferred to 12 kDa cellulose membrane and purified by dialysis against deionized water for 3 days with water change once a day. The purified keratin samples were lyophilized to dryness. The chemical structure of isolated keratin was examined by Fourier transform infrared spectrometer at METU Merlab. The molecular weight distribution of keratin was detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie Blue staining assay (Agarwal et al., 2019). Proteins in the isolated samples were denatured with 10% SDS and run in 12% polyacrylamide gel. The protein in the bands was stained with Coomassie blue dye and molecular weights of the proteins were analyzed due to the protein ladder. Band formation of the marker protein at 40-50 kDa and 55-60 kDa shows the presence of keratin (Rogers et al., 2006).

2.2.2 Preparation of Xanthan Protein Based Hydrogels in Different Compositions

Nomenclature and component ratios of the hydrogel groups containing xanthan and protein components to be prepared are given in Table 2.1. Firstly, glycerol was added into the beaker then weighed xanthan, gelatin, and keratin were added into the glycerol, mixed, and then heated at 130˚C for 2 hours (Bilanovic et al., 2015). After cooling hydrogels at room temperature, they were kept at 4 degrees. The same procedure was applied to the production of xanthan/gelatin hydrogels. The physical and mechanical properties, adhesiveness, biodegradability, and *in vitro* biocompatibility of hydrogels of different compositions were investigated and compared. The hydrogel group with the highest potential for wound dressing in terms of degradation, porosity, cell viability, water vapor transmission, was determined and immersed into vitamin C solution.

Table 2. 1 Composition of xanthan and protein-containing hydrogels by-weight ratios

Groups	Xanthan Ratio (w/w)	Gelatin Ratio (w/w)	Keratin Ratio (w/w)	Crosslinker Ratio (w/w)	VC Solution (g/ml)
X1:GEL1:GLY1	1	1	-	1	-
X1:GEL1:GLY2	1	1	-	2	-
X1:GEL1:GLY3	1	1	-	3	-
X:GEL:K/VC0.1	1	1	0.04	2	0.1/100
X:GEL:K/VC0.05	1	1	0.04	2	0.05/100
X:GEL:K/VC0.1	1	1	-	2	0.1/100
X:GEL:K/VC0.05	1	1	-	2	0.05/100

2.2.3 Characterization of Xanthan Protein Based Hydrogels

2.2.3.1 Differential Thermal Analysis-Thermogravimetric Analysis

Differential Thermal Analysis (DTA) -Thermogravimetric Analysis (TGA) of XGHs and KXGHs was performed by TA Instruments SDT 650 Simultane DSC/TGA in Central Laboratory at METU. The analysis was done in a heating mode from 25 to 600°C and a heating rate of 20°C/min in a nitrogen atmosphere.

The crystallinity of hydrogels was calculated using the formula below (M.R. Kessler, 2004):

$$Degree\ of\ Crystallinity = \frac{\Delta H_m + \Delta H_c}{\Delta H_f}$$

ΔH_m is the heat of fusion, ΔH_c is the heat of cold crystallization (endothermic) and ΔH_f is the heat of fusion for 100% crystalline material.

2.2.3.2 Attenuated Total Reflectance Fourier Transform Spectrophotometry Analysis

Crosslinking of the hydrogels was verified by the attenuated total reflectance Fourier transform (ATR-FTIR) spectrophotometry using the device (IFS/66S, Hyperion 1000) in Central Laboratory at METU. The spectra of the polymers and the hydrogels were obtained with the transmittance mode on a ZnSe ATR crystal cell due to the accumulation of 256 scans with a resolution of 4 cm^{-1} and a spectral range of 4000-400 cm^{-1}.

2.2.3.3 Morphology of The Hydrogels

The microstructure properties of the prepared xanthan protein based hydrogel groups were examined by scanning electron microscope (SEM, QUANTA 400F Field Emission) from METU Merlab. Before analysis, the samples were covered with gold-palladium by vacuum.

2.2.3.4 Porosity

The porosity of the XGHs was determined by mercury porosimeter (Quantachrome Corporation, Poremaster 60) in high-pressure analysis mode (0- 50.000 psi) in Central Laboratory at METU.

2.2.3.5 Water Uptake Capacity

The gravimetric method was used to determine the percent liquid retention capacity of hydrogels in PBS. After drying of hydrogels at 40°C in an oven, hydrogel samples (30x40 mm^2) in the dry state (n = 3) were weighed. The weighed samples were incubated in 5ml 0.10 M phosphate buffer (PBS, pH 7.4) for 7 days at 37°C. At certain time intervals (day 1, 3, and 5), hydrogel samples were removed from PBS, and hydrogels were weighed after excess water from the surface was removed.

The percent liquid retention capacity of the hydrogels in PBS solution was determined using the following formula (Balakrishnan et al., 2005):

$$\%water\ uptake\ capacity = \frac{m_{wet} - m_{dry}}{m_{dry}} x100$$

2.2.3.6 *In Vitro* Degradation Study

Hydrolytic degradation of xanthan protein based hydrogel samples (n = 3) was studied at 37°C in PBS (0.1M PBS solution (pH 7.4) (Ren et al., 2005; Huang et al., 2015) for 1 week. Dry weights hydrogel samples (30x40 mm^2) were weighed ($m_{initial}$) and samples were placed in incubation media. At certain intervals (1 h, 24h, 3, and 5 days) hydrogel samples were removed from the solutions, washed with distilled water, and weighed after removal of excess liquid. The samples were then dried in the oven at 40°C. After drying, sample weights were weighed (m_{final}). The remaining weight percentages of the hydrogel groups will be determined using the following formula:

$$Weight\ loss\ percentage = \frac{m_{final} - m_{initial}}{m_{initial}} x100$$

Additionally, total protein amounts released from xanthan protein based hydrogel samples in PBS were determined using the BCA protein assay (Smith et al., 1985). Shortly, hydrogels were placed in PBS and incubated at 37°C. Aliquots from PBS were taken after 1 and 24 hours, 3, and 5 days of incubation and 175 microliter aliquot was mixed with 25 microliter BCA solution and left in the dark at 60°C for 25 minutes. At the end of the incubation, the absorbance of the solution at 562 nm was measured by the microplate reader (Biotek Instruments Inc.,μOuantTM, USA). To determine the total amount of protein in the samples, the calibration curve constructed standard with different concentrations of BCA in PBS (2-50 μg / mL) was used. The cumulative protein amount released was plotted concerning time.

2.2.3.7 Water Vapor Transmission Rate

The water vapor permeability of the prepared hydrogel groups was measured according to ASTM E 96-95 standard. During the analysis, a closed system was used to ensure proper environmental conditions. This sealed container contained a digital hydrometer, a beaker containing saturated magnesium chloride solution or silica beads, and a beaker containing deionized water sealed with hydrogel groups inside. Hydrogels were placed in a beaker containing a certain amount of distilled water with a water level of 5 mm (n = 3). After measuring the effective area (S) of the hydrogel, the total weight (m_1) of the beaker containing deionized water sealed with hydrogel was measured. The weighing apparatus was kept at 37°C for 24 hours. A closed device containing a hydrometer and a beaker containing distilled water-sealed was used as the control group. At the end of 24 hours, the weight of the beaker containing deionized water sealed with hydrogel was weighed (m_2). The water vapor permeability of the hydrogels was determined using the following formula (Doulabi et al., 2015; Pan et al., 2017):

$$water\ vapor\ permeability\ = (\frac{m_2 - m_1}{S})\ \times 24$$

2.2.3.8 Oxygen Permeability

To determine the oxygen permeability of hydrogels, a two-headed bottle, a dissolved oxygen meter, and a digital stirrer were used (n = 3). While one head of the bottle was covered with hydrogels, the probe of oxygen was put into the other head and covered with paraffin to hinder the entrance of air. During measurement, the probe was kept in the water. The water in the bottle was mixed with a stirrer at 800 rpm. At the end of 24 hours, the amount of dissolved oxygen in deionized water was determined for each group. Besides, the control group was repeated by leaving the other mouth of the balloon containing no oxygen meter open (positive control) or completely closed with parafilm (negative control) to create control groups.

2.2.3.9 Mechanical Properties

Dry hydrogel samples (30x40 mm^2) were subjected to a tensile test with a tensile speed of 2mm/min on the Lloyd LS500 material tester (Lloyd, UK) (n = 3) at room temperature. Tensile strength and percent elongation at break of specimens were calculated using stress-strain curves (Rottmar et al., 2015).

2.2.3.10 Bacterial Barrier and Antibacterial Tests

Microbial penetration was analyzed by covering a vial involving 5mL of nutrient broth with hydrogels. While negative control, vial closed by cotton was used, positive control was an open vial. The vials were waited in an open environment for one week to let bacteria grow in the medium and observe turbidity. The vials containing cloudiness were considered to have microbial contamination. The cloudiness was measured at 600 nm in the microplate reader (μQuant, Biotek, USA) (n=3)(Ehterami et al., 2018).

2.2.3.11 Vitamin C Loading of The Hydrogels

XGHs and KXGHs were immersed into VC solutions (0.1% and 0.05 %(w/v)) incubated at room temperature in the dark for 24 hours. After 24h, VC-loaded hydrogels were removed from the solution and washed with distilled water to get rid of excess VC on the surface of the hydrogel. The amount of VC loaded to hydrogels was determined by measuring the VC concentration in VC solutions and remnant solutions after removing the hydrogel due to UV spectrophotometric analysis. The absorbance of the solutions was measured at 265nm and the amount of VC was determined using the calibration curve constructed with different concentrations of VC (Xinyu Hu et al., 2020).

2.2.3.12 Vitamin C Release Analysis

In vitro release of VC from the hydrogels was studied by immersing them into 5 ml PBS (pH 7.4) solution and incubating them in a water bath at 37°C at 150 rpm. Aliquots from the release media were collected after 1, 6, 24, 48,72, and 96 h, and release media were replaced with fresh PBS. 100 µl of release media from each sample was placed on a 96-well plate, the absorbance of media was measured at 265 nm (Bose et al., 2019). The amount of vitamin C released by hydrogels was determined by using the calibration curve (Figure A.1). The release profile of VC was presented in terms of cumulative percent release.

2.2.3.13 *In Vitro* Cytotoxicity Tests

Cytotoxicity of hydrogels was tested with an extract test according to ISO 10993-12 standard using the L929 mouse fibroblast cell line. Hydrogel (6 cm^2) samples were sterilized by standing under ultraviolet (UV) irradiation for 2 hours and kept in one mL of cell culture medium at 37°C. After 1, 4, and 7 days of incubation, 100 µL aliquots were taken and added to cells seeded in the 96-well cell culture plate and incubated for 24 hours at 37°C in 5% carbon dioxide incubator. At the end of incubation, the media were removed and replaced with a serum-free DMEM cell culture medium containing 10% Alamar blue. After 4 hours of incubation at 37°C in the dark, the media absorbance was measured with a microplate spectrophotometer (µQuant, Biotek, USA) at 570 nm (reduction) and 600 nm (oxidation). As a positive control group, L929 cells incubated under the same conditions but not interacted with hydrogels were used. DMEM cell culture medium containing Alamar blue was taken as the negative control group. The percentage of Alamar Blue Reduction, which corresponds to cell viability was calculated according to the formula below.

$$Percentage\ of\ Alamar\ Blue\ Reduction = \frac{(O_{600} \times A_{570}) - (O_{570} - A_{600})}{(O_{600} \times P_{570}) - (O_{570} \times P_{600})} \times 100$$

O570 = molar extinction coefficient of oxidized Alamar blue at 570 nm

O600 = molar extinction coefficient of oxidized Alamar blue at 600 nm

A570 = absorbance of the test sample at 570 nm

A600 = absorbance of the test sample at 600 nm

P570 = Absorbance of negative control sample at 570 nm

P600 = absorbance of the negative control sample at 600 nm

2.2.3.14 Collagen Staining

The effect of Vitamin C released from the hydrogels on collagen synthesis of fibroblasts was analyzed by collagen staining with Sirius red. Firstly, 6 cm^2 hydrogel samples were sterilized by ultraviolet (UV) irradiation for 2 hours then they were kept in 1mL cell culture medium for 1, 4, and 7 days at 37°C. Extract media was collected on 1^{st}, $4^{th,}$ and 7^{th} day and added to seeded L929 fibroblast cells. 100 µL aliquots of the extract were taken and added to cells seeded in the 96-well cell culture plate and incubated at 37°C in 5% carbon dioxide incubator. At the end of 4^{th} and 7^{th} days, fibroblasts (L929 cells) and the control group were stained with Sirius red to observe collagen synthesis by the cells. As a positive control group, L929 cells were incubated in the growth media only. For Picrosirius Red solution, solution A was prepared by dissolving 0.5 g Sirius red F3B in 500 mL saturated picric acid solution. Separately, solution B was prepared by adding 5 mL acetic acid to 1 liter of distilled water. Firstly, fibroblast cells were fixed by immersing in 4% paraformaldehyde solution prepared in phosphate-buffered saline (PBS) for 20 minutes at 37°C, and cells were washed with PBS. After fixation, cells waited in 100 µl of solution A for 1 hour at room temperature in a shaker. Then, solution A was removed and cells were washed with solution B twice. Finally, fibroblast cells were dehydrated with %100 ethanol twice (Kiernan, n.d.). Stained cells were observed under the phase-contrast microscope and images from 5 different regions were taken under a 4X lens of a light microscope. The percent of the stained area was calculated by ImageJ software (Onat et al., 2019).

2.2.3.15 Statistical Analysis

Data are presented with mean and standard deviation values. Statistical analysis of the data was performed by using the ANOVA test of the SPSS-15.0 program (SPSS Inc., USA). Differences between groups were considered statistically significant at $p \leq 0.05$ level

CHAPTER 3

RESULTS&DISCUSSION

3.1 Preparation and Characterization of Xanthan Gelatin Containing Hydrogels

Firstly, optimization studies of the polymer/crosslinker ratio of hydrogels were performed for xanthan gelatin hydrogels (XGHs) containing different weight ratios of xanthan, gelatin, and glycerol. The hydrogels were then analyzed in terms of mechanical and chemical properties. The hydrogel that showed the highest porosity and cell viability was selected as the composition for preparing keratin xanthan gelatin hydrogel (KXGH). Then, keratin xanthan gelatin hydrogels (KXGHs) with different keratin amounts were produced and characterized. KXGH that showed higher cell proliferation, lower weight loss, and higher water uptake was loaded with different VC concentrations for preparing the hydrogels as a temporary wound dressing. The nomenclature and composition of these hydrogels prepared are given in Table 2.1.

For preparing the hydrogels the pre-weighed polymers were added to glycerol. Then, the solution was thoroughly mixed with mechanical stirring and heated at 130 °C for 2 h (Bilanovic et al., 2015). After cooling, hydrogels were kept at 4 °C. The physical and mechanical properties, adhesiveness, degradability, and in vitro biocompatibility of hydrogels of different compositions were investigated.

3.1.1 Crosslinking Mechanism of Xanthan, Gelatin, Keratin by Glycerol Crosslinking

In a waterless environment, the hydroxyl groups of glycerol can cross-link functional groups of xanthan (Bilanovic et al., 2015). Glycerol reacts not only with functional groups of xanthan backbone (hydroxyl groups) but also its side chains (acetyl and carboxyl groups) in the branches. Glycerol can crosslink other polymers due to their reactive functional groups. During the crosslinking mechanism of xanthan and gelatin, the water is released. However, water produced by the reaction stays in the bulk of the xanthan/glycerol polymer network and makes xanthan chains unwind. Due to the unwinding, functional groups of xanthan become available for glycerol crosslinking. It was shown that glycerol doesn't react with $-NH_2$ and $-CH_3$, polymers should have carboxyl groups to be crosslinked by glycerol as shown in Fig 3.1 (Bilanovic et al., 2015). Gelatin has both carboxylic and amino groups in its structure with its ionizable groups such as carboxyl groups of aspartic acid and carboxylic acids, the ε-amino group of lysine, the guanidinium group of arginine, the imidazolium group of histidine, and the terminal α-carboxyl and α-amino groups (Djagny et al., 2001). Moreover, keratin has functional groups such as carboxyl, hydroxyl, and amino groups. Extracted hair has both ionizable (4.7% aspartic acid, 7.7% glutamic acid, and 5.4% arginine) and non-ionizable (9.3% serine, 8.8 proline, and 3.5% threonine) amino acids (Giubertoni et al., 2020). Functional groups of the polymers used in this study are presented in Table 3.1. The carboxyl group of amino-acids can form an intramolecular hydrogen bond with the -OH of a carboxyl group and the amide C=O group of the polymers, which assists crosslinking. Therefore, H-bonds can be formed by the interactions between glycerol and keratin (Rocha Plácido Moore et al., 2006). It was claimed that hydroxyl groups of plasticizers form hydrogen bonds between polymer and plasticizer by replacing the polymer-polymer interactions (Gennadios et al., 1993)

Not only molecular size configuration but also a total number of functional hydroxyl groups of the plasticizer such as glycerol can affect the interactions. It was shown

that the effectiveness of glycerol could be related to its small size, which lets the glycerol molecule inserted between the polymer chains (Donhowe & Fennema, 1993).

Table 3. 1 The functional groups of xanthan, glycerol, gelatin, and keratin

Functional Groups	XANTHAN (Bilanovic et al., 2015)	GLYCEROL	GELATIN (Deshmukh et al., 2017)	KERATIN (Watt & Leeder, 2007)
R-COOH	+	-	+	+
R-OH	+	+	+	+
R-O-R	+	-	-	
R-CO-R	+	-	-	
R-H	+	-	-	
$R-NH_2$	-	-	+	+

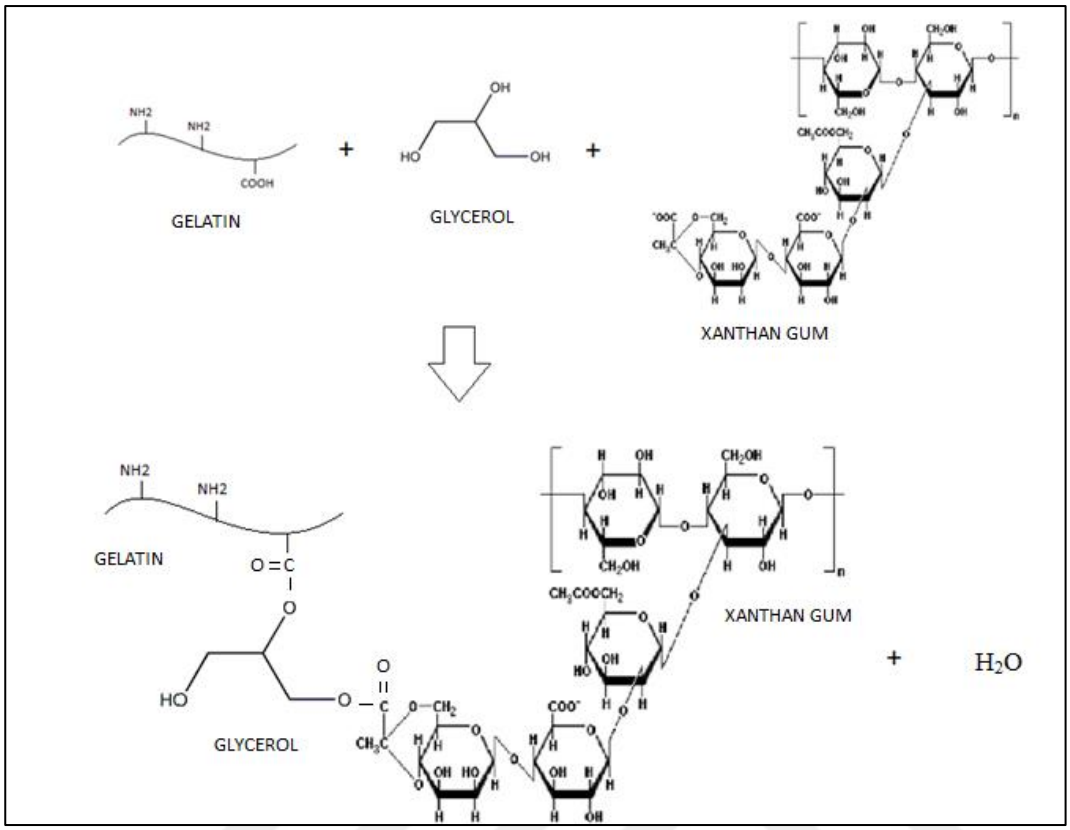

Figure 3. 1 Illustration of the crosslinking mechanism of glycerol

(Modified from Garcia et al., 2013; Murad et al., 2019)

The chemical structure of XGHs was also studied with FTIR. The FTIR spectra of xanthan protein hydrogel wound dressings are shown in Fig 3.2**.** XGHs had a characteristic diffraction peak at 3277.7 cm^{-1}, which shows strong (O-H stretching) peak of alcohol intermolecular bonding. The shift to higher wavenumbers in XGH can be caused by strong electrostatic attractions between the biopolymers as previously reported (Sow et al., 2018). O-H group stretching bands are defined in the range 3600-2900 cm^{-1}, while asymmetric COO^- stretching are in 1640-1590 $cm^{-1,}$ and symmetric COO^- stretching are between 1440-1400 cm^{-1} ("NIST chemistry WebBook," 2014). The area under the peak of C-O-C bands observed in the FTIR spectrum of XGH was much larger than xanthan's bands, which proved crosslinking of xanthan with the glycerol.

The pure xanthan has bands at 3246 and 2881 cm^{-1}, which is relatable to O-H stretching vibrations with intermolecular H-bonding and C-H stretching, respectively (Kumar et al., 2017). After the reaction, the peak became broader and shifted to 3277 cm^{-1}, which represents the acetal formation. Acetal formation is expected because during crosslinking glycerol's OH groups bonded to –COOH groups of gelatin and acetyl, –COOH groups of xanthan. Both spectra demonstrate broad band in the range of 3600–3100 cm^{-1} attributed to OH stretching vibrations.

Gelatin has characteristic absorption bands at 1653, 1541, 1238 cm^{-1}, which correspond to C=O and C-N stretching vibration of groups (amide carbonyl) in Amide I, to N-H and C-N vibration of groups in Amide II, and C-N and N-H vibrations in the Amide III band, respectively (Derkach et al., 2020).

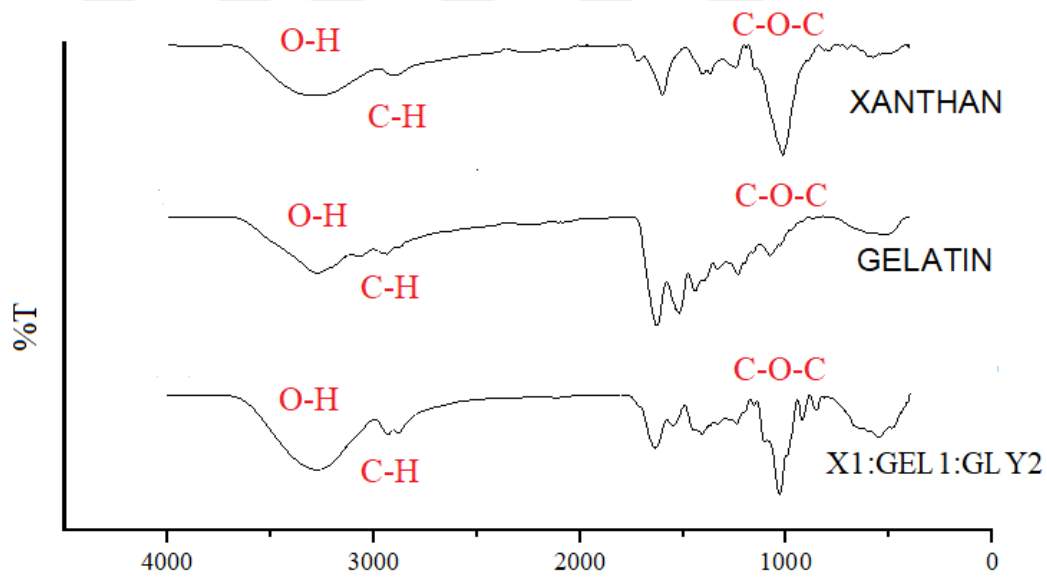

Figure 3. 2 FTIR spectra of the xanthan and gelatin polymers and X1:GEL1:GLY2 hydrogels crosslinked with glycerol.

3.1.2 TGA and DSC Analysis of Xanthan Gelatin Hydrogels

DSC analyzes changes in physical or chemical properties in a material because of exposure to temperature by determining the heat changes and TGA measures the rate of change of the material weight depending on temperature or time (Ma et al., 2013). TGA was applied to study thermal stability and analyze weight loss and the rate of weight loss change according to temperature, time, and atmosphere.

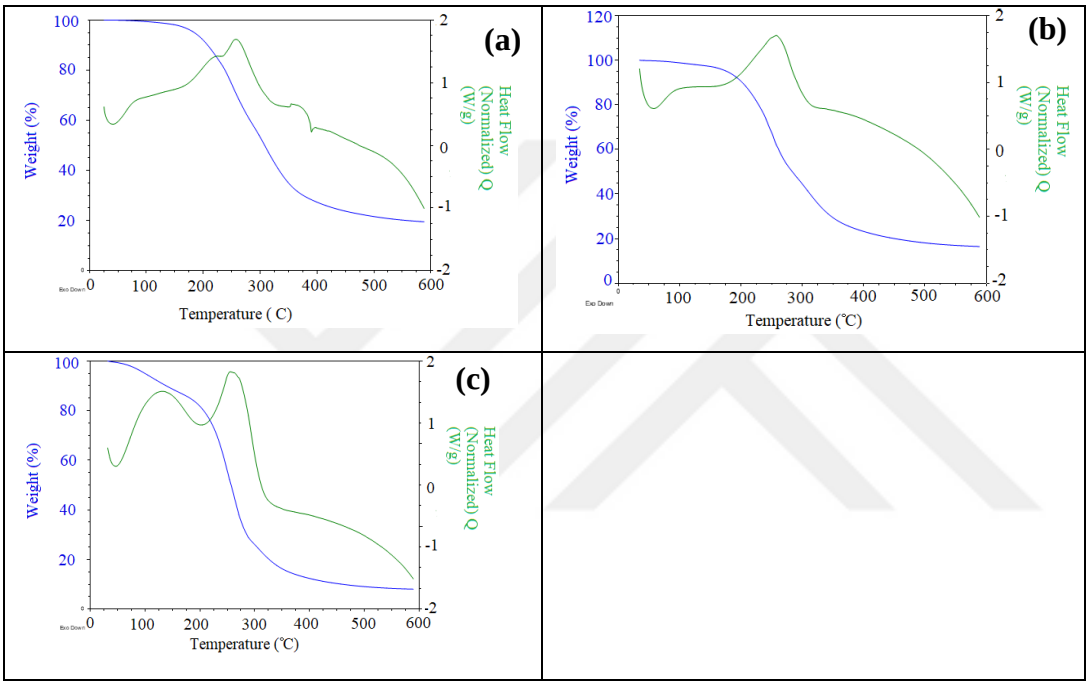

Figure 3. 3 TGA and DSC (exodown) spectra of (a) X1:GEL1:GLY1, (b) X1:GEL1:GLY2, (c) X1:GEL1:GLY3

For gelatin, the first decomposition stage is seen between approximately 80–90°C because of water loss, the second stage occurs about 260°C due to enhanced thermal degradation gelatin (Rahman et al., 2011). In a previous study, xanthan showed 14% weight loss which was caused by the release of moisture and volatile matters. While 60% weight loss was observed between 212°C -299°C, weight loss of 81% was observed in the range 325°C –555°C (Sethi et al., 2020b). To analyze the stability of hydrogels their weight loss percentage was measured in different temperature ranges.

Between 0°C -130°C, X1:GEL1:GLY1 and X1:GEL1:GLY2 showed 2% and 3% weight loss. However, the weight of the loss of X1:GEL1:GLY3 was 9%, which can be caused by the loss of water and glycerol vaporization (Castelló et al., 2009). In the range of 130°C -280°C, the weight loss was increased to 40%, 49%, and 68% X1:GEL1:GLY1, X1:GEL1:GLY2 and X1:GEL1:GLY3 respectively. When temperature increases from 280°C to 320°C, the weight loss percentage was 63%, 69%, and 83% for X1:GEL1:GLY1, X1:GEL1:GLY2 and X1:GEL1:GLY3 respectively. Depending on these results, the increase in glycerol resulted in enhanced weight loss and decreased the thermal stability of XGHs. Moreover, in DSC results, multiple endothermic peaks with characteristic shapes for each composition were observed in the thermal curve of xanthan-gelatin hydrogels. The endothermic event of glycerol in TGA in the range of ~130 and 230 °C is attributed to thermal decomposition (Barbosa et al., 2020). Xanthan has an exothermic peak at 275 °C, which is attributed to the degradation of polysaccharides (Maia et al., 2012). The broad endotherms beyond 280°C are related to the endothermic degradation of the gelatin backbone (Subramanian & Vijayakumar, 2013). Depending on DSC results, X1:GEL1:GLY1, X1:GEL1:GLY2, and X1:GEL1:GLY3 showed 0.4%, 15.6%, and 11.8% crystallinity, respectively. It was claimed that a higher amount of plasticizer decreases the glass transition temperature because it enhances the mobility of polymer chains. The rate of crystallization enhances with an increase in the water content. However, because of the hygroscopic feature of glycerol, the water content can increase, which decreases the glass transition temperature with a higher crystallization rate (Van Soest & Vliegenthart, 1997). During glycerol crosslinking, water is released, which can increase the heterogeneity of the system. This situation can cause the formation of ice nanocrystals at low temperatures in water-rich glycerol water mixtures (Bilanovic et al., 2015; Hayashi et al., 2006). The further addition of glycerol can cause lower viscosity and the melting points of mixtures (Maugeri & Domínguez De María, 2012). X1:GEL1:GLY3, in the 30–200 °C temperature range, showed initiation of weight loss, which can be caused by the evaporation of physically and chemically

bound water. The second region of TGA was observed in the 200–400 °C temperature range, which a peak is also present in the DSC curves. The reason for this can be glycerol degradation in the X1:GEL1:GLY3. The third region is about 400 °C and weight loss became stable, which can be caused by the degradation backbone of polymer membrane (Germán et al., 2012). For TGA analysis at 600˚C, weight loss of hydrogels was higher than 80% for X1:GEL1:GLY1, X1:GEL1:GLY2, and higher than 90% for X1:GEL1:GLY3 as shown in Fig 3.3 .

3.1.3 Morphology and Porosity of The Hydrogels

High porosity structure is a beneficial effect on the diffusion of nutrients and oxygen (Khademhosseini & Langer, 2007). The degree of porosity can affect the mechanical properties, as an example, stiffness of the hydrogel can decrease with an increase in the porosity (Gerecht et al., 2007). It was demonstrated that pore size 5–15 μm and 20–125 μm were sufficient for fibroblast ingrowth, for the regeneration of adult mammalian skin, respectively (Whang et al., 1999). An average pore diameter of 20–125 μm is considered satisfactory for contraction-inhibiting activity in collagen–glycosaminoglycan graft copolymers for dermal repair (Yannas et al., 1989)

It was reported that an increase in glycerol amount caused an increased porosity in a study on plasticizer containing polymeric film (Rabek et al., 2014). However, a decrease in porosity was observed for X1:G1:GLY3 which had high glycerol amount (Fig 3.4). The reason for this can be the loss of plasticizing effect of glycerol, depending on further increase in glycerol content. It was claimed that the higher interaction of the polymer with glycerol doesn’t depend on the increase in glycerol content (Basiak et al., 2018). That can be the reason for lower porosity in X1:GEL1:GLY3 because the higher glycerol may disrupt the stabilized structure of the polymer network.

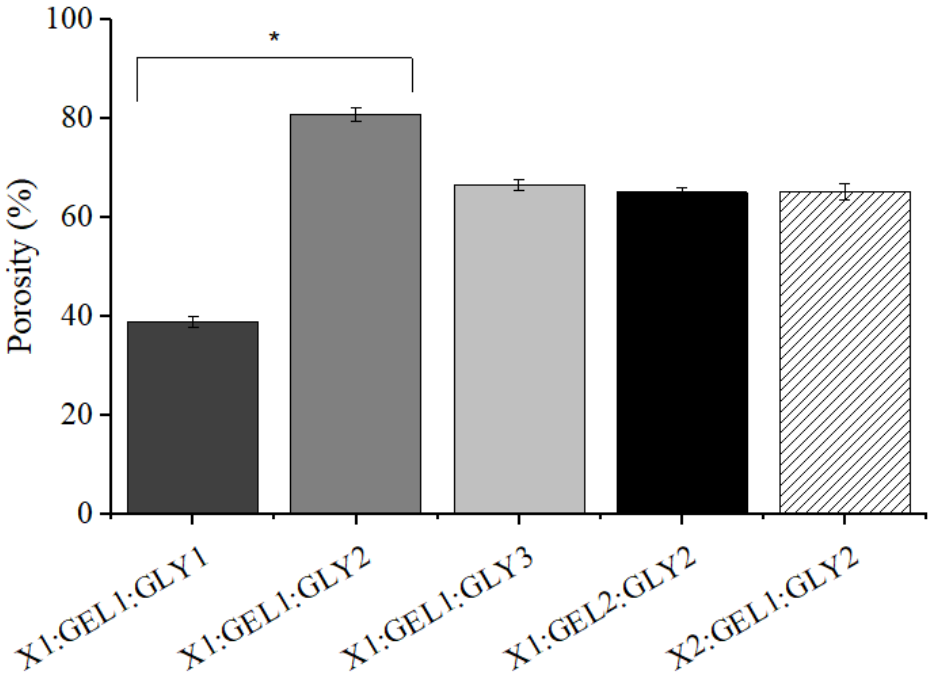

Figure 3. 4 The porosity percentage of xanthan/gelatin hydrogels (XGHs)

*indicates the groups statistically different than other groups (n=3).

Higher polymer concentrations lead to a higher cross-linking degree; therefore, the hydrogels prepared with high polymer concentrations tended to have low porosity and high density (Cheng et al., 2020). This was convenient with our results. X1:GEL2:GLY2 and X2:GEL1:GLY2 groups showed lower porosity compared to X1:GEL1:GLY2. The morphology of XGHs was examined by SEM. As shown in Fig 3.5 and Fig 3.6, XGHs and KXGHs showed a rough uneven topography, respectively. Similarly, Momoh et al., 2015 claimed that the presence of glycerol affects the morphology of the film. The presence of glycerol caused rough morphology, on the other hand, non-glycerol films showed clear and uniform morphology. The pore size of the hydrogels was in the range of 20–123 μm. Interconnected pores can increase gas exchange and pore size of 20–120 μm is convenient for the wound healing process (J. Li & Mooney, 2016). Moreover, it was observed that the surface of the xanthan gelatin hydrogels was rough. It was reported

that a rough surface can accelerate the first of the clotting reactions by activating more platelets and releasing more platelet factors (Patton & Thibodeau, 2019).

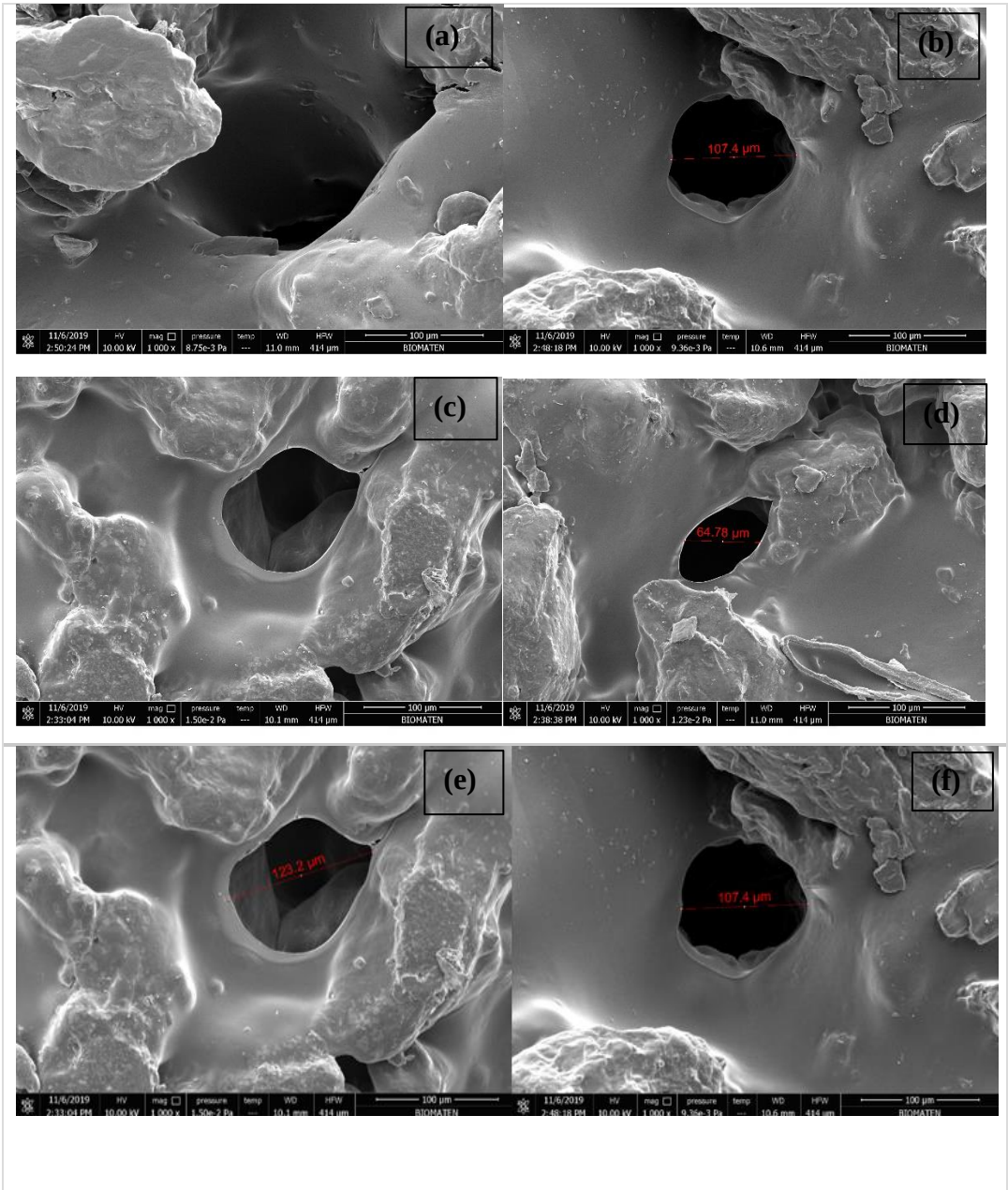

Figure 3. 5 SEM images of xanthan-gelatin hydrogels. (a) X1:GEL1: GLY1, (b) X1:GEL1: GLY2, (c-e) X1:GEL1: GLY3, (d)X2:GEL1: GLY2, (f)X1:GEL2: GLY2

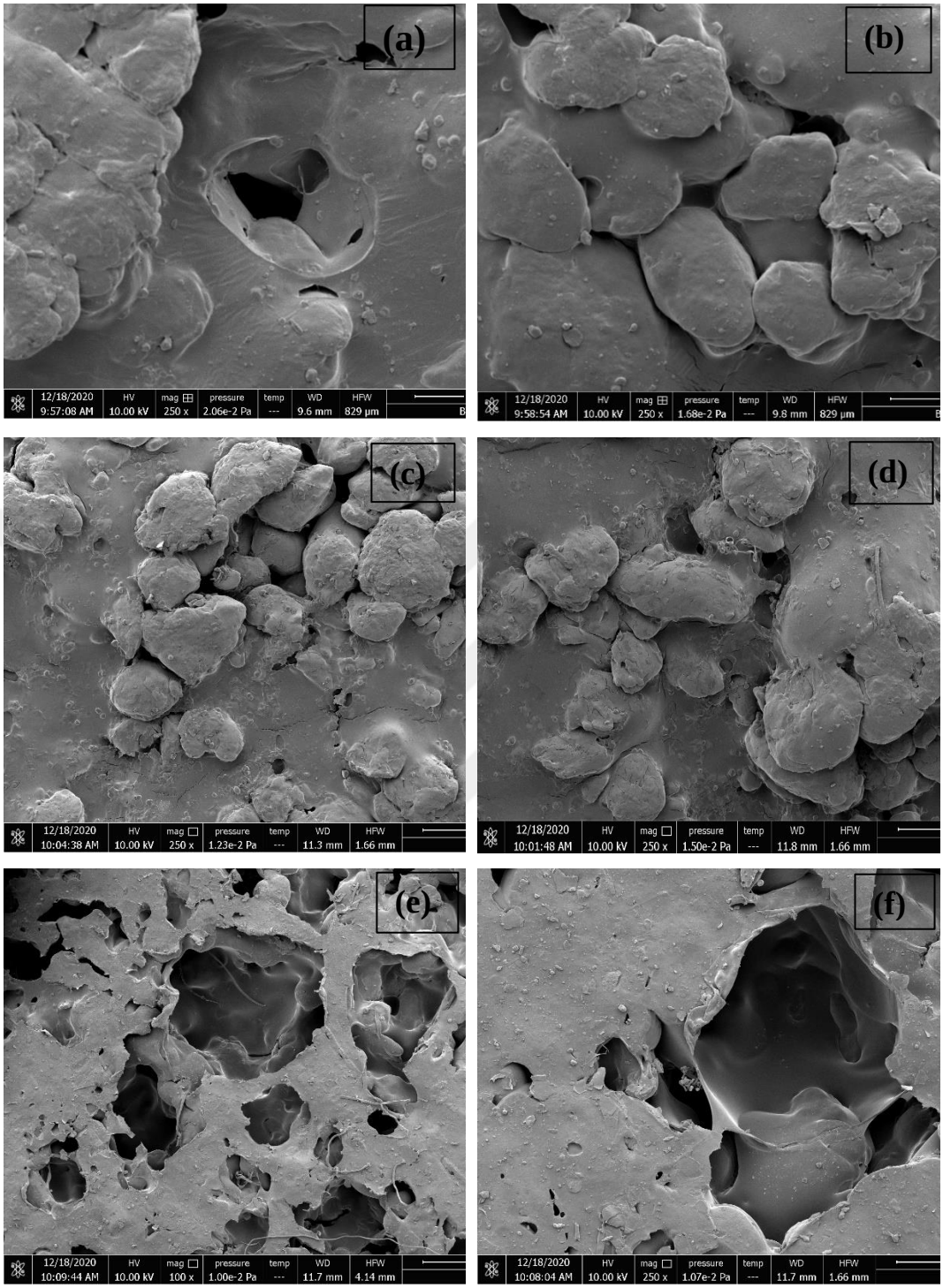

Figure 3. 6 SEM images of keratin-xanthan-gelatin hydrogels (a-b) X1:GEL1:GLY2:K0.08, (c-d)X2:GEL1:GLY2:K0.04, (e-f) X2:GEL1:GLY2:K0.008

3.1.4 Weight Loss, Water Uptake and Cumulative Protein Release Profiles of Xanthan Gelatin Hydrogels

A moist environment at the wound site not only lets the penetration of the active substances but also protects wounds against bacterial infection with the decrease of pain feel (Shezad et al., 2010). Besides, an ideal wound dressing should be able to absorb the exudate without losing its integrity. Alongside their integrity, they should be easily applied to the skin, which needs a flexible structure. However, lower glycerol/higher polymer containing hydrogels such as X1:GEL1:GLY1, X2:GEL1:GLY2, and X1:GEL2:GLY2 showed a stiffer structure, which makes them difficult to apply on the skin. Water uptake (WU) and weight loss (WL) properties of hydrogels represent the ability of wound dressing in terms of the absorption of exudates and the integrity of the dressing. Water uptake and weight loss of xanthan gelatin hydrogels are given in Fig 3.7-3.8, respectively.

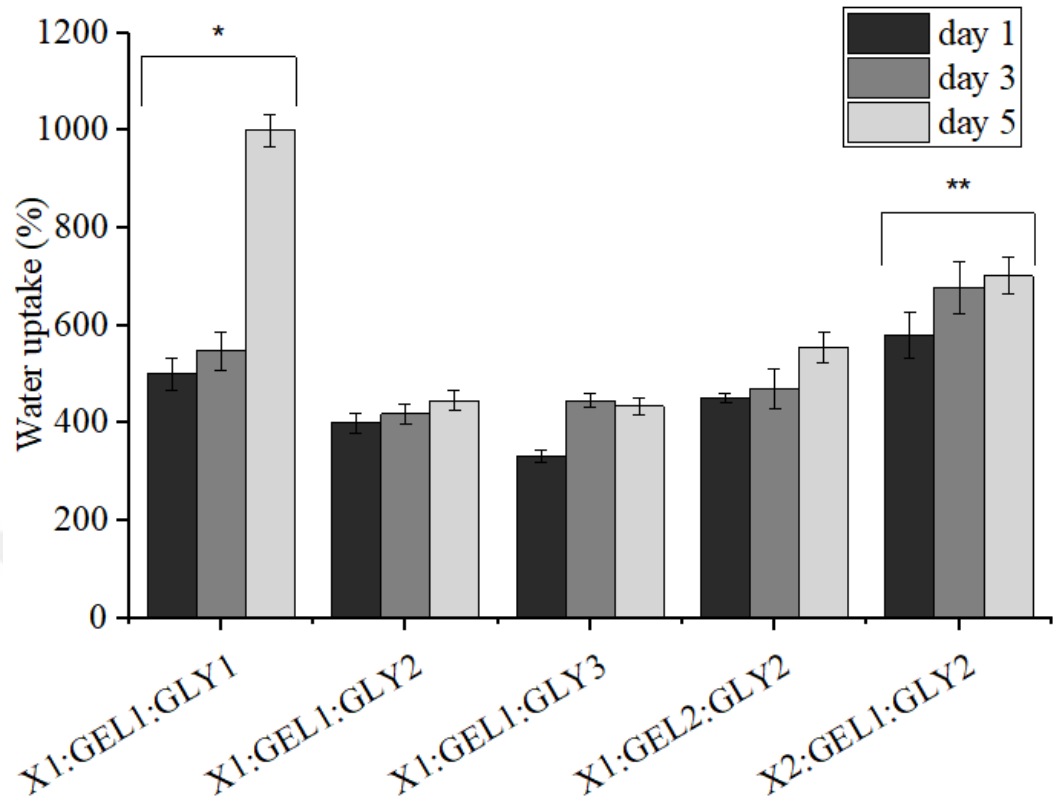

Figure 3. 7 Water uptake capability of xanthan gelatin hydrogels in PBS (pH 7.4) at 37°C * indicates the group that is significantly different than X1:GEL1:GLY2 and X1:GEL1:GLY3 at all time points. ** indicates X2:GEL1:GLY2 is statistically different than other groups at all time points (n=3). ($p<0.05$)

The increased glycerol content caused a decrease in swelling percentage in XGHs. X1:GEL1:GLY1 showed a higher swelling property compared to X1:GEL1:GLY2 and X1:GEL1:GLY3 groups that have higher glycerol crosslinker. Higher crosslink can reduce free amino groups, which resulted in decreased water absorption. (J. P. Chen et al., 2008). Moreover, higher crosslink density/cross-linking points can decrease swelling capacity (Kiti & Suwantong, 2020). Moreover, the excess crosslinker may cause a sharp decrease in swelling because of higher crosslinking points generated and the closer three-dimensional networks, which restricts the entrance of water (X. Li et al., 2015). The swelling capability of the hydrogel can gradually decrease with an enhanced reswelling cycle. This can be caused by the

breakage of physical crosslinking (Q. Li et al., 2012; Xu et al., 2015). This can be the explanation for the small increase in swelling percentage on 3rd and 5th days.

Compared to X1:GEL1:GLY2, while X1:GEL2:GLY2 hydrogels showed slightly higher swelling capacity, X2:GEL1:GLY2 showed statistically higher water uptake at all time points. Swelling can be affected by the hydrophilicity of the additional polymer (Berger et al., 2004). An increasing amount of xanthan enhanced the water uptake until the 5th day, which can be caused by its hydrophilic nature and anionic characteristics of xanthan (Sethi et al., 2020a). Moreover, in an aqueous environment, glycerol-xanthan interaction loses its strength when absorption increases, the higher absorption of water can make the interaction weaker. Swelling is ion-sensitive as the presence of ions makes ionic interactions weaker due to a shielding effect, which enhances swelling and delivery (Shu & Zhu, 2002). In citric acid crosslinked xanthan hydrogels, it was observed that the initial water uptake mechanism does not rely on the hydrogel composition it depends on preferential passage of the solvent by interconnected pores without interaction with polymeric chains (Sethi et al., 2020a).

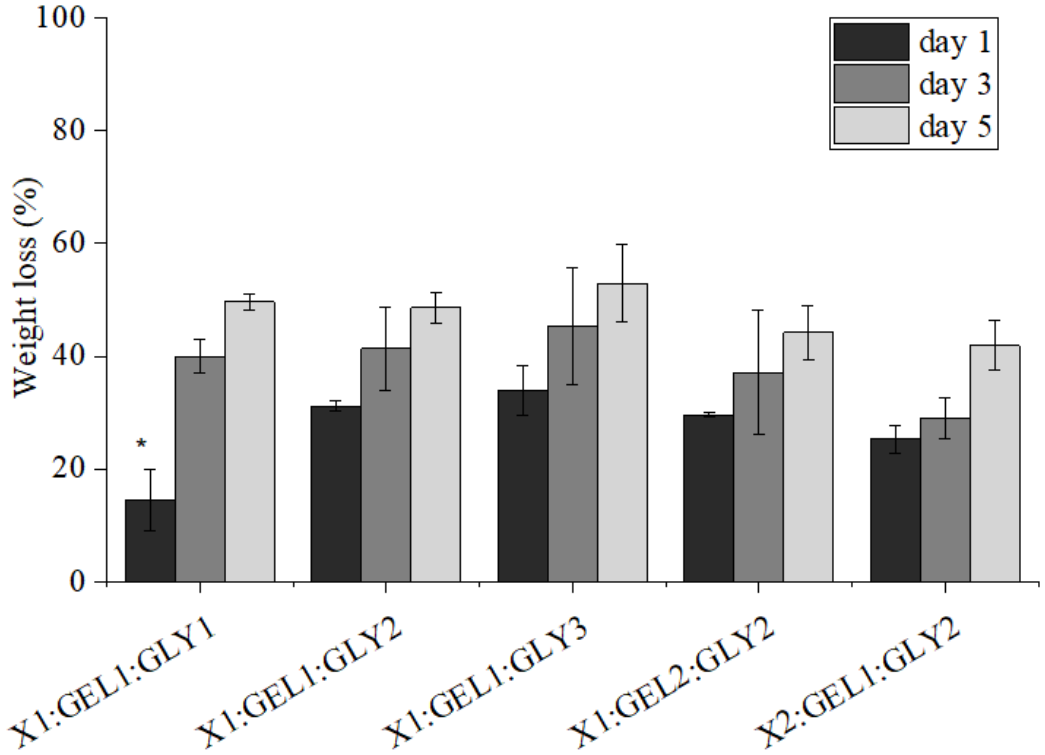

Figure 3. 8 Weight loss of XGHs in PBS (pH 7.4) at 37 °C. * indicates the group which is statistically different than other groups on 1st day ($p<0.05$) (n=3).

The weight loss and medium uptake feature are based on the hydrophilicity and the three-dimensional structure of the hydrogels. All XGHs lost weight almost linearly with incubation time. Higher gelatin concentration resulted in a decrease in the weight loss percentage (X. Wu et al., 2010). The lowest weight loss was observed for X1:GEL1:GLY1 hydrogels on day 1. For all days, weight loss of X1: GEL1:GLY2 and X1:GEL1: GLY3 was similar. In previous studies, it was observed that the presence of oil (also plasticizer) in protein-based or polysaccharide-based films can prevent polymer chain-to-chain interactions, which results in a flexible structure in the films (Hoque et al., 2010; Limpisophon et al., 2010). This kind of detainment of polymer interaction could have caused a higher weight loss percentage in X1:GEL1:GLY2 and X1:GEL1:GLY3. Another reason for the faster degradation of X1:GEL1:GLY2 and X1:GEL1:GLY3 groups compared to X1:GEL1:GLY1 could be the loss of surplus glycerol. When the weight of xanthan and gelatin was

higher than the weight of glycerol, hydrogels became stiffer. The stiffer hydrogels can keep their integrity for a longer time, which can sustain a slower degradation rate until the stiffness is lost. This can be a reason for the lower weight loss of X1:GEL1:GLY1 on the first day. However, when hydrogels lost their stiffness, weight loss increased. Therefore 1:1, and 2:3 polymer/glycerol ratios were thought to be suitable to use in applications that require reduced stiffness and faster degradation rates e.g. skin repair (Grover et al., 2012). On the other hand, X2:GEL1:GLY2 showed the lowest weight loss on day 5 even though the polymer to glycerol ratio was 3:2 w/w. The reason for this can be the higher xanthan amount in the X2:GEL1:GLY2. The study on xanthan hydrogels has reported that a high concentration of xanthan (i.e. 25 gL^{-1}) was needed to obtain a gel because of the low availability of carboxylic groups in the helical structure of the xanthan (Bejenariu et al., 2008). The increase in the xanthan concentration can enhance the availability of the carboxylic groups to react with the glycerol. Xanthan and carboxymethyl cellulose hydrocolloids showed that xanthan had water retention, which causes weight retention (Mohammadi et al., 2014). Moreover, Gambuś et al., (2007) claimed that xanthan accretion can be the reason for the reduction in weight loss.

While protein release is based on passive diffusion through the hydrogel, the rate of protein release depends on mesh size and homogeneity of hydrogel (Aimetti et al., 2009; C. C. Lin & Anseth, 2009; Zustiak & Leach, 2011). X1:GEL1:GLY2 and X2:GEL1:GLY2 showed lower cumulative protein release than other XGHs as shown in Fig 3.9. The hydrogel can selectively release the lower molecular weight monomer as keeping larger proteins. A similar result was observed for PLGA microspheres and other hydrogels (Pérez-Rodriguez et al., 2003). While the molecular weight of gelatin is between 15 to 400 kDa, xanthan gum has a higher molecular weight (2. 10^6–2. 10^7 Da)(Benjakul & Kittiphattanabawon, 2018; Xiaolong Hu et al., 2019). This could be the reason for the increase in cumulative protein release.

X1:GEL2:GLY2 showed higher weight loss and cumulative protein release than X2:GEL1:GLY2. It was expected that higher protein-containing hydrogels should release higher protein, which can be the reason why X1:GEL2:GLY2 showed higher protein release compared to X2:GEL1:GLY2.

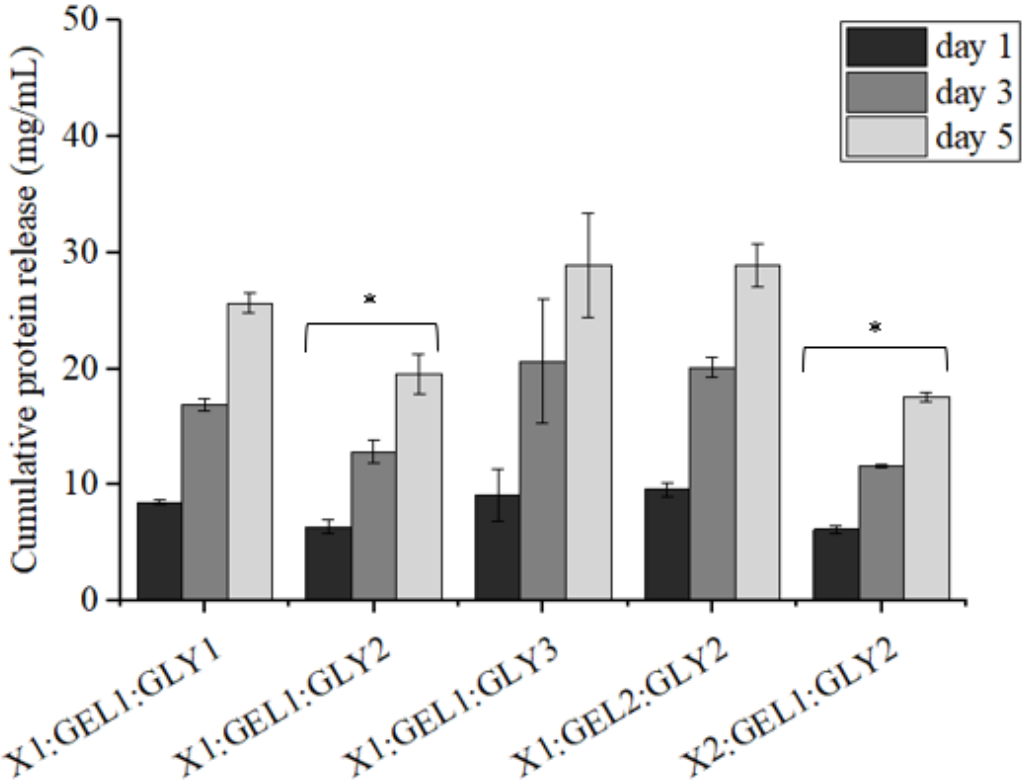

Figure 3. 9 Cumulative protein release from XGHs in PBS (pH 7.4) at 37 °C

* indicates groups statistically different than other groups at all time points.

3.1.5 Mechanical Tests

Young's modulus of materials is an important factor that affects cell proliferation and differentiation, which are related to wound healing (Schneider et al., 2006). Ideal wound dressings should not lose their integrity during use. The tensile test results of XGH are shown in Table 3.2. For X1:GEL1: GLY1, the tensile strength was about 0.467±0.09 MPa. When glycerol concentration was increased, there was a significant decrease in Young's Modulus as shown in Fig 3.10. This result is in agreement with a previous study that analyzed glycerol's effect on mechanical properties (Corradini, De Medeiros, Carvalho, Curvelo, & Mattoso, 2005). It was reported that the glycerol concentration ranging from 10% to 20% was not flexible sufficient to show different effects based on its concentration in terms of mechanical properties for glassy composite systems and biopolymer-based edible films (Ferreira & Andrade, 2015; Nguyen Vu & Lumdubwong, 2016). When the plasticizer amount was increased, Young's modulus and tensile strength at break decreased, on the other hand, elongation increased. Glycerol can reduce the rigidity of the network, by forming a less-ordered structure and enhancement of polymer chain movement (Sothornvit & Krochta, 2005).

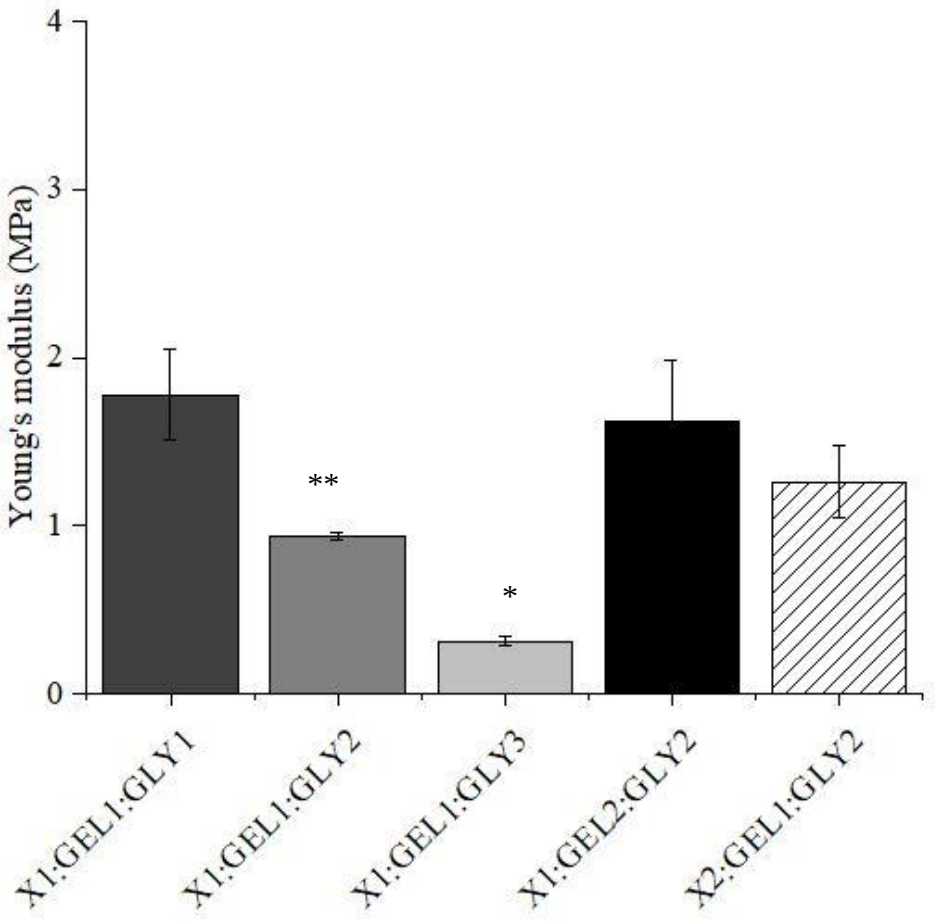

Figure 3. 10 Young's modulus (MPa) values of XGHs (n=3) *indicates the groups statistically different than other groups **indicates the group statistically different than X1:GEL1:GLY1, X1:GEL1:GLY3, and X1:GEL2:GLY2

X1:GEL2:GLY2 showed a statistically higher Young's modulus than X1:GEL1:GLY2 ($p<0.05$). Higher gelatin content resulted in a higher young modulus of the hydrogels. The addition of gelatin was previously reported to increase Young's modulus of hydrogels produced for skin engineering (Karimi & Navidbakhsh, 2014). Among XGHs, X2:GEL1:GLY2 was significantly different than X1:GEL1:GLY2 and X1:GEL1:GLY3 in terms of tensile strength (TS). X1:GEL1:GLY3 had higher elongation at break (EB) than X1:GEL1:GLY1 and X1:GEL1:GLY2.

An increase in gelatin or xanthan content also caused higher TS and EB as shown in Table 3.2. The increase in both TS and EB can be caused by the electrostatic interaction between opposite charged functional groups of gelatin and xanthan. Farris et al. reported that higher TS and EB can be caused by the oppositely charged interaction of pectin and gelatin (Farris et al., 2011).

Table 3. 2 Tensile Strength (MPa) and Elongation at break (%) results of XGHs (n=3)

Name of the group	**Ultimate tensile strength (MPa)**	**Elongation at break**
X1:GEL1:GLY1	0.467±0.09	0.68±1.15
X1:GEL1:GLY2	0.226±0.01	0.706±3.7
X1:GEL1:GLY3**	0.220±0.04	1.82±59.34**
X1:GEL2:GLY2	0.418±0.16	1.64±70.6
X2:GEL1:GLY2*	0.498±0.08*	0.728±9.2

*indicates the group that is different than X1:GEL1:GLY2 and X1:GEL1:GLY3 in terms of TS in XGHs

**indicates X1:GEL1:GLY3 is statistically different than all groups in terms of EB

XGHs have shown favorable mechanical properties for skin tissue since the tensile strength of hydrogel samples was in the range between 25 kPa (0.025MPa) and 140 MPa, which is typical for the tensile test of human skin (Kalra et al., 2016). Additionally, their young modulus values were in the range of 4.5 kPa and 8 kPa (Young's modulus of skin) (Pailler-Mattei et al., 2008).

3.1.6 Water Vapor Transmission Rate of Xanthan Gelatin Hydrogels

Hydrogels which are designed as wound dressing materials should keep the high humidity in the wound area to assist healing. Water vapor transmission rate (WVTR) is an important factor that shows the potential of the hydrogel in terms of the transmission of body liquid. While higher WVTR causes faster drying of the wound that can cause eschars, low WVTR results in the accumulation of exudates which may laten the healing process and increase the risk of bacterial growth (Kokabi et al., 2007). These are the reasons showing the importance of moisture balance that is very critical for the wound healing process (McColl et al., 2007; Xu et al., 2016)

Arvanitoyannis et al. (1998) claimed that the reason for the increase in WVTR due to glycerol was the reduction in polymer packaging density (Arvanitoyannis et al., 1998). Depending on this, higher WVTR on X1: GEL1: GLY3 group was expected compared to X1: GEL1: GLY2 group. However, X1: GEL1: GLY1, X1: GEL1: GLY2, and X1: GEL1: GLY3 showed similar WVTR. This can be explained by the interaction of hydroxyl groups of gelatin and glycerol to form a hydrogen bond, which could have reduced the number of sorption sites for water. Reduced sorption sites can decrease the diffusion rate of water molecules into a hydrogel (Talja et al., 2007). It has been reported that an increase in the amount of plasticizers like glycerin results in decreased WVTR in biopolymer films (Gontard et al., 1993).

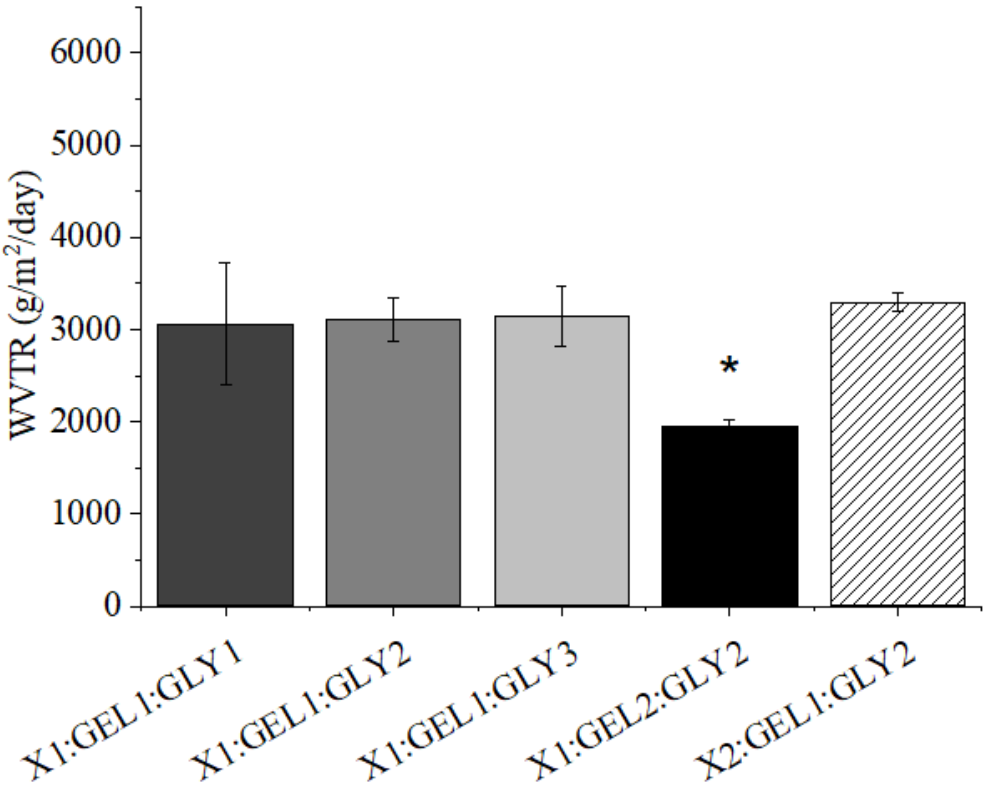

Figure 3. 11 WVTR values of XGHs. * indicates the group which is significantly different than other groups (n=3).

X1:GEL1:GLY1, X1:GEL1:GLY2, X1:GEL1:GLY3, and X2:GEL1:GLY2 didn't have a significant difference. Moreover, the hydrophilic property of glycerol makes the hydrogels more polar. This added polarity may increase intermolecular distance, which results in fewer hydrogen bonds and intermolecular interaction (Yulistiani et al., 2020). Depending on this, it is expected to observe higher WVTR for X1:GEL2:GLY2. However, it was observed significantly lower WVTR for the X1:GEL2:GLY2 as shown in Fig 3.11. The lower WVTR can be caused by the ionic interaction between gelatin and xanthan, which caused a denser structure, therefore hindering water vapor transfer through the hydrogel. Similar results were observed in gelatin films containing polysaccharides such as gellan and K-carrageenan showed a barrier effect against water vapor because of ionic complexes (Pranoto et al., 2007). Lamke et al. have reported that the evaporative water loss rate for normal

skin is about 204±12 g m^{-2} d^{-1} at a skin temperature of 35.8±0.2 °C. Depending on the first degree and granulating wounds, the evaporative water loss rate was 278.4±1.1 and 5138±202 g m^{-2} d^{-1}, respectively. Ideal wound dressings' WVTR should have higher than normal skin WVTR (Y. M. Lee et al., 2000). The ideal WVTR is accepted between 2000 and 2500 g/m^2/24h (Rezvanain et al., 2017). However, many commercial dressings are not between this range and have a very large spectrum of WVTR, ranging from 6512 ± 445, 9009 ± 319, 9360±34 for Biofilmex®' Geliperm® and Vigilon®(no films) respectively (P. Wu et al., 1995). As XGHs are in the range 3059.09±126 and 4523 ±133, they can be suitable for granulating, low to moderate exudate wounds.

3.1.7 Oxygen Permeability of Xanthan Gelatin Hydrogels

For the wound healing process, oxygen is needed for matrix synthesis, cell migration, and proliferation (Hong et al., 2014). García et al., have reported that oxygen permeability can increase when glycerol concentration increases (García et al., 2011). Glycerol ratio has an important effect on oxygen permeability because glycerol can allow the migration of oxygen molecules by enhancing molecular mobility (Suppakul et al., 2013). Permeation of dissolved oxygen (DO) results of hydrogels are shown in Fig 3.12. While the open control's DO value was 5.23±0.03 mg/L, DO for the closed control was 5.02 ± 0.06 mg/L. DO of hydrogel groups and the open control were almost the same (about 5.23 mg/L) and they were higher than the DO of the closed control. Additionally, the closed group had a significantly lower DO value compared to the xanthan gelatin hydrogels.

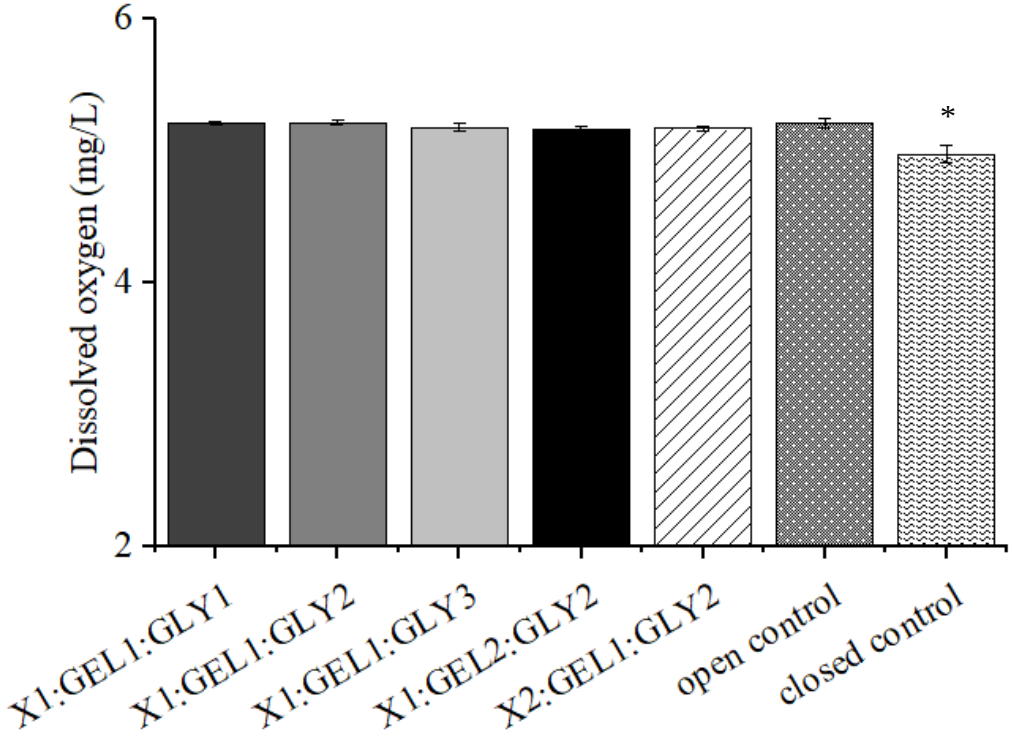

Figure 3. 12 Oxygen permeability of XGHs *the closed control group is significantly different than all groups (n=3)

3.1.8 Cytotoxicity Tests of Optimized Xanthan Gelatin Hydrogels

Cell culture studies were conducted with L929 cells which are widely used in studies on wound healing and tissue regeneration (Thangapazham et al., 2014). The extract of the hydrogels was used to investigate the cytocompatibility of the hydrogels by the cell viability assay and microscopy images of cells exposed to extracts of the hydrogels are shown in Fig 3.13 -Fig 3.14, respectively. Relative cell viability in all hydrogel groups was above 100% on day 1. An increase in the glycerol content caused a decrease in the boost effect on the relative cell viability (X1:GEL1:GLY2: 120.6±2.4, X1:GEL1:GLY3: 104.7±2.4). The reason for the decreased boost effect can be released glycerol which makes the cell media more viscous. It was claimed the media that has high viscosity can prevent the cells from taking nutrients from the media (Ulubayram et al., 2002). For hydrogel groups with the same glycerol content,

change in gelatin or xanthan did not affect the cell viability significantly. On 4th day, relative cell viability observed in X1: GEL1: GLY2 (130.9±0.08%) was significantly higher than other groups. Results showed that all the produced XGHs were cytocompatible.

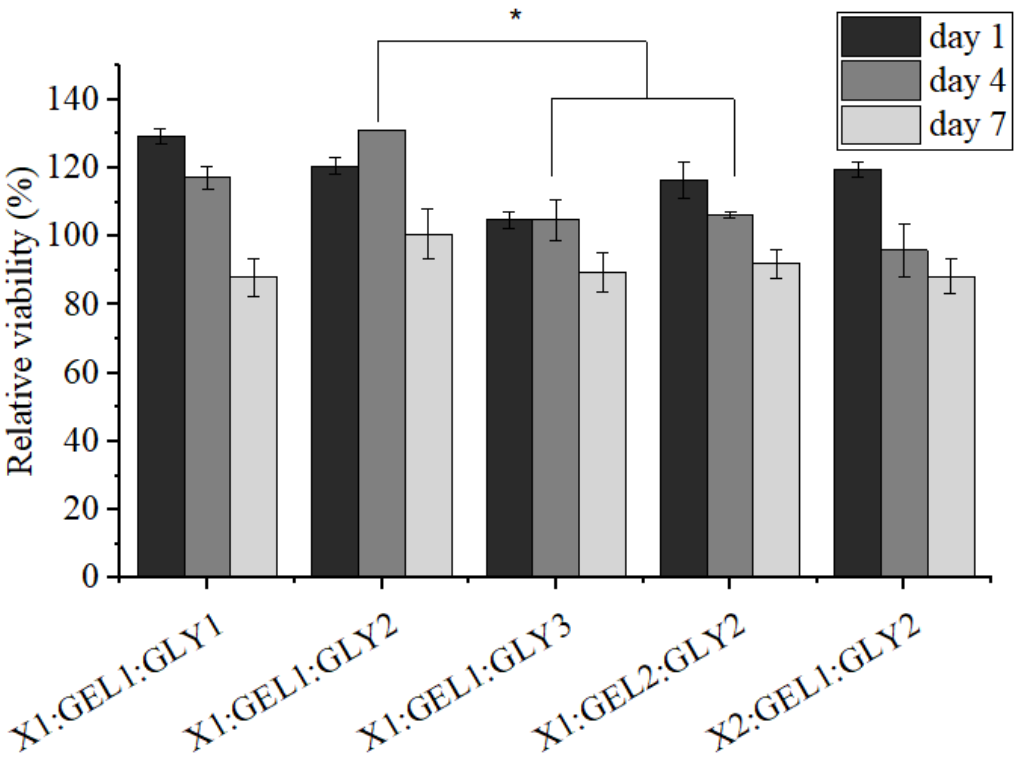

Figure 3. 13 Effect of xanthan/gelatin hydrogels on the viability of L929 fibroblasts

* indicates the groups statistically higher than X1:GEL1:GLY3 and X1:GEL2:GLY2 on day 4 (n=3).

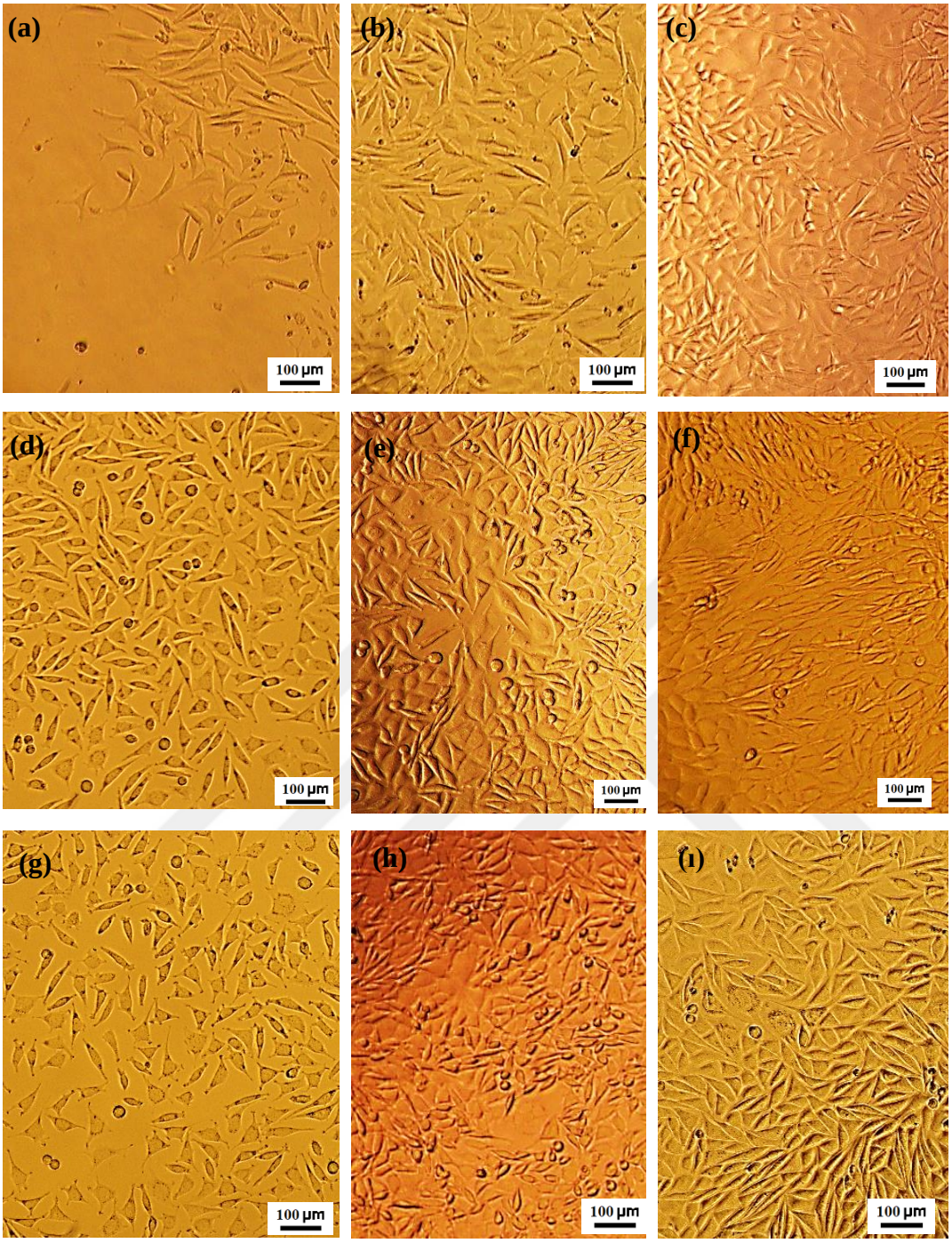

Figure 3. 14 Phase contrast micrographs of XGHs (a-b-c) X1:GEL1:GLY1 on 1st , 4th and 7th day, (d-e-f) X1:GEL1:GLY2 on 1st, 4th and 7th day, (g-h-i) X1:GEL1:GLY3 on 1st, 4th and 7th day, (i-j-k) X1:GEL2:GLY2 on 1st, 4th and 7th day, and (l-m-n) X2:GEL1:GLY2 on 1st, 4th and 7th day

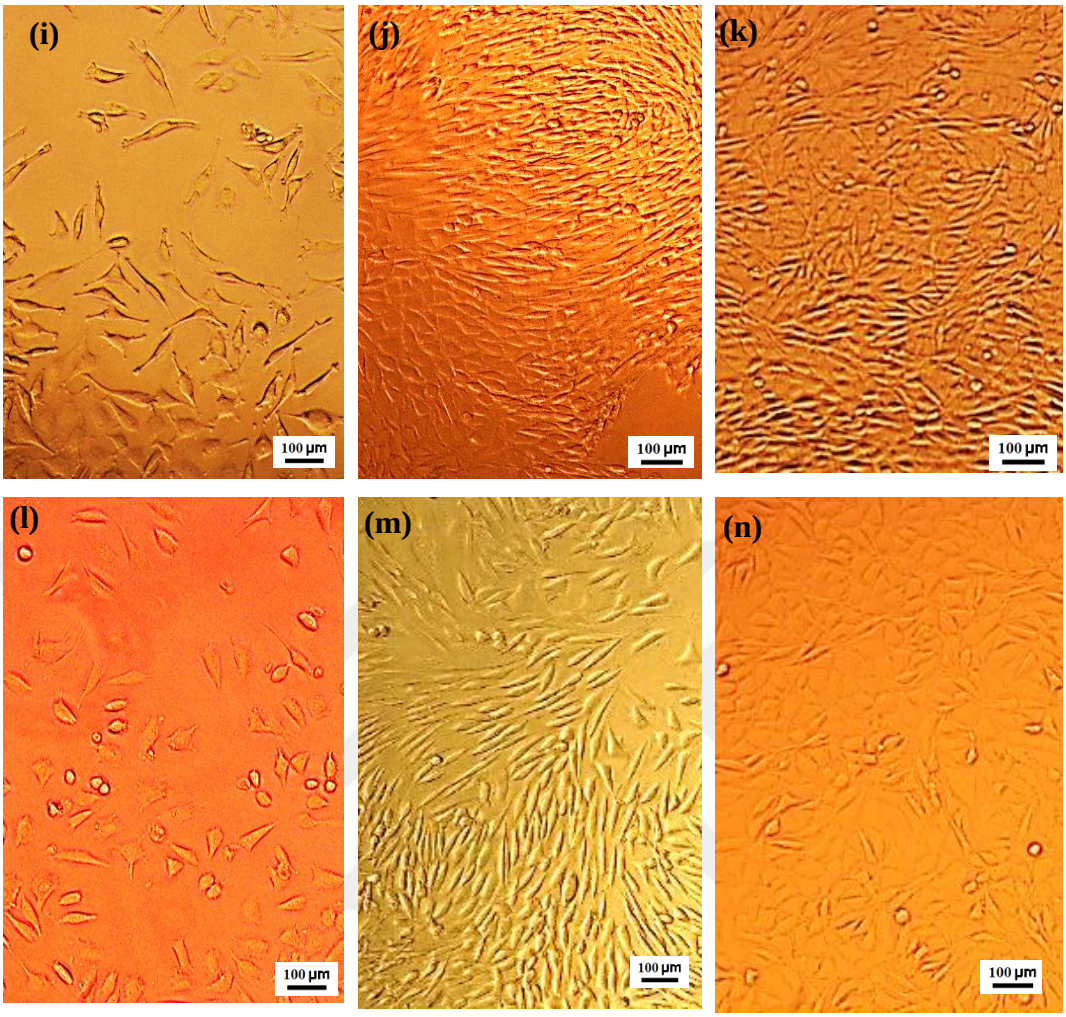

Figure 3. 14 (cont) Phase contrast micrographs of XGHs (a-b-c) X1:GEL1:GLY1 on 1st , 4th and 7th day, (d-e-f) X1:GEL1:GLY2 on 1st, 4th and 7th day, (g-h-i) X1:GEL1:GLY3 on 1st, 4th and 7th day, (i-j-k) X1:GEL2:GLY2 on 1st, 4th and 7th day, and (l-m-n) X2:GEL1:GLY2 on 1st, 4th and 7th day

3.1.9 Antibacterial Tests of Xanthan Gelatin Hydrogels

The reason for microbial proliferation on wounds is the moist, warm, and nutritious environment of the wound (Greenhalgh et al., 1986). Infection of wounds can postpone wound healing, however, it can be prevented by a proper wound dressing that blocks bacterial transmission to the wound. As shown in Fig 3.15, except for positive control, negative and xanthan gelatin hydrogels prevented the bacterial contamination of the nutrient broth.

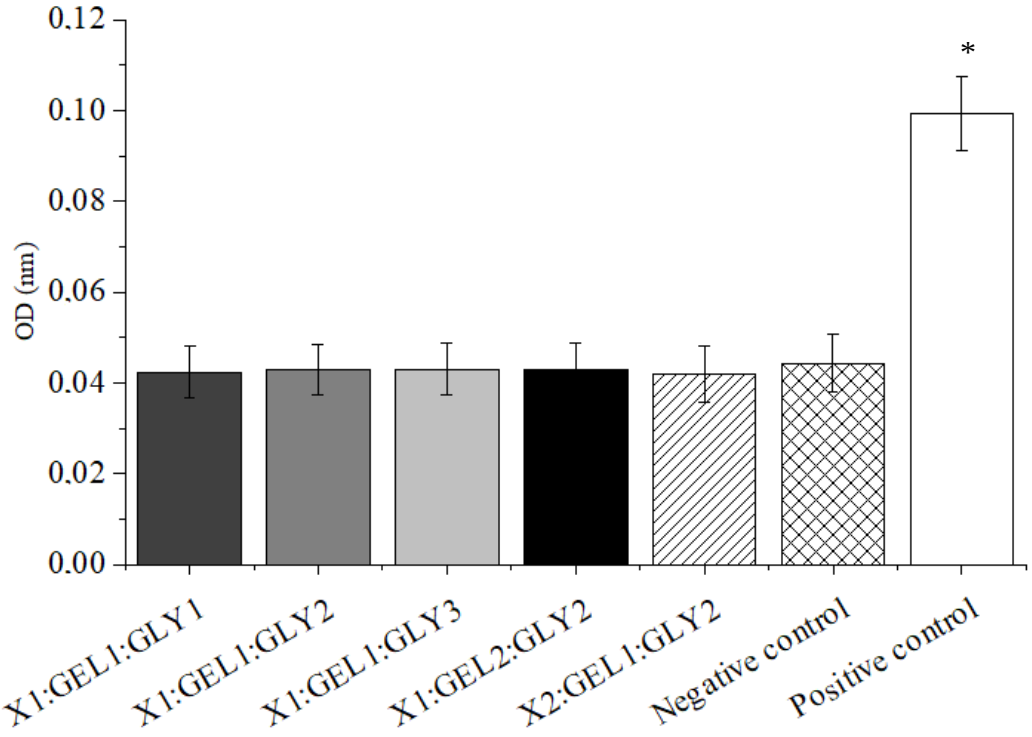

Figure 3. 15 Bacterial permeability analysis of XGHs.

*Positive control is significantly different than other groups (n=3).

3.2 Preparation and Characterization of Keratin Containing Xanthan Protein Hydrogels

3.2.1 Keratin Isolation

For isolation of keratin, the sodium sulfide method was used. Isolated keratins had a brown color protein solution, after lyophilization, they became brown colored proteins. The result was in agreement with previous sodium sulfide extraction results (Agarwal et al., 2019a).

Studies related hair derived keratin biomaterials have proved that keratin assists cellular attachment because of their cell-binding motifs, such as glutamic acid-aspartic acid-serine and leucine-aspartic acid-valine binding residues which are found in several extracellular matrix proteins such as fibronectin (Patrucco et al., 2019; Verma et al., 2008). The keratin addition into XGHs aimed to increase the proliferation of fibroblast cells. Before the addition of keratin into XGHs, the isolated proteins were analyzed to clarify that if they were keratin. SDS-PAGE analysis showed that there was no denaturation in the structure of keratin obtained; alpha-keratins (around 48 kDa), and beta keratins (63-75kDa) gave bands in the kDa values, which are characteristics for keratin. It has also been shown in previous studies that the molecular weight of alpha-keratin protein in human hair samples is 40-50 kDa and the molecular weight of beta-keratin is 55-60 kDa (Rogers et al., 2006) as represented in Fig. 3.16.

Keratin, which is a fibrous structural protein, is found in skin, hair, wool, nails, horns, and feathers. Due to the disulfide bond, keratin has mechanical durability (Rouse & Van Dyke, 2010). It was observed that the addition of keratin into gelatin caused better mechanical strength (Ramadoss et al., 2017). Disulfide bonds occur between cysteine residues. Besides, disulfide bonds have a role in hair fiber stabilizing which leads to relatively high wet strength, insolubility, and thermal stability (Popescu &

Höcker, 2007). During the keratinization process in hair, the structural transition can mainly govern by disulfide bridges (H. Wang et al., 2000).

To extract keratin from animal tissue harsh conditions can be needed, which can cause a xenogeneic response, restricting its applicability in biomedical applications. To inhibit xenogenesis, human hair was chosen as substrates to extract keratin. Different methodologies have been developed to isolate keratin from human hair mainly depending on the cleavage of disulfide linkages to increase its solubility. The effectiveness of several compounds such as sodium sulfide, peracetic acid, urea, and thioglycolic acid in keratin extraction was studied (Agarwal et al., 2019b). Results showed that the sodium sulfide method extracted the highest concentration of keratin protein. Also, it was reported that bands around 48 kDa are visible if keratin is isolated with the sodium sulfide extraction (Agarwal et al., 2019b).

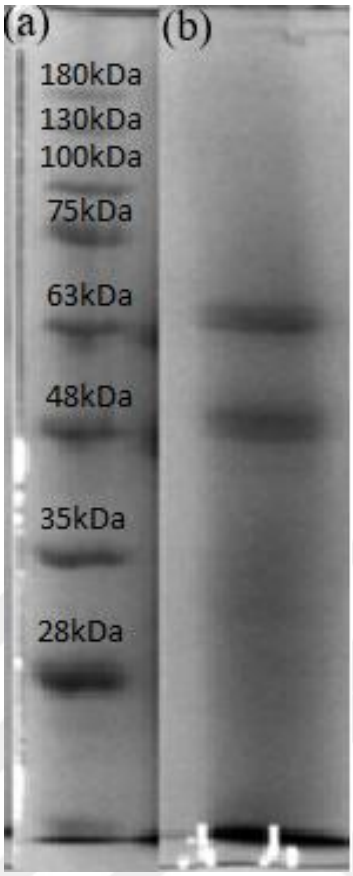

Figure 3. 16 SDS-PAGE analysis results of keratin isolated from human hair :(a) protein ladder, (b) isolated keratin protein assay

3.2.2 Characterization of Keratin Xanthan Gelatin Hydrogels

3.2.2.1 FTIR Analysis

The presence of keratin in the hydrogels was approved by the presence of the peaks at 3259 cm^{-1}, 1638 cm^{-1},1544 cm^{-1},1033cm^{-1} (Swati Sharma et al.). Due to the presence of β-sheets, amide I, II, and III were observed at peaks of 1672, 1598, and 1276 cm^{-1} (Swati Sharma et al.,) While peaks at 1659–1660 cm^{-1} were attributed to α-helix, the intramolecular β-sheet was observed at 1633 cm^{-1} (Selmin et al., 2012). The peaks of 1633, 1559, and 1392 cm^{-1} correspond to peptide bonds which are amides I, II, and III related to the protein structure of keratin. While both α-helix and β-sheet structures involve amide I, amide III bonds occur in β-sheets (Y. X. Wang & Cao, 2012). Majority of α helical structure causes a peak of amide I in the 1655 cm^{-1} region. The peak of amide in X1:GEL1:K0.08:GLY2 shifted to 1638 cm^{-1}, which was attributed to the random structures (Hartrianti et al., 2017). The presence of keratin also increased intensity in the O-H and C-H regions of the spectrum, which proved the crosslinking of keratin by glycerol as shown in Fig 3.17.

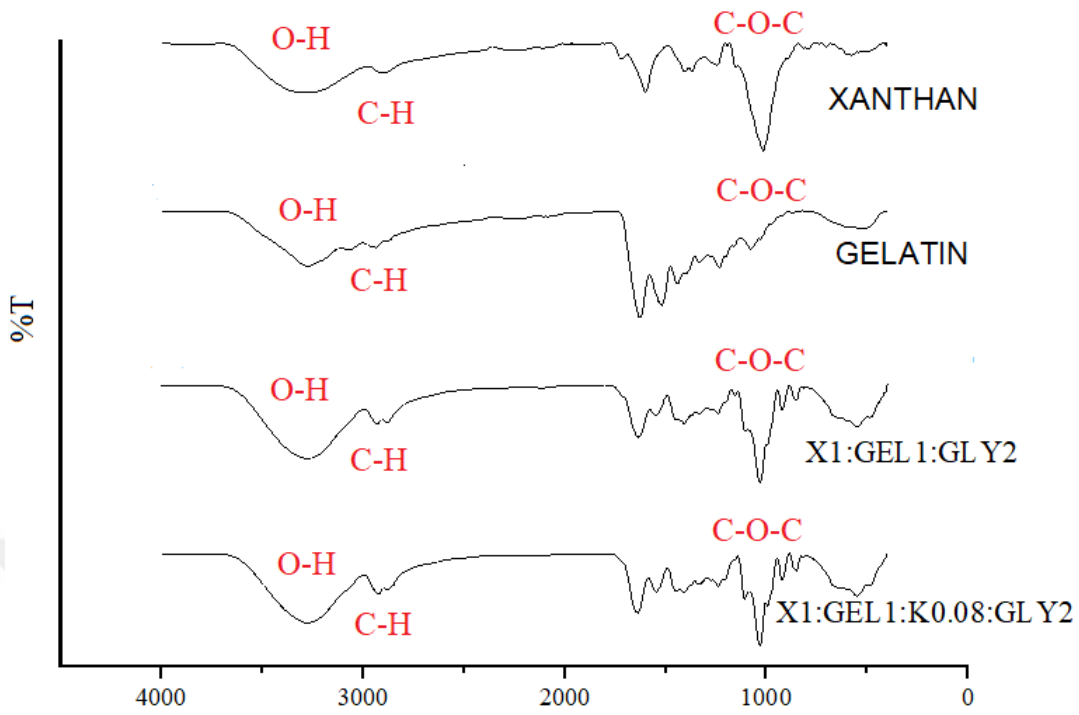

Figure 3. 17 FTIR analysis of xanthan, gelatin polymers, and X1:GEL1:GLY2, X1:GEL1:K0.08:GLY2

3.2.2.2 TGA and DSC Analysis of Keratin Xanthan Gelatin Hydrogels

For TGA analysis at 600˚C, the weight loss of hydrogels was higher than 80% for X1:GEL1:GLY1 and X1:GEL1:GLY2:K0.04 (Fig 3.18). The first decomposition stage for X1:GEL1:GLY2 was between 0˚C - 150˚C and X1:GEL1:GLY2 showed approximately 3% weight loss. The addition of keratin didn't cause a significant change in weight loss, which was 4% in the first decomposition temperature between 0-150˚C. Moreover, second decomposition range between 150˚C -400˚C, the weight loss percentage was 77% and 76% for X1:GEL1:GLY2 and X1:GEL1:GLY2:K0.04, respectively.

For DSC analysis, the addition of keratin didn't significantly change endotherm peaks which were observed at 58°C and 61°C for X1:GEL1:GLY1 and X1:GEL1:GLY2:K0.04. Moreover, it decreased the exoderm peak value from 263°C

to 257°C. The peaks higher than 240°C can be attributed to the thermal degradation of the keratin (Barone et al., 2005). In a conclusion, the addition of keratin didn't cause a significant change in TGA and DSC results.

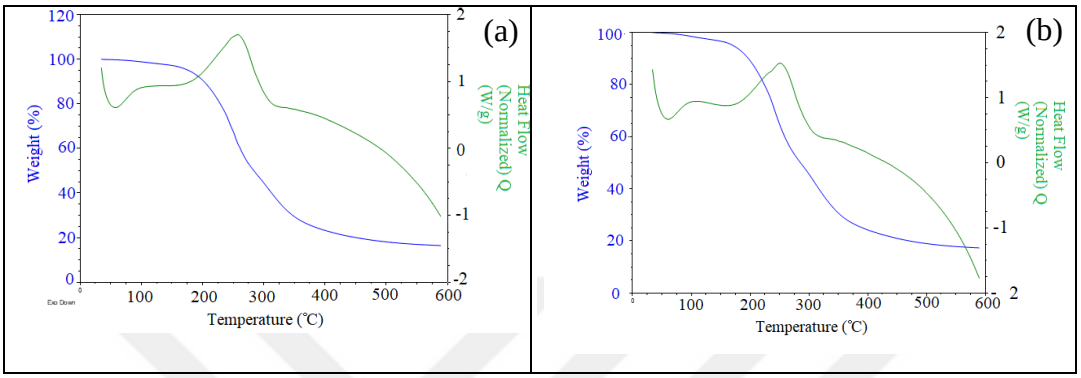

Figure 3. 18 TGA and DSC (exodown) spectra of (a) X1:GEL1:GLY2, (b) X1:GEL1:GLY2:K0.04

3.2.2.3 Weight Loss, Water Uptake Profiles and Cumulative Protein Release of Keratin Xanthan Gelatin Hydrogels

Swelling behavior and weight loss of the KXGHs were studied. When the keratin concentration was increased, the water uptake percent of hydrogel slightly decreased on 1st day (Fig 3.19). Compared to X1:GEL1:GLY2, all KXGHs had lower water uptake capacity on day 1 and day 3. The reason for lower water absorption could be the hydrophobic amino acids in keratin. Keratin includes 60% hydrophobic and 40% hydrophilic amino acids in its structure (Staroń et al., 2011). This result is convenient with the previous study which showed that the increase in the keratin amount decreased the water uptake capacity of alginate-keratin sponges (Hartrianti et al., 2017). The used keratin was isolated hair and hair contains hard α-keratins (Gillespie, 1990). It was claimed that hard α-keratins in water can swell less, which is controlled by the hydration state of their constituent intermediate filaments, which can be the reason for lower swelling in KXGHs (Greenberg & Fudge, 2013). The decrease of water uptake in KXGHs may be caused by the denser hydrogel structure

and hindering the reaction of active groups in the polymers with the water molecules (Pinkas et al., 2017).

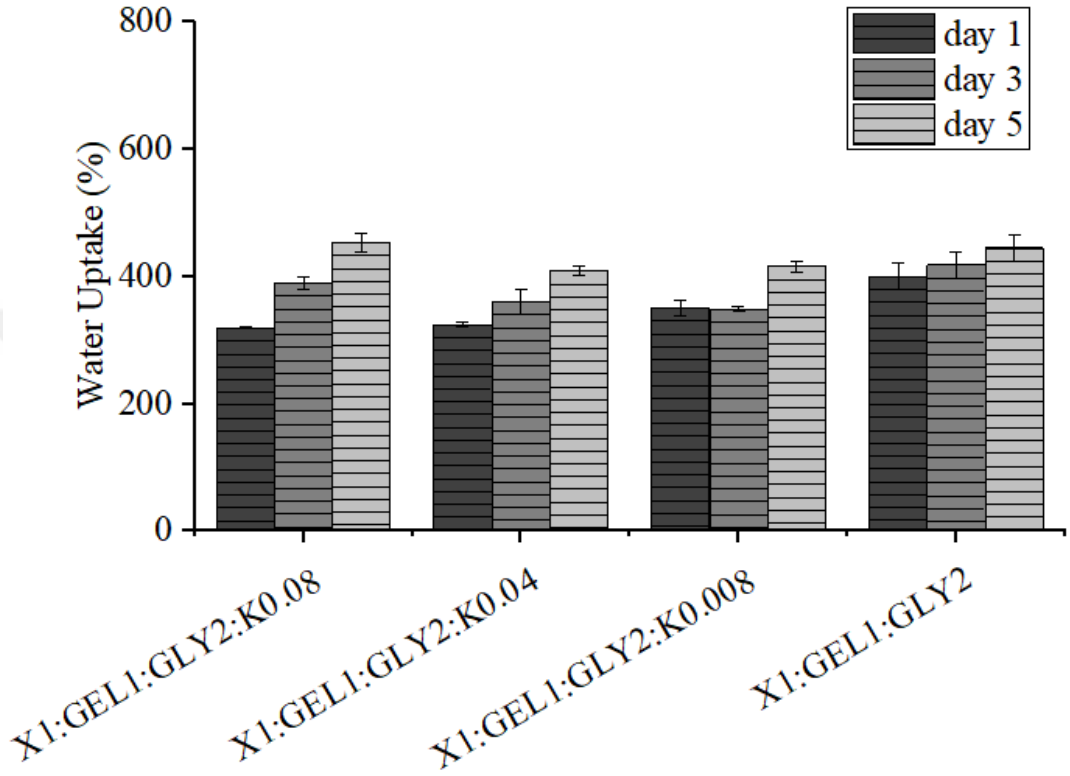

Figure 3. 19 Water uptake capability of KXGHs hydrogels in PBS (pH 7.4) at 37 °C (n=3)

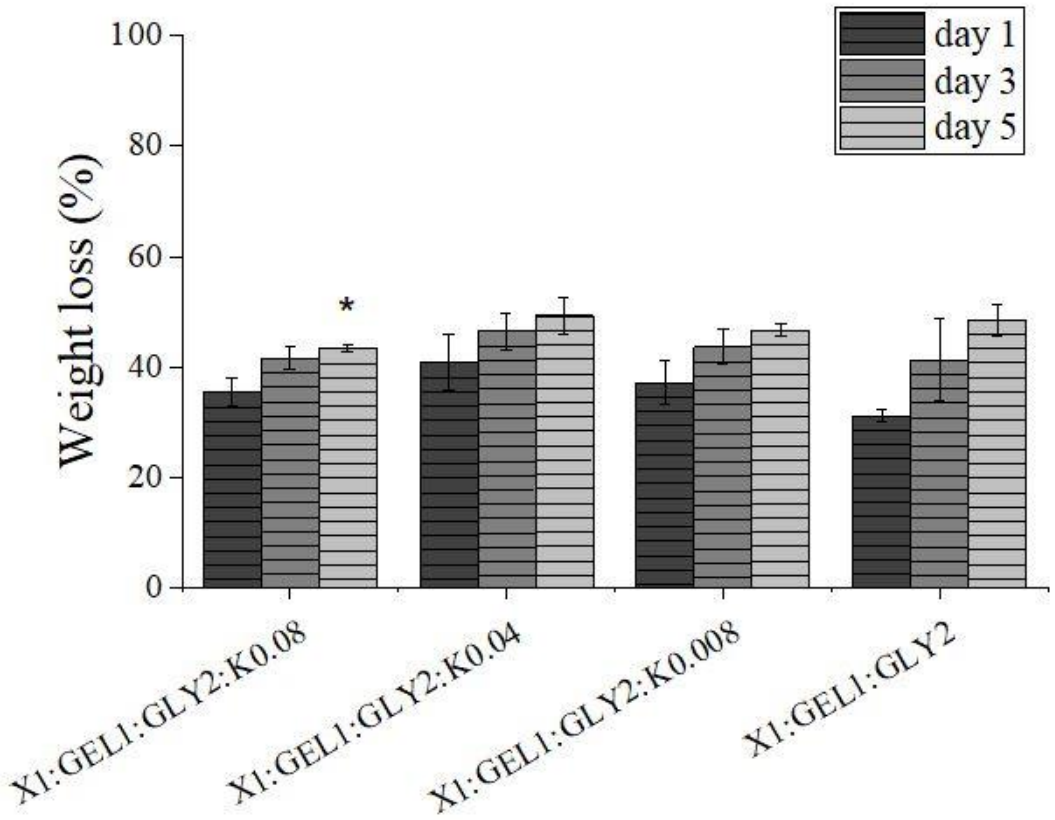

Figure 3. 20 Weight loss percentage of KXGHs in PBS (pH 7.4) at 37ºC for 5 days. *indicates the groups statistically different than other groups on day 5 (n=3).

In terms of percent weight loss, the presence of keratin concentration slightly increased the weight loss on 1st day (Fig. 3.20**)**. High molecular weight and higher amount of α-keratin in hair structure can cause weaker mechanical stability (Esparza et al., 2018). The α-keratins on the surface of KXGHs might have been released into the PBS media because of their unstable structure, which can be the reason for higher weight loss. Until 200 °C, XGHs and KXGHs showed lower than 15% weight loss. On the other hand, while the crystallinity of X1:GEL1:GLY2 was 15.6%, the presence of keratin decreased the crystallinity to 12.9%. A similar result was observed for Poly(ε-caprolactone)/keratin-based composite nanofibers. The increase of keratin higher than 10 wt %, caused lower crystallinity, which can cause enhanced degradation rate (Edwards et al., 2015).

However, on the 5th day, X1:GEL1:GLY2:K0.08 showed lower weight loss. This could be due to the higher keratin content which might have made the crosslinking network denser owing to carboxylic acid side groups of amino acids that can interact with glycerol, xanthan, and gelatin. It was claimed that in every keratin level the presence of higher cysteine can cause effective crosslink density. Moreover, the degradation of the hydrogel can be inversely proportional to cysteine level, in other words, a high cysteine level causes lower weight loss (Cao et al., 2019).

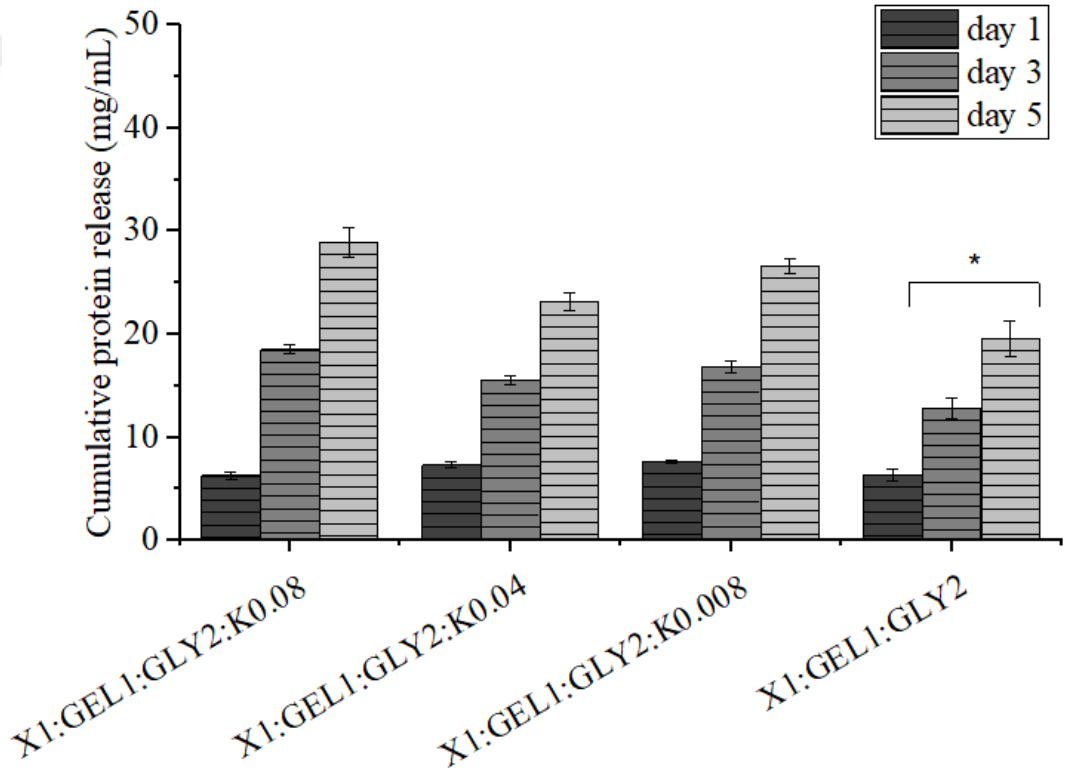

Figure 3. 21 Cumulative release of KXGHs in PBS (pH 7.4) at 37 °C *indicates the group statistically different than other groups (n=3).

As expected, the addition of keratin, compared to X1:GEL1:GLY2, increased the cumulative protein release. Higher protein release can be caused by the increase in total protein content. Moreover, it was claimed that the drug release rate depends on a positive relationship with gel degradation rate (Cao et al., 2019). Because KXGHs

showed slightly higher weight loss on the 1st day, it was expected to increase in terms of protein release as shown in Fig 3.21.

The difference in cumulative protein release can be caused by the relative difference in solubility between gelatin and keratin and their interactions with glycerol and xanthan. Moreover, the initial release can be related to the dissolution and rapid release of the surface protein of XGHs with initial swelling. Overall, the protein release of XGHs showed sustained release for 5 days, which can be reasoned by diffusion from the hydrated and swollen gel (Momoh et al., 2015).

Type A (acid) gelatin has a net negative charge at pH 7.4. Also, the isoelectric point of human hair is around 3.7, meaning that hair has a negative net surface charge at pH 7 (*CAS Reference Linking Service*, n.d.; Cruz et al., 2017). During weight loss and protein release studies, they were in PBS whose pH is 7.4 because of this, gelatin and keratin have similar charges and therefore they are prone to electrostatically repel each other. This situation can cause a higher cumulative release from KXGHs. A similar result was seen with electrically charged drugs loaded with alginate beads (Mumper et al., 1994).

3.2.2.4 Mechanical Tests of Keratin Xanthan Gelatin Hydrogels

Among KXGHs, X1:GEL1:GLY2:K0.04 had the highest TS compared to X1:GEL1:GLY2:K0.08 and X1:GEL1:GLY2:K0.008 as shown in Table 3.3. Moreover, X1:GEL1:GLY2:K0.04 had a higher TS value than X1:GEL1:GLY2, X1:GEL1:GLY3, X1:GEL2:GLY2. The presence of keratin increased both TS and EB. Among KXGH, only X1:GEL1:GLY2:K0.04 showed a statistically higher TS value. On the other hand, they didn't have a significant difference in terms of EB. The increase in TS can be caused by higher crosslinking. It was observed that the crosslinking might have caused higher TS for polymer-based and other graphene-based composites (Tao et al., 2017). Moreover, wool keratin/poly (vinyl alcohol) blended fiber had better mechanical properties when they contain 5 wt% keratin,

however, it showed a sharp decrease in the tensile strength at keratin concentration higher than 15% (Liu et al., 2018).

Table 3. 3 Tensile Strength (MPa) and Elongation at break (%) results of X1:GEL1:GLY2 and KXGHs (n=3)

Name of the group	**Ultimate tensile strength (MPa)**	**Elongation at break**
X1:GEL1:GLY2	0.226±0.01	0.706±3.7
X1:GEL1:GLY2:K0.08	0.386±0.05	0.98±40.03
X1:GEL1:GLY2:K0.04***	0.668±0.05	1.26±2.5
X1:GEL1:GLY2:K0.008	0.411±0.05	1.01±5.8

*** indicates the group statistically different than the groups in terms of TS in KXGHs

3.2.2.5 Water Vapor Transmission Rate of Keratin Xanthan Gelatin Hydrogels

The addition of keratin slightly increased the WVTR values of XGHs shown in Fig 3.22. Also, X1:GEL1:GLY2:K0.008 showed a statistical difference than other groups. The more hydrophilic nature of a polymer tends to increase water vapor permeation (Zhou et al., 2014). Because keratin is a hydrophilic polymer, it can increase the hydrophilicity of XGHs, which resulted in higher WVTR. Similarly, it was claimed that the addition of keratin made the PCL nanofiber more hydrophilic (Panpan Wu et al., 2018).

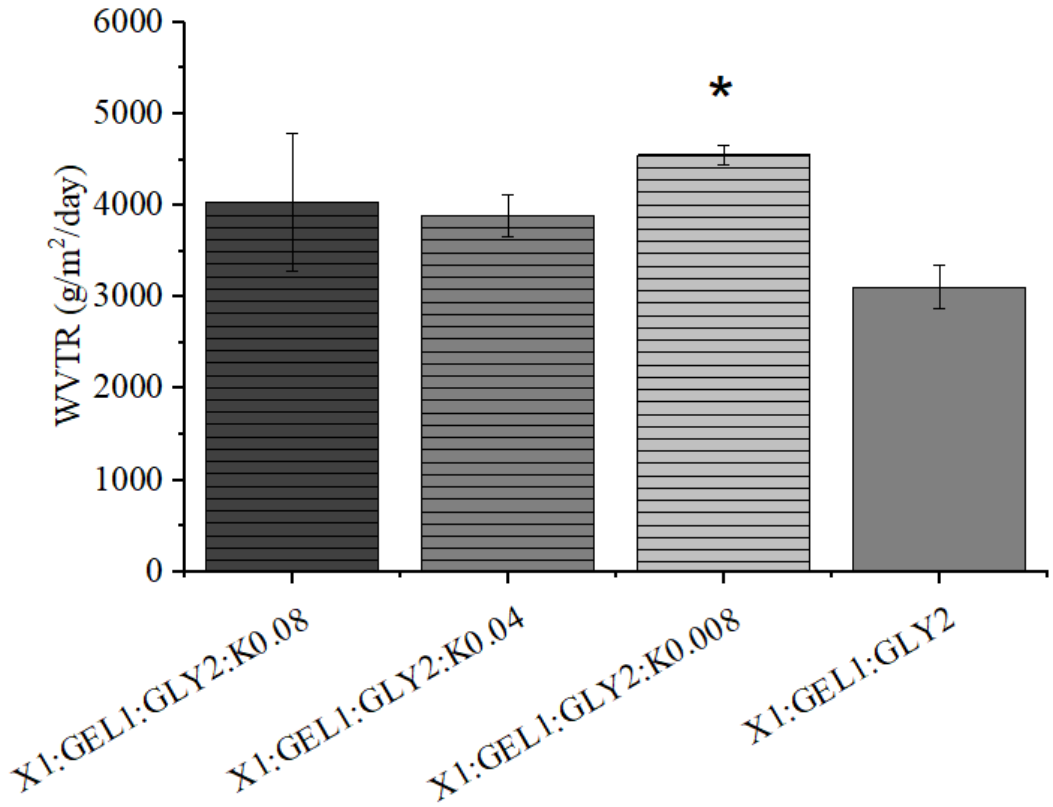

Figure 3. 22 WVTR values of KXGHs (n=3)

*indicates the group statistically different than other groups.

3.2.2.6 Oxygen Permeation Analysis of Keratin Xanthan Gelatin Hydrogels

Oxygen has a role not only in multiple wound healing stages but also in bacteria-killing, reepithelization, and collagen synthesis (Knighton et al., 1983). That is the reason why it was observed if the keratin concentrations affect oxygen permeability. The addition of keratin didn't affect the oxygen permeability of XGHs significantly as shown in Fig 3.23. It can be explained that keratin is permeable to dissolved oxygen. Oxygen permeability was observed for keratin membranes (Aiba et al., 1985). As a result, xanthan gelatin hydrogels can be considered permeable to oxygen (Khorasani et al., 2019).

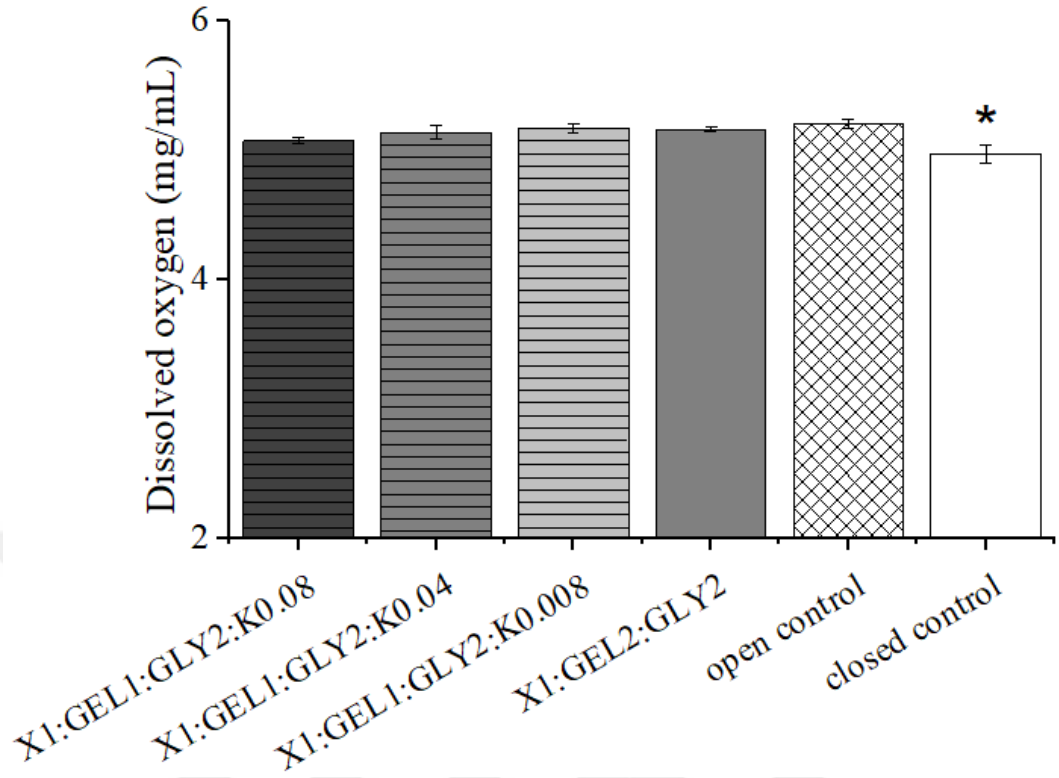

Figure 3. 23 Oxygen permeability of KXGHs

*indicates closed (positive) control is different than other groups (n=3).

3.2.2.7 Cytotoxicity Tests of Keratin Optimization and Keratin-Xanthan-Protein Hydrogels

In the literature, different keratin concentrations were studied for testing dose-dependent cytotoxicity. It was reported that 0.042g/mL keratin–chitosan and keratin–gelatin composite scaffold showed cell proliferation on the scaffold with improved cell attachment (Balaji et al., 2012). In another study, 10, 15, and 20 w/v% keratin hydrogels were shown to facilitate cell proliferation, which proves the biocompatibility of keratin hydrogel (Cao et al., 2019). Different percentage weight ratios, 10:0, 10:2, 10:3, 10:4, 10:5, and 10:6 (gelatin: keratin), were studied to produce gelatin keratin glue (Thirupathi Kumara Raja et al., 2013). 0%, 2%, and 4%

keratin containing PLA/chitosan/keratin composites were designed for biomedical applications (Tanase & Spiridon, 2014). 10 mg/ml keratin solution was used by mixing with 1 M $CaCl_2$ solution at a ratio of 50:1 (v/v) to produce keratin hydrogel (Wang et al., 2012). 10% w/v keratin poly(vinyl alcohol) nanofiber solution was produced and results showed that 20% keratin showed superior biological properties and a higher surface area to volume ratio than 30% (C. C. Lin & Anseth, 2009). In previous studies, because different keratin concentrations were tested for films hydrogels, and foams, we tried to find the optimum keratin concentration for XGHs. %20, %10, %4, %2 and 0.4% (w/w) keratin concentration containing hydrogels were produced. (%20, %10, %4, %2 and 0.4% (w/w) keratin concentrations correspond to X1:GEL1:K0.4:GLY2, X1:GEL1:K0.2:GLY2, X1:GEL1:K0.08:GLY2, X1:GEL1:K0.04:GLY2 and X1:GEL1:K0.008:GLY2 respectively. To optimize keratin concentration for XGHs, different keratin concentrations were tested in terms of mechanical and chemical properties.

Then, sterilized KXGHs hydrogels were kept in a cell culture medium at 37°C for 24h. Media extracts were used on fibroblasts and cell viability was analyzed by Alamar Blue assay as shown in Fig 3.24. On 1st day, %20 and %10 keratin (w/w) showed cytotoxicity on L929 fibroblast cells. On the other hand, %4, %2, and 0.4% (w/w) keratin concentrations caused a boosted proliferative effect on cells. However, on 4th day, %4 (w/w) keratin concentration started to show the cytotoxic effect. Also, while %2 (w/w) keratin concentrations continued to show boost effect and 0.4% (w/w) didn't caused boosted proliferation. On 7th day, both 2% (w/w) and 0.4% (w/w) didn't cause toxic effect. The microscopic views of fibroblasts are presented in Fig 3.25.

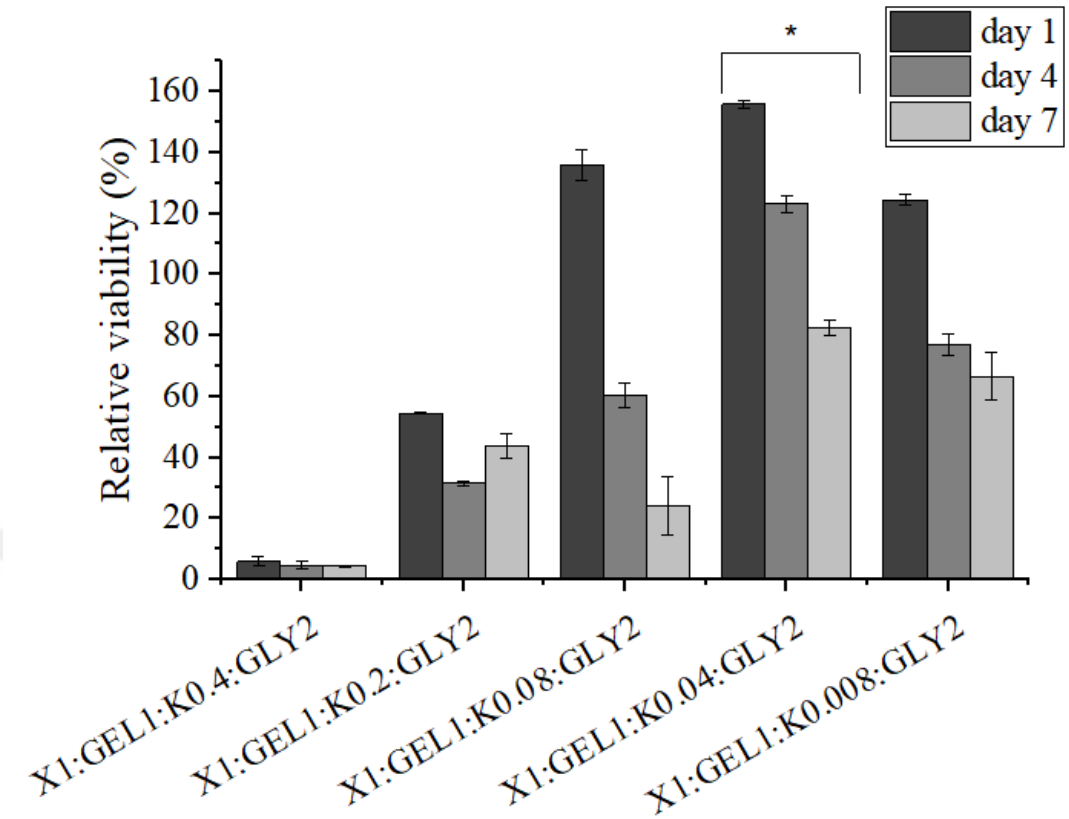

Figure 3. 24 Effect of XGHs containing different concentrations of keratin on the viability of the fibroblasts *indicates the group statistically different than the other groups (n=3).

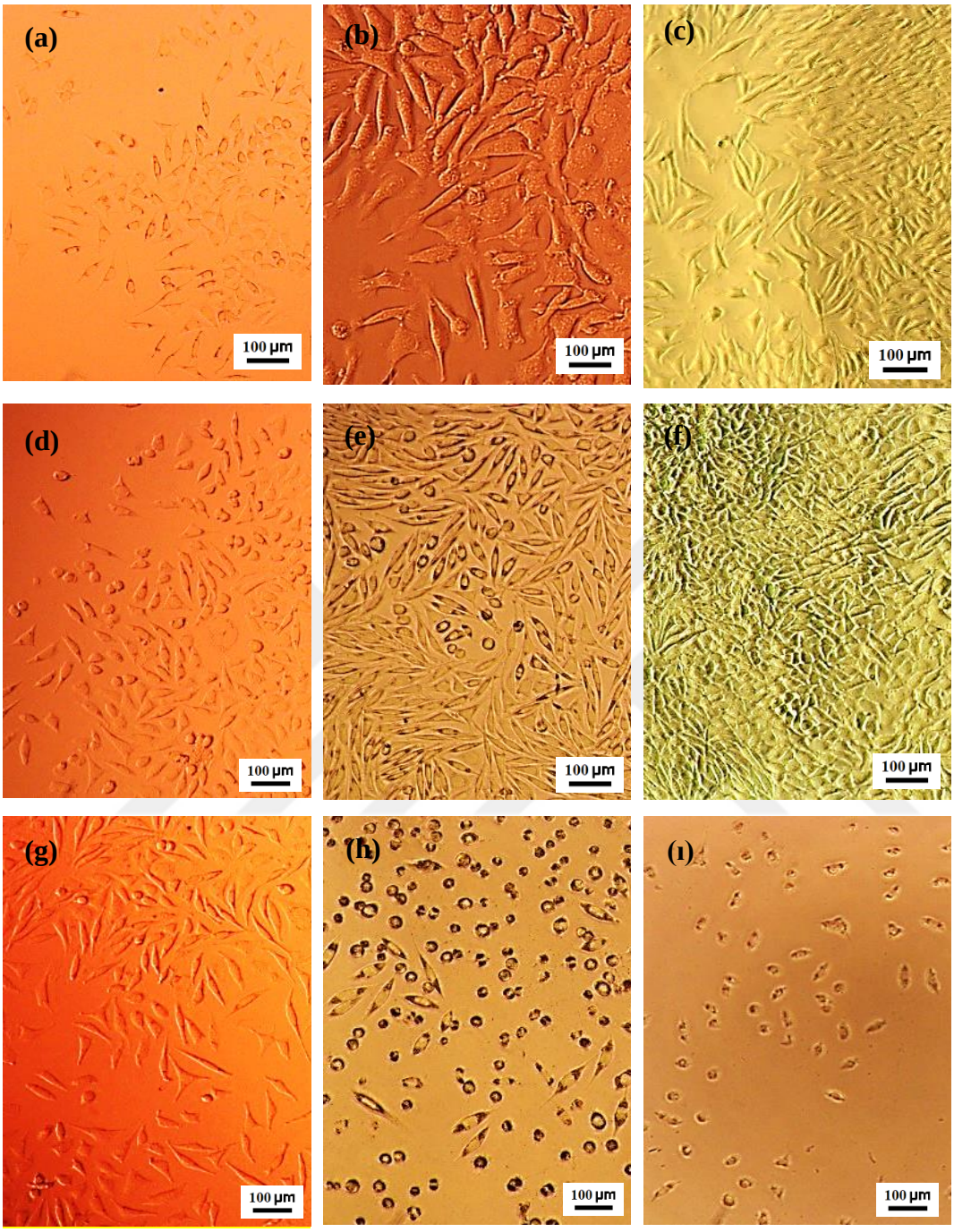

Figure 3. 25 Phase contrast micrographs of the cells which were treated with the extract of different keratin containing XGHs (a-b-c) X1:GEL1:K0.008:GLY2 on 1st, 4th and 7th days, (d-e-f) X1:GEL1:K0.04:GLY2 on 1st, 4th and 7th day, (g-h-ı) X1:GEL1:K0.08:GLY2 on 1st, 4th and 7th day.

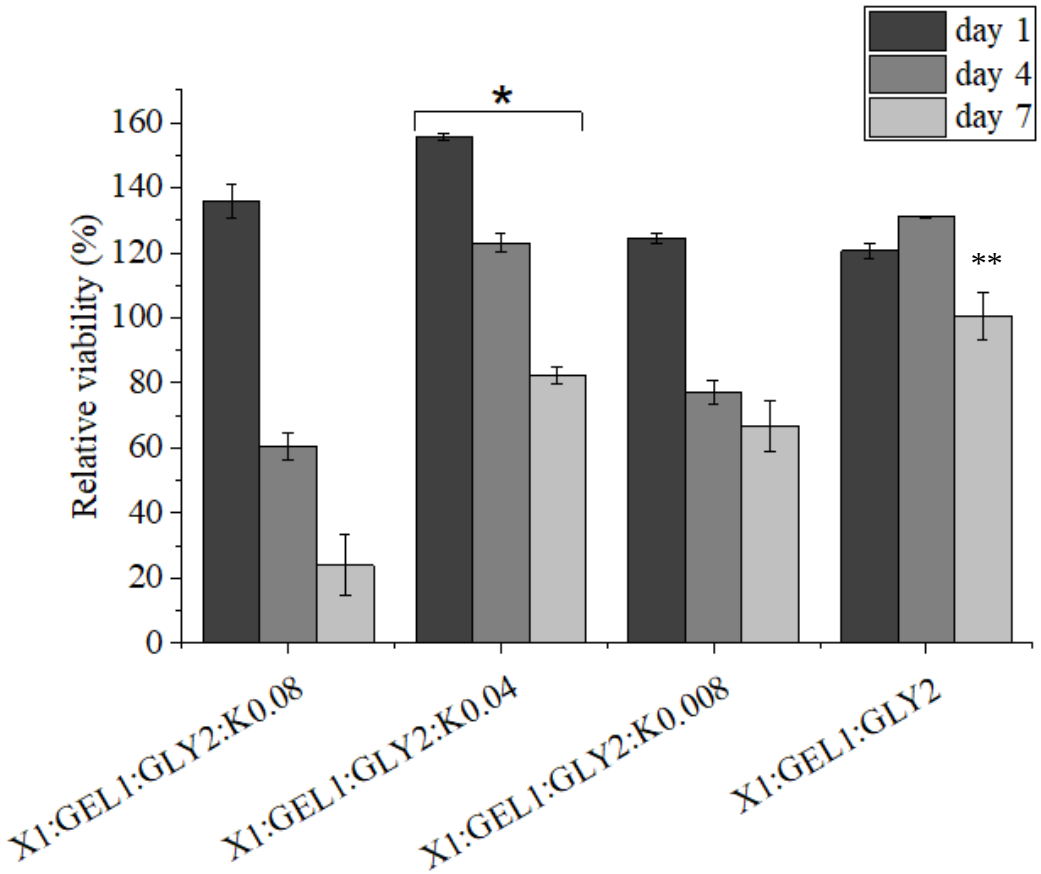

Figure 3. 26 Effect of the hydrogels with different keratin concentrations on the viability of fibroblasts (L929) (n=3).

* indicates the group which is different than other KXGHs at all time points, **indicates the group statistically different than other groups on day 7

Cell culture studies were conducted to test the cytocompatibility of keratin containing hydrogels (Fig 3.26). X1:GEL1:K0.04:GLY2 group showed higher cell proliferation than other groups. On 1st day, X1:GEL1:K0.08:GLY2, X1:GEL1:K0.04:GLY2 and, X1:GEL1:K0.008:GLY2 showed 135+5.1%, 153.3±1.05%, and 124.5±1.6% relative cell viabilities, respectively. However, on the 4th day, X1:GEL1:K0.08:GLY2 (60.3%±4.09) showed a cytotoxic effect on the fibroblasts. On 7th day, X1:GEL1:K0.008:GLY2 showed 76.9%±3.7, however, a

15% reduction in cell viability can be acceptable for wound dressing applications (W. C. Lin et al., 2013). Compared to other groups, X1:GEL1:GLY2:K0.04 showed statistically highest cell viability on 1st day. On the 4th day, X1:GEL1:GLY2:K0.04 showed higher cell viability than other KXGHs and similar cell viability to X1:GEL1:GLY2. For KXGHs, the optimum keratin concentrations in hydrogel enhanced the proliferation of cells. These results are parallel with the previous study. Wang et al. reported that keratin hydrogel significantly increased L929 mouse fibroblasts proliferation and they can be a template for skin regeneration applications(S. Wang et al., 2012).

The cytotoxic effect of X1:GEL1:K0.08 can be caused by higher keratin released into the media. The released keratin may increase the viscosity of the feeding medium, high viscous medium can hinder the nutrient diffusion to the cells (Ulubayram et al., 2002).

3.3 Preparation and Characterization of VC Containing Keratin Xanthan Gelatin Hydrogels

3.3.1 Weight Loss, Water Uptake and Cumulative Protein Release Profiles of VC-Containing Keratin Xanthan Gelatin Hydrogels

Immersion of hydrogel into VC solution may increase the hydrophilicity of KXGHs due to VC hydrophilic nature, which resulted in higher swelling capacity. This result is convenient with the previous study that showed the enhancement in hydrophilicity (especially surface of membrane) also increases the water uptake (Rossi et al., 2017). On the 1st and 3rd days, X:GEL:GLY:K/VC0.05 showed higher swelling capacity than the other groups.

It was observed that VC immersion caused both higher swelling and cumulative protein release compared to X1:GEL1:GLY2 as shown in Fig 3.27-Fig 3.29. Protein release can be related to swelling ability. Reduced crosslink density showed higher water uptake ability, which results in higher protein release. Oppositely, better cross-linked hydrogels have slower erosion process, make the entrance of water molecules more difficult, and decrease the rate of protein release, which was observed in chitosan/pectin particles (Chang & Lin, 2000). X:GEL:GLY:K/VC0.05 showed statistically higher swelling on 1st and 3rd day. The reason for the higher swelling can be caused by water entrance during immersion. As the water molecule is polar, it can form hydrogen bonds with hydroxyl groups, by disrupting interchain hydrogen bonds (Deanin R.D., 1972; Kwei, 1966).

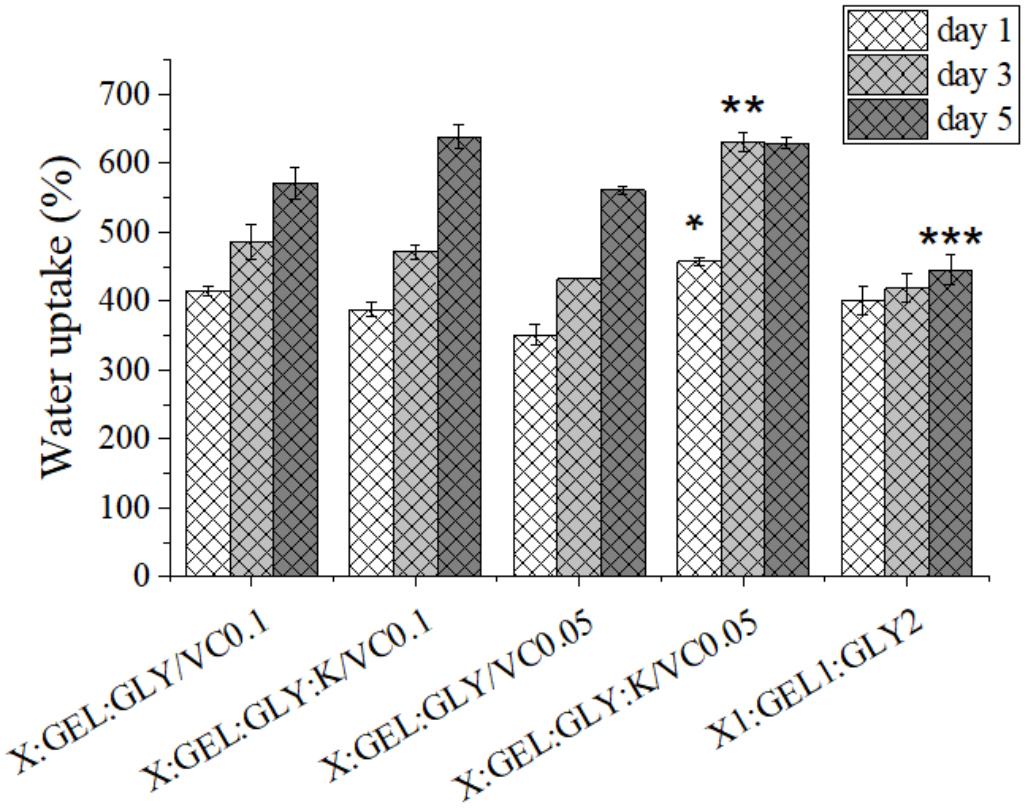

Figure 3. 27 Water uptake capability of the hydrogels in PBS (pH 7.4) at 37 ºC * indicates the groups statistically different than other groups on 1st day, ** indicates the groups statistically different than other groups on 3rd day, *** indicates the groups statistically different than the other groups on 5th day(n=3).

In terms of weight loss X:GEL:GLY/VC0.1, X:GEL:GLY:K/VC0.1, and X:GEL:GLY/VC0.05 didn't show a significant difference from each other on the 1st day, however, they showed higher weight loss than X:GEL:GLY:K/VC0.05 as shown in Fig 3.28.

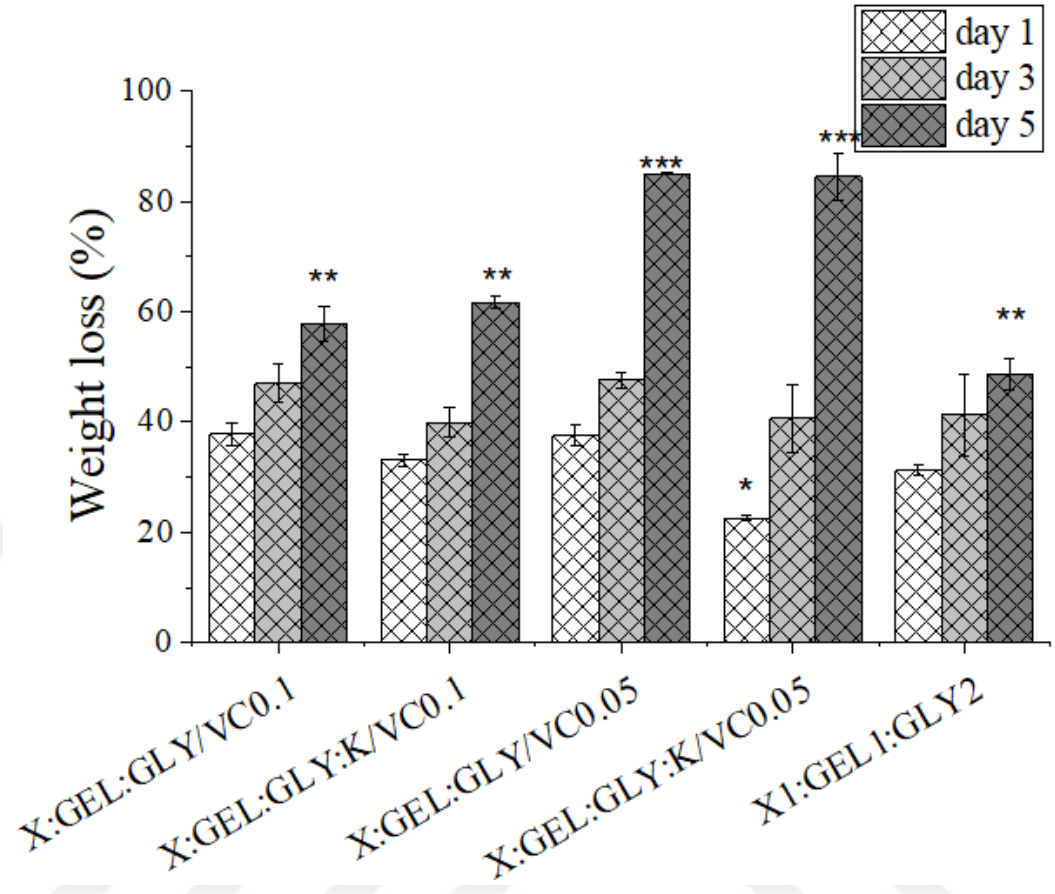

Figure 3. 28 Weight loss percentages of VC-KXGHs in PBS (pH 7.4) at 37 °C *indicates the group statistically different than the other groups on 1st day.** indicates groups different than X:GEL:GLY/VC0.05 and X:GEL:GLY:K/VC0.05 on 5th day *** indicates groups statistically different than X1:GEL1:GLY2 on 5th day. (n=3)

X:GEL:GLY/VC0.1 and X:GEL:GLY/VC0.05 showed slightly higher weight loss than X1:GEL:GLY2 on 1st day. Moreover, all VC-containing groups showed higher weight loss than X1:GEL1:GLY2 on 5th day. X:GEL:GLY/VC0.05 and X:GEL:GLY:K/VC0.05 caused higher weight loss than X:GEL:GLY/VC0.1 and X:GEL:GLY:K/VC0.1 on 5th day.

During immersion, hydroxyl ions bind to carboxyl groups of the polymer. In the higher VC concentration, more hydroxyl groups can bind to more carboxyl groups in the hydrogel and decrease the available carboxyl groups in hydrogels. On the other hand, in lower VC concentration, because of fewer hydroxyl groups, there can be more carboxyl groups that are available to form bonds. Water molecular in the environment is prone to bind these available carboxyl groups rather than hydroxyl. A previous study claimed that the formation of free carboxylic acid groups can enhance the solubility of polymer (Ramirez-Barron et al., 2021). This situation can cause higher swelling and weight loss.

In terms of cumulative protein release, X1:GEL1:GLY2 showed lower release than other VC-KXGHs and VC-XGHs as shown in Fig 3.29. One of the reasons for this can be higher weight loss. Higher weight loss can be the reason for the higher gelatin and keratin release environment, which increases the protein concentration in the PBS solution. The higher swelling may cause a higher protein release because when the polymer network swells, the network becomes larger, which can make the release easier (am Ende et al., 1995)

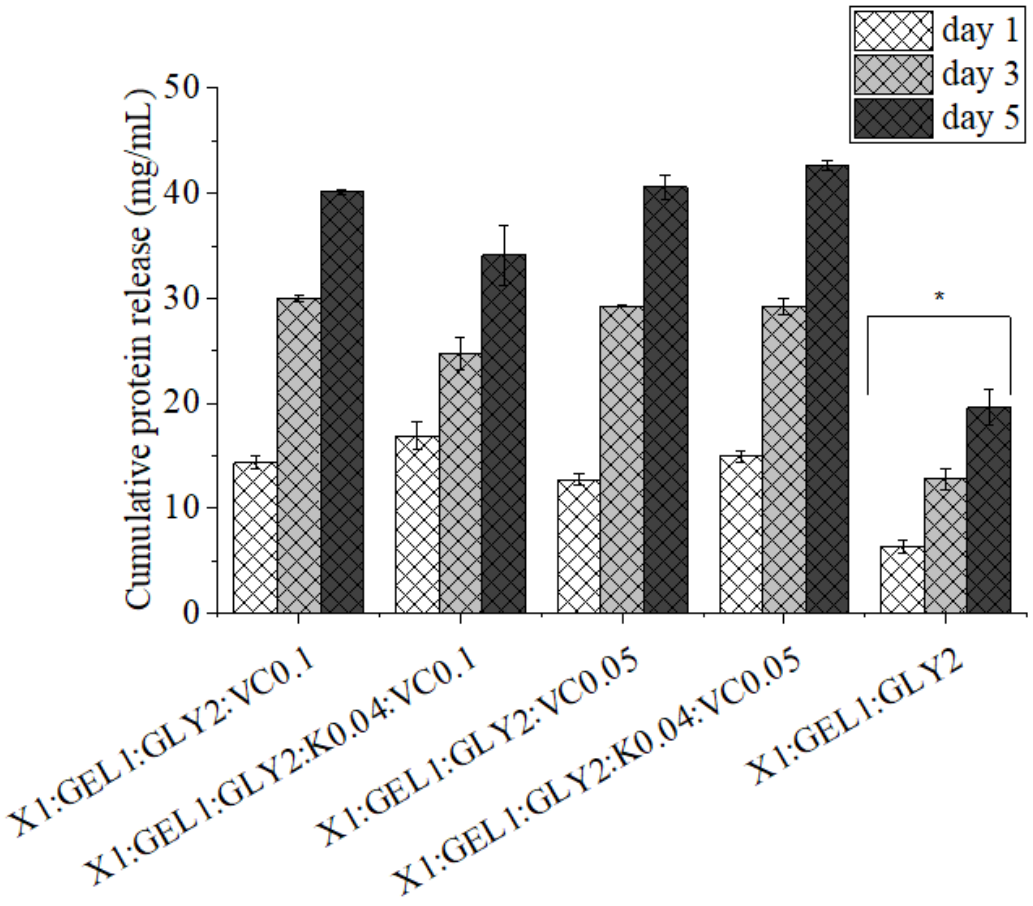

Figure 3. 29 Cumulative protein release from the hydrogels in PBS (pH 7.4) at 37 °C

*indicates the groups statistically lower than the other groups at all time points (n=3).

3.3.2 Water Vapor Transmission Rate of VC Containing Keratin Xanthan Gelatin Hydrogels

After VC immersion, water vapor transmission rates of hydrogel groups were similar (Fig 3.30). The reason for this can be that VC didn't act like a crosslinker. The higher crosslinking decreases the space between macromolecules, resulting in reduced WVTR (Parris et al., 1995). The lower WVTR was observed with an increasing crosslinking degree for films that were produced by immersing Ca solution (Pavlath et al., 1999). The presence of glycerol as a plasticizer decreases the density of the

protein network becomes more permeable. The free volume of the polymer network eases solvent mobility, makes the diffusion of water into the matrix easier. Moreover, the hygroscopic feature of the plasticizers, which causes higher water content may enhance the mobility of the molecules. Higher water may be a factor that affects the permeate solubility (Sobral et al., 2001). The presence of higher free carboxyl groups in X1:GEL1:GL2:K/VC0.05 can enhance WVTR because carboxyl and hydroxyl functional groups increase the hydrophilicity (Y. Li et al., 2011)

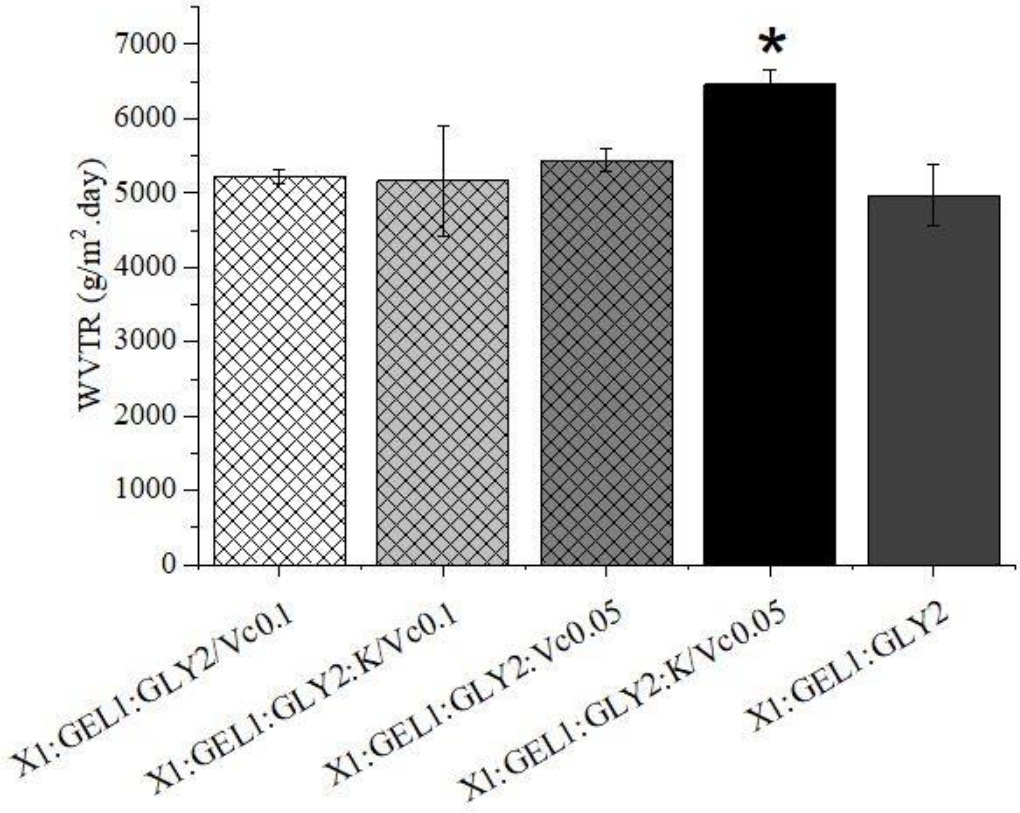

Figure 3. 30 WVTR of VC-KXGHs and XGH.

*indicates the groups statistically different than X1:GEL1:GL2:K/VC0.1 and X1:GEL1:GLY2 (n=3)

3.3.3 Cytotoxicity Test of VC Containing Keratin Xanthan Gelatin Hydrogels

On 1st day, X1:GEL1:GLY2/VC0.1 showed higher cell viability compared to X1:GEL1:GLY2/VC0.05 as presented in Fig 3.31. Depending on release results, it was observed that X1:GEL1:GLY2/VC0.1 had a higher release percent compared to the other groups. The higher cell viability can be caused by exposure to higher VC which was released by the X1:GEL1:GLY2:VC0.1. It was proved that the physiological concentration of Vitamin C (60 μM) stimulates not only DNA-synthesis but also proliferation in endothelial cells (Ulrich-Merzenich et al., 2007). On the other hand, a high dose of VC can cause a toxic effect on neural stem/progenitor cells and DNA damage (Kim et al., 2018). Furthermore, X1:GEL1:GLY2:K/VC0.1, X1:GEL1:GLY2/VC0.05, and X1:GEL1:GLY2:K/VC0.05 didn't show a significant difference on 1st day. Besides, on 4th day, the highest cell viability belonged to X1:GEL1:GLY2:K/VC0.1. The reason can be the increased VC release in X1:GEL1:GLY2:K/VC0.1. On 7th day, the lowest relative cell viability was seen in X1:GEL1:GLY2:K/VC0.05. Even though X1:GEL1:GLY2:K/VC0.05 caused lower viability compared to other groups, its relative viability percentage was 92.7%+2.1. Depending on results, it was concluded that VC containing KXGHs and XGHs are biocompatible. Phase-contrast images of the cells are presented in Fig 3.32.

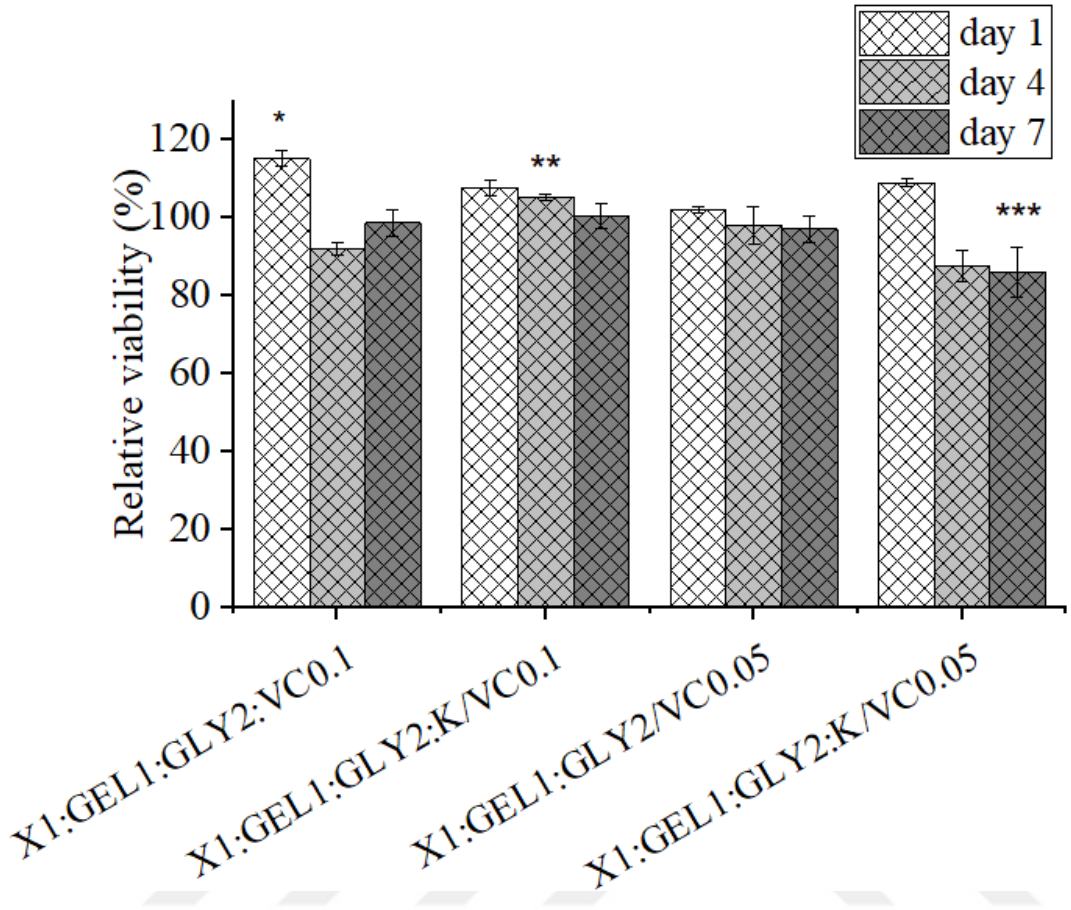

Figure 3. 31 Viability of fibroblasts (L929) exposed to the extract media of VC-KXGHs (n = 3). * indicates the group statistically different than X1:GEL1:GLY2/VC0.05 and control,**indicates the group statistically different than X1:GEL1:GLY2/VC0.1 and X1:GEL1:GLY2:K/VC0.05, ***indicates the group statistically different than other groups on 7th day.

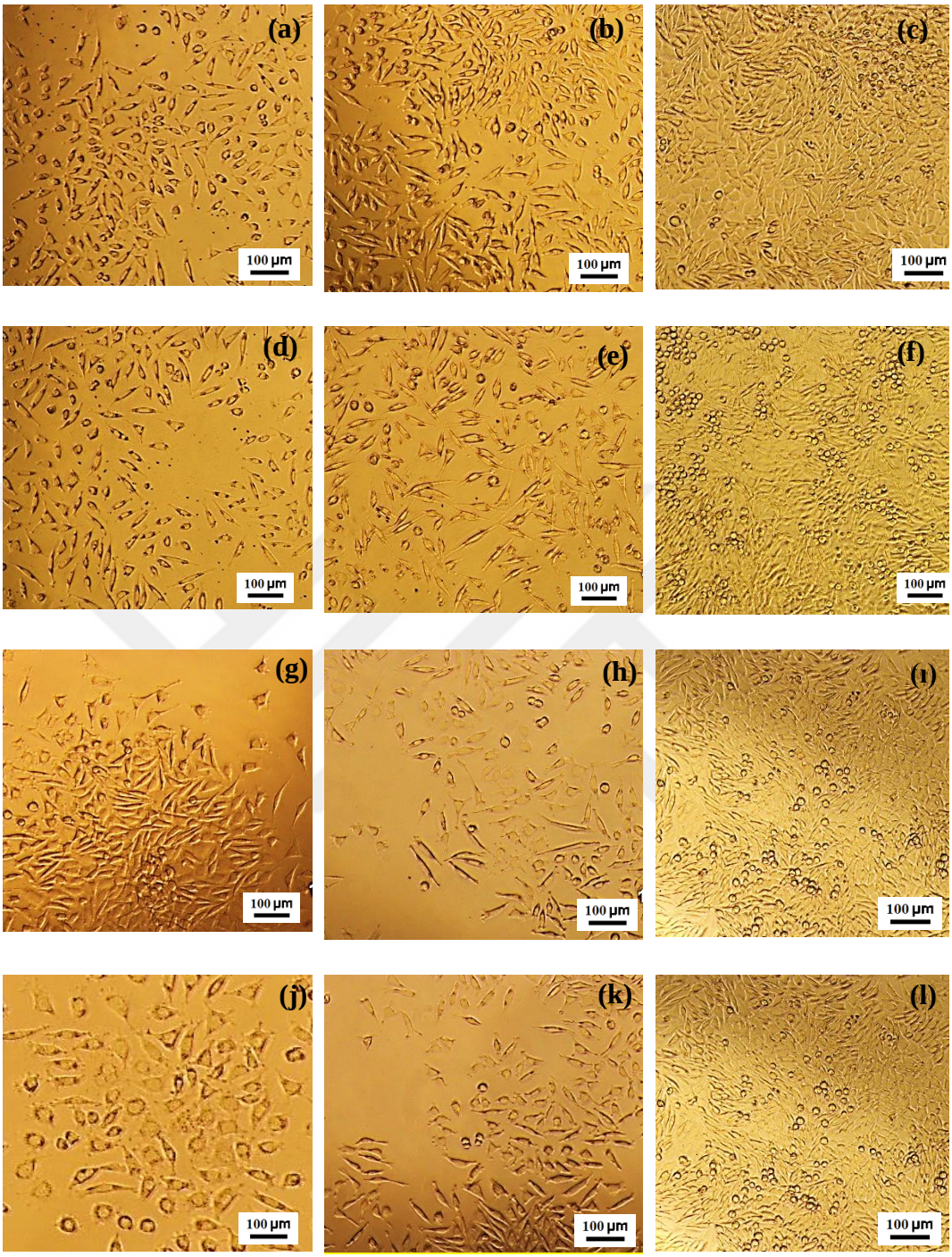

Figure 3. 32 Phase contrast micrographs of cells treated with the extract of VC containing hydrogels (a-b-c) X1:GEL1:GLY2/VC0.1, (d-e-f) X1:GEL1:GLY2:K/VC0.1, (g-h-ı) X1:GEL1:GLY2/VC0.05, (j-k-l) X1:GEL1:GLY2:K/VC0.05 on 1st, 4th, and 7th day, respectively.

3.3.4 Vitamin C Release from VC Containing Keratin Xanthan Gelatin Hydrogels

It was claimed that the drug release from hydrogel is dominated by the rate of water diffusion, crosslinking density, and interchain interaction (Brazel & Peppas, 1999; W. F. Lee & Chen, 1998). The VC release from KXGHs supplies information about its ability to provide local controlled drug release. Drug-loaded hydrogels can release the drug due to their swelling mechanism which causes relaxation of polymeric segments and diffusion of water into the polymer network (Dengre et al., 2000). Fig 3.33 represents in vitro VC-release profile of the KXGHs at 37 °C in a phosphate buffer (pH 7.4). The rate of VC release was faster in one hour. The reason for the faster release can be the contact between the outer surface of KXGHs and PBS because of the entrance of the PBS into the polymer network. The polyelectrolyte nature of xanthan and its acidic feature makes it less soluble in lower pH (Talukdar & Kinget, 1995). At pH 7.4, in PBS, the number of ionized acid groups is higher and electrostatic repulsion between xanthan chains is enhanced with increased solubility of xanthan, which can make its chains more flexible when water interacts (Mikac et al., 2010). Increased solubility of a polymer may increase also the rate of release of the agent (Carbinatto et al., 2014). The higher release rate can be caused by the molecular weight of VC. The study about drug transport in crosslinked polymeric materials proved that the rate of release increased with decreasing solute molecular weight (Brazel & Peppas, 1999). During the release of VC, the environment of the medium may become basic depending on the released hydroxyl group. The increase of pH may help the release of VC because ionized carboxyl groups can cause repulsion thanks to similar charges and swelling (Khalid et al., 2009).

The swelling property of hydrogels affects the release behavior of both drugs and nutrients in medical/food engineering applications (X. Hu et al., 2019). Moreover, the degree of swelling can affect the diffusion of solute, surface mobility, and mechanical properties (Nicholas A. Peppas & Hoffman, 2013). As shown in Fig 3.27, the VC immersion of KXGHs increased the water uptake percentage. This can

be caused by the hydrophilic nature of VC enhanced the hydrogel affinity to water molecules. In this situation, the higher water uptake may increase water transfer, which creates a driving force to assist VC diffusion from KXGHs (S. H. Park et al., 2018). VC release was gradually increased. While the swelling ability of a neutral hydrogel depends on the polymer-solvent mixing, the swelling of polyelectrolyte hydrogels can be explained by environmental conditions such as pH and ions belonging to ionizable groups of the hydrogel network (Brannon-Peppas & Peppas, 1991; Flory & Rehner, 1943). VC released from salecan and chitosan hydrogel showed that the release of VC depends on the pH of the media. In pH 1.2 media, protonated carboxyl groups can form hydrogen bonds, which make the polymer network denser, thus prevent VC release from KXGHs. On the other hand, in pH 6.8, the VC release rate enhanced and reached 92.3 % (Xinyu Hu et al., 2020). In our experiment, PBS was selected as the media whose pH is 7.4 (close to 6.8) thus can be a reason for a higher percent of VC release. Except for X1:GEL1:GLY2:K/VC0.1, all VC-KXGHs showed rapid initial release, which can be reasoned by a crosslinked network of the hydrogel that can't control the surface VC release. When VC-KXGHs were subject to the medium, the medium can let free dissolution of VC (B. J. Kong et al., 2016).

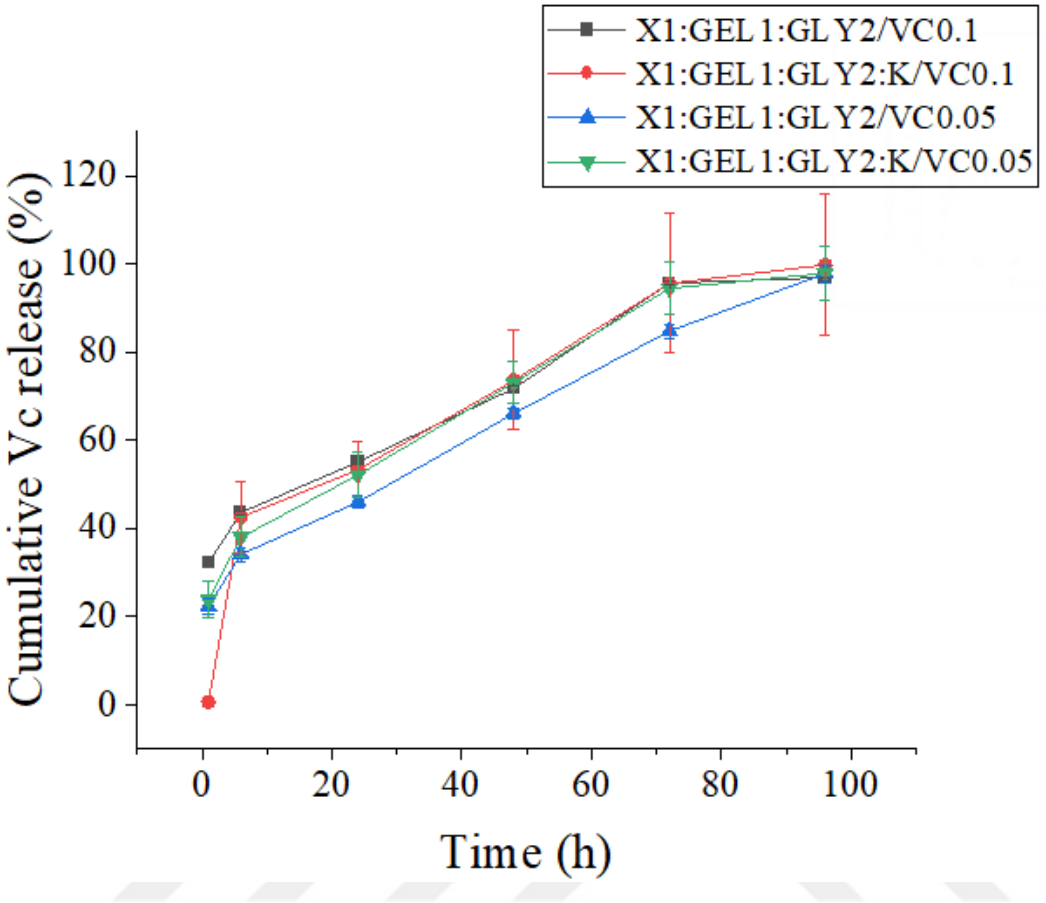

Figure 3. 33 *In vitro* Vitamin C release profile of the hydrogels in PBS at 37 °C (n=3).

3.3.5 Collagen Staining

In skin health, Vitamin C has a role similar to the co-factor for the proline and lysine hydroxylases, which promotes not only collagen synthesis but also stabilizes the collagen tertiary structure (Pullar et al., 2017). VC-XGHs and VC-KXGHs released VC to cell media, it was expected that VC release will increase the collagen synthesis of fibroblasts. After cells were seeded into 96 well plates, they were incubated with the extract of VC-XGHs and VC-KXGHs. At the end of 4^{th} and 7^{th} days, L929 fibroblast cells were stained by Direct Red 80 staining. Direct Red 80, which is an anionic dye, binds to the [Gly-X-Y] triple helical structure in collagen fibrils. (Commonly X corresponds to proline, Y is attributed either hydroxyproline or

hydroxylysine) (Junqueira et al., 1979). It was observed that VC solution increased the collagen synthesis of the cells, compared to the control groups as shown in Fig 3.35. On 4th day, while the control groups' stained area was 30%$\pm$2, collagen stained areas were 53%$\pm$4, 49%$\pm$9, 47%$\pm$7, and 41%$\pm$3 for X1:GEL1:GLY2/VC0.1, X1:GEL1:GLY2:K/VC0.1, X1:GEL1:GLY2/VC0.05, and X1:GEL1:GLY2:K/VC0.05, respectively. On 7th day, the percent of stained areas were 60%$\pm$9.5, 61%$\pm$3, 59.8%$\pm$2.1, 63.9%$\pm$2.5, and 41.8%$\pm$3 for X1:GEL1:GLY2/VC0.1, X1:GEL1:GLY2:K/VC0.1, X1:GEL1:GLY2/VC0.05, X1:GEL1:GLY2:K/VC0.05, and control group, respectively. The control group showed a statistically lower stained area than the other groups on 4th and 7th days ($p<0.05$) as shown in Fig 3.34. X1:GEL1:GLY2/VC0.1 showed a statistically higher stained area than X1:GEL1:GLY2:K/VC0.05 on 4th day. The reason for this can be X1:GEL1:GLY2/VC0.1 showed statistically higher relative cell viability than other groups on 1st day than others and X1:GEL1:GLY2:K/VC0.05 caused lower relative cell viability on 4th and 7th days than other groups. Moreover, X1:GEL1:GLY2/VC0.1 showed higher VC release than the other groups. After the first release, all groups showed similar release behavior. On 4th day, the higher collagen stained area for X1:GEL1:GLY2/VC0.1 can be caused by higher VC release and higher relative cell viability. On 7th day, no statistical difference was observed between VC-XGHs and VC-KXGHs. In conclusion, these results showed that VC containing XGHs and KXGHs increased the collagen synthesis in L929 fibroblast cells. A microscopic view of red-stained collagens on L929 fibroblast cells is shown in Fig3.35. The result was convenient with the previous study which showed that hyaluronic acid and collagen sponges including vitamin C derivative enhanced collagen deposition, compared to the control group in vivo (diabetic mice)(Niiyama & Kuroyanagi, 2014). Furthermore, polysaccharide film loaded with VC and propolis also showed enhanced wound healing in vivo (diabetics/non-diabetics mice) with homogeneous collagen distribution (Voss et al., 2018).

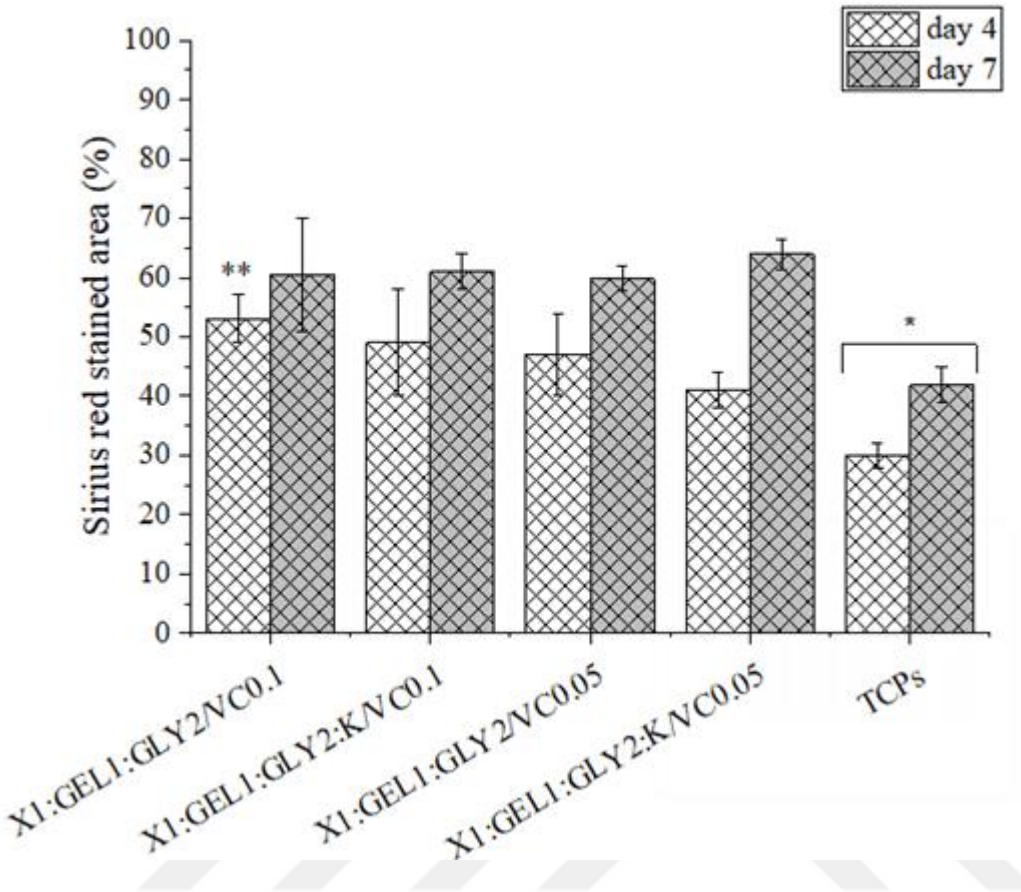

Figure 3. 34 Percent stained area for collagen on the wells seeded with fibroblasts on 4^{th} and 7^{th} days * indicates the group statistically different than the other groups on 4^{th} and 7^{th} day ** indicates the group statistically different than X1:GEL1:GLY2:K/VC0.05 on 4^{th} day.

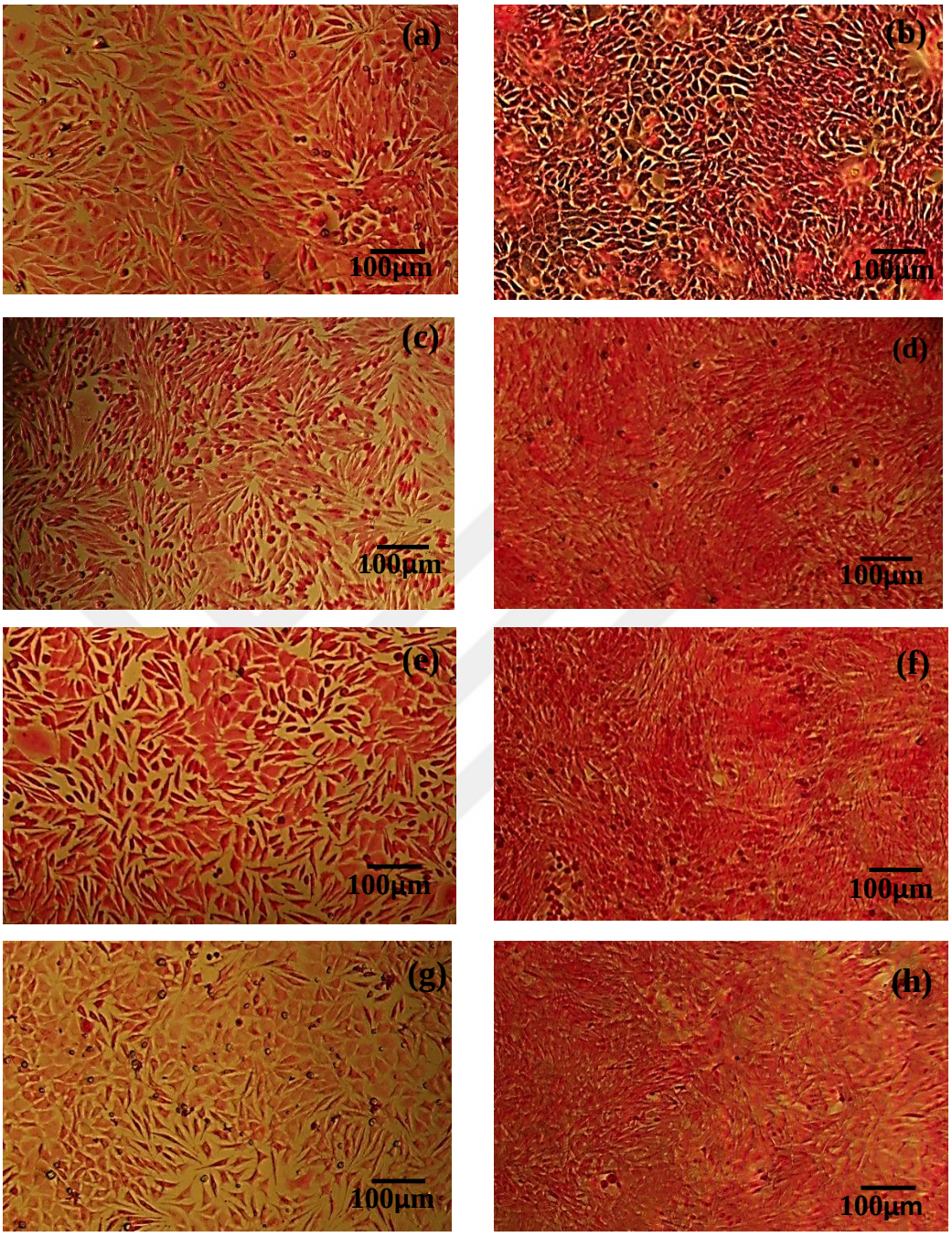

Figure 3.35 Percent stained area for collagen on the wells seeded with fibroblasts (a-b) X1:GEL1:GLY2/VC0.1, (c-d) X1:GEL1:GLY2:K/VC0.1, (e-f) X1:GEL1:GLY2/VC0.05, (g-h) X1:GEL1:GLY2:K/VC0.05 and (ı-i) TCPS on 4th and 7th day, respectively.

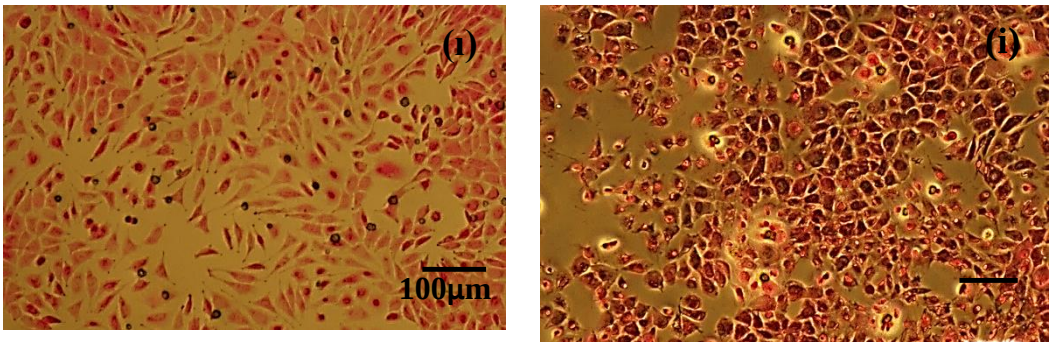

Figure 3. 35 (cont.) Percent stained area for collagen on the wells seeded with fibroblasts (a-b) X1:GEL1:GLY2/VC0.1, (c-d) X1:GEL1:GLY2:K/VC0.1, (e-f) X1:GEL1:GLY2/VC0.05, (g-h) X1:GEL1:GLY2:K/VC0.05 and (ı-i) TCPS on 4th and 7th day, respectively.

CHAPTER 4

CONCLUSION

In this study, novel keratin containing xanthan gelatin hydrogels were produced as a temporary wound dressing by glycerol crosslinking for local delivery of vitamin C for low to moderate exudate wounds. Firstly, xanthan gelatin hydrogels were analyzed in terms of toxicity, and all XGHs were cytocompatible. Then total polymer to glycerol ratio was optimized depending on cell culture studies, porosity, and mechanical properties in terms of applicability of hydrogels to skin. (1:1w/w) polymer to glycerol ratio was selected as the optimum ratio. To increase cellular growth, keratin was combined with XGHs. To determine optimum keratin concentration, biocompatibility of different keratin concentrations (20%, 10%, 4%, 2% and 0.4% (w/w)) was analyzed. The presence of keratin increased both tensile strength and elongation at break. Moreover, it was observed increased weight loss and protein release, on the contrary, lower water uptake ability. To the best of our knowledge, for delivery of vitamin C, keratin containing xanthan gelatin hydrogel was designed by glycerol crosslinking for the first time. Vitamin C was chosen as a bioactive agent to assist the healing process by increasing collagen synthesis. Compared to the control group, L929 fibroblast cells incubated with VC-XGHs and VC-KXGHs not only showed enhanced proliferation of fibroblast cells but also increased collagen synthesis with enhanced water uptake and protein release.

As a conclusion, future studies are offered to be improved the use of keratin containing xanthan gelatin hydrogels for vitamin C local delivery as a potential product as a temporary wound dressing for low to moderate exudates.

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APPENDICES

A. Calibration Curve

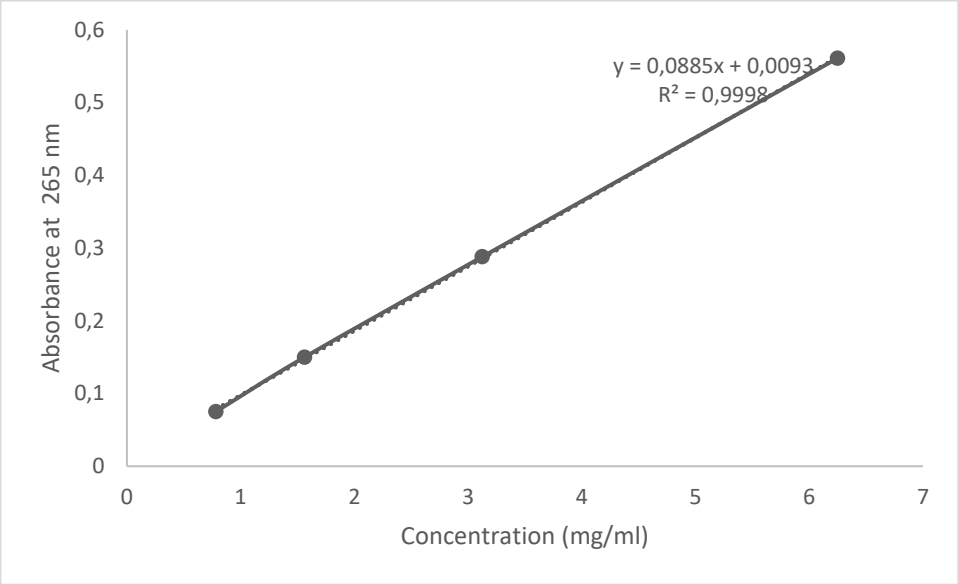

Figure A. 1 The calibration curve of Vitamin C