REPUBLIC OF TÜRKİYE
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PREPARATION OF KARAYA GUM CRYOGELS WITH ANTIBACTERIAL PROPERTIES FOR DENTAL APPLICATIONS

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MASTER OF SCIENCE THESIS
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Program of Bioengineering

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Başak AKIN

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Dedicated to my late father

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LIST OF SYMBOLS

CFU	Colony Forming Unit
σ_f	Fracture Strength
σ_{nom}	Nominal Stress
OD	Optical Density
ε	Strain
q_w	Swelling Ratio
W	Toughness
m_{dry}	Weight of Dry Cryogels
m_s	Weight of Swollen Cryogels
E	Young's Modulus

LIST OF ABBREVIATIONS

AgNPs	Silver Nanoparticles
ATR	Attenuated Total Reflectance
AUNPs	Gold Nanoparticles
BHI-A	Brain Heart Infusion Agar
BHI-B	Brain Heart Infusion Broth
BMP	Bone Morphogenic Protein-2
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DVS	Divinyl Sulfone
ECM	Extracellular Matrix
FBS	Fetal Bovine Serum
FESEM	Field Emission Scanning Electron Microscopy
FTIR	Fourier Transform Infrared Spectroscopy
KG	Karaya Gum
MRSA	Methicillin-resistant *Staphylococcus Aureus*
PEG	Polyethylene Glycol
PVA	Polyvinyl Alcohol

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ABSTRACT

Preparation of Karaya Gum Cryogels with Antibacterial Properties for Dental Applications

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Tooth extraction, although seemingly simple, comes with several intra-operative and postoperative complications. Among them, excessive bleeding and postoperative bleeding are the most prominent complications often encountered in patients with underlying diseases. Current treatment options involve the use of hemostatic dental sponges and antibiotics to prevent infections. However, dental sponges currently available in the market mostly lack antibacterial activity and have limited functionality. Cryogels are macroporous polymeric networks commonly used in biomedical applications due to their rapid and superior swelling properties, a function highly in demand for dental applications. Macroporous structure of cryogels creates a favorable environment for the absorption of bodily fluids such as blood, slowing down bleeding and assisting hemostasis as a result. In addition, cryogels can be functionalized to impart antibacterial properties, mitigating the need to use antibiotics following dental operations. In this study, karaya gum cryogels were prepared in three different polymer concentrations to evaluate the effects of polymer concentration on the porosity of the cryogels and determine the optimal parameters for the treatment of postoperative complications in dentistry. Further, KG cryogels were loaded with vancomycin to impart antibacterial activity and prevent postoperative infections. The resulting cryogels exhibited antibacterial efficacy against *Streptococcus pyogenes* and *Streptococcus mutans*. Compression tests revealed that karaya gum cryogels can be used as alternatives to commercial dental sponges owing to their strong mechanical integrity. Additionally, karaya gum cryogels show remarkable biocompatibility against L929 mouse fibroblast

cells. Overall, this study introduces novel karaya gum cryogels with antibacterial properties and offers an innovative solution to meet the needs of dental operations.t

Keywords: Karaya gum, cryogel, antibacterial, vancomycin, dental biomaterial.

ÖZET

Antibakteriyel Özelliğe Sahip Karaya Sakızı Kriyojellerin Dental Uygulamalar için Hazırlanması

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Yüksek Lisans Tezi

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Diş çekimi her ne kadar basit bir prosedür gibi görünse de intra-operatif ve postoperatif birçok komplikasyonu beraberinde getirir. Bunlar arasında aşırı kanama ve ameliyat sonrası kanama, mevcut hastalıkları olan hastalarda sıklıkla karşılaşılan en belirgin komplikasyonlardır. Güncel tedavi seçenekleri, enfeksiyonları önlemek için hemostatik dental süngerlerin ve antibiyotiklerin kullanımını içerir. Ancak şu anda piyasada bulunan dental süngerler çoğunlukla antibakteriyel aktiviteye sahip olmamakla birlikte sınırlı işlevselliğe sahiptir. Kriyojeller, dental uygulamalar için oldukça talep gören bir özellik olan hızlı ve üstün şişme özellikleri nedeniyle biyomedikal uygulamalarda yaygın olarak kullanılan makrogözenekli polimerik ağlardır. Kriyojellerin makro gözenekli yapısı, kan gibi vücut sıvılarının emilimi için uygun bir ortam yaratarak kanamayı yavaşlatır ve sonuç olarak hemostaza yardımcı olur. Buna ek olarak, kriyojeller antibakteriyel özellikler kazandırmak üzere işlevselleştirilebilir ve dental operasyonların ardından antibiyotik kullanma ihtiyacını azaltabilir. Bu çalışmada, polimer konsantrasyonunun kriyojellerin gözenekliliği üzerindeki etkilerini değerlendirmek ve diş operasyonları sonrası komplikasyonların tedavisi için en uygun parametreleri belirlemek amacıyla üç farklı polimer konsantrasyonunda karaya gam kriyojelleri hazırlanmıştır. Ayrıca, kriyojellere antibakteriyel aktivite kazandırmak ve ameliyat sonrası enfeksiyonları önlemek için vankomisin eklenmiştir. Elde edilen kriyojeller *Streptococcus pyogenes* ve *Streptococcus mutans*'a karşı antibakteriyel etkinlik sergilemiştir. Basma testleri sonucunda karaya gam kriyojellerin güçlü mekanik dayanımları sayesinde ticari dental

süngerlere bir alternatif olarak kullanılabileceği saptanmıştır. Ayrıca, karaya gam kriyojeller L929 fare fibroblast hücrelerine karşı kayda değer biyouyumluluk göstermiştir. Sonuç olarak bu çalışma antibakteriyel özellik gösteren yeni karaya gam kriyojelleri, dental operasyonların ihtiyaçlarını karşılayacak inovatif bir çözüm olarak tanıtmaktadır.

Anahtar Kelimeler: Karaya gam, kriyojel, antibakteriyel, vankomisin, dental biyomalzeme.

1
INTRODUCTION

1.1 Literature Review

Tooth extraction is a common dental operation that involves the removal of the tooth from the socket. While the complications related to tooth extraction are generally manageable, patients with underlying diseases such as diabetes suffer greatly from intraoperative and postoperative complications due to delayed wound healing and proneness to infections [1]. Complications can be caused by inflammatory or iatrogenic factors such as nerve injury and bone fraction. Excessive bleeding is an inflammatory complication that may occur even in healthy patients during or after the operation. It usually stems from local factors such as soft tissue bleeding due to traumatic extraction or systemic factors like coagulation defects and medicine [2].

Dental sponges are hemostatic biomaterials used for the treatment of excessive bleeding. The porous structure of the sponges allows the filtration of blood and assists with clot formation while also slowing down, and eventually stopping the blood flow [3]. Therefore, an interconnected macroporous morphology is highly desired when producing a dental sponge. Current research is focused on the production of hydrogels to prevent and treat complications related to dental operations [4, 5]. Hydrogels are three-dimensional crosslinked polymer networks that can absorb large amounts of water due to their hydrophilic nature. Although hydrogels are compatible with biological tissues, they lack macroporous structure and mechanical integrity [6]. While several methods are available in the literature in order to mitigate these drawbacks, cryogelation excels with ease of application and the advantage of not requiring additional chemicals.

Cryogelation is a process in which a monomer or polymer solution is reacted at subzero temperatures, and the solvent crystals act as porogens to create a macroporous structure. The resulting product is referred to as “cryogel”, a polymeric gel with relatively enhanced mechanical strength and elasticity [7]. Several studies reported that the superior morphology of cryogels allows

enhanced cell migration and angiogenesis [8, 9]. For instance, Chang et al. introduced a gelatin/hydroxyapatite/β-tricalcium phosphate cryogel composite integrated with bone morphogenetic protein-2 (BMP) for osseointegration [9]. Similarly, Qayoom et al. demonstrated that BMP-loaded collagen/gelatin-based cryogels functionalized with nanohydroxyapatite can be used as antibiotic carriers for the treatment of osteomyelitis [8]. Cryogels are also studied for the regeneration of the periodontal tissue. It was previously reported that platelet-derived growth factor-loaded gelatin cryogels promote periodontal osteogenesis [10].

Dental sponges currently available in the market are predominantly produced using natural polymers such as gelatin, collagen, and cellulose [11]. Studies show that gelatin-based sponges crosslinked with glutaraldehyde decrease the pain experienced by the patients and the incidence of dry socket, which is a postoperative complication that occurs due to the absence of a clot in the tooth socket after extraction [3]. However, the poor mechanical properties of gelatin and the risk of anaphylactic reactions remain as crucial drawbacks. In addition to gelatin, collagen poses similar risks to patients who are allergic to bovine or porcine products [12]. On the other hand, cellulose is generally used in the form of oxidized regenerated cellulose due to its hemostatic and antibacterial properties [13]. These beneficial properties are due to the ability of oxidized regenerated cellulose to lower the pH in the environment. Conversely, it was also reported that low pH levels cause inflammation via foreign body reactions [14].

Biopolymers currently dominate the biomedical market owing to their high biocompatibility and accessibility. Natural gums are polysaccharides that can be derived from plants, algae, and bacteria [15]. All natural gums have distinctive features and can be characterized according to their configuration, conformation, molecular weight, monosaccharide composition, position of glycosidic linkages, solubility, rheological properties, and arrangement of polysaccharides. They have been utilized as thickening agents in the food industry due to their ability to form viscous solutions. Recently, natural gums have been considered attractive alternatives for use in biomedical applications owing to their non-toxicity and inexpensiveness [16]. Bearing high molecular weight, natural gums can form stable gels in aqueous solutions. Natural gums such as xanthan gum, tragacanth gum, and gum arabic have been used to produce cryogels for a variety of biomedical applications. Demir et al. introduced tragacanth gum/chitosan-based cryogels crosslinked with glutaraldehyde for tissue engineering. Tragacanth gum-based cryogels exhibited enhanced cell adhesion and controlled degradation behavior [17]. In another study, locust bean gum-based cryogels were prepared with the addition of cysteine-modified chitosan and oxidized dextran [18]. Crosslinking occurred due

to the Schiff base reactions between chitosan and dextran, and the resulting cryogels demonstrated high swelling capability as well as hemocompatibility, indicating great potential as wound dressings. Similarly, Raschip et al. prepared xanthan gum-based cryogels as monoliths, films, and microspheres using several different crosslinkers [19]. The results indicated that the cryogelation process allows the preservation of the porous structure, and properties such as swelling capacity can be controlled in chemically crosslinked cryogels by changing the crosslinker concentration.

Karaya gum (KG) is a branched polysaccharide with L-rhamnose, D-glucuronic acid, and D-galacturonic acid units. Its easily modifiable structure allows the production of hydrogels via grafting method. Majority of KG-based hydrogels in the literature are grafted with acrylic acid, and the cross-linking takes place with the addition of N,N'–methylenebisacrylamide [20]. KG can also be blended with other polysaccharides such as chitosan for further functionalization [21]. Further, a previous study reported KG-based injectable hydrogels for the treatment of Methicillin-resistant Staphylococcus aureus (MRSA) infections [22]. Herein, hydrogels prepared using deacetylated KG and polyvinyl alcohol were used as delivery systems for phage therapy, and the resulting hydrogels did not exhibit side effects. Overall, it can be stated that the studies on KG-based hydrogels are quite limited in the literature, and there were no reports available on chemically crosslinked KG cryogels.

Cryogels can be functionalized with bioactive molecules for antibacterial activity, which is an essential property for the prevention and treatment of postoperative infections. Currently, infections following dental operations are treated with antibiotics such as amoxicillin [23]. However, the systemic administration of antibiotics induces side effects and increases the risk of resistance [24]. In order to mitigate these disadvantages, antibiotics can be integrated into cryogels, allowing the sustained and controlled release of the drug while avoiding systemic side effects. For instance, Kolosova et al. introduced Ceftriaxone-loaded polyvinyl alcohol-based cryogels for the treatment of infected wounds [25]. It was concluded that polyvinyl alcohol-based cryogels exhibited significant antibacterial effects and reduced inflammation. Similarly, Ari et al. prepared ciprofloxacin-loaded pullulan cryogels, and determined that the toxicity of the cryogels increased with the increasing concentration of ciprofloxacin [26].

Vancomycin is a potent glycopeptide antibiotic used to treat serious infections caused by Gram-positive bacteria. It was previously loaded to silk fibroin hydrogels for diabetic wound healing, and the results suggested that the silk fibroin-based

hydrogels allow the sustained release of vancomycin for 56h [27]. Vancomycin was also loaded to polysaccharide-based hydrogels and tested against MRSA [28]. Resulting hydrogels demonstrated high biocompatibility as well as antimicrobial activity, and significantly prevented immediate biofilm formation. Meanwhile, studies on vancomycin-loaded cryogels are quite limited in the literature. It is important to investigate vancomycin within a cryogel system to understand how the distinctive morphology of cryogels can help elevate both its release profile and antibacterial efficacy.

1.2 Objective of the Thesis

Currently, a multifunctional dental biomaterial is in high demand for the treatment of postoperative complications such as excessive bleeding, dry socket, and osteomyelitis. However; an all-in-one solution is not currently available in the market. Traditional dental sponges are not sufficient to treat bacterial infections, and the systemic administration of antibiotics can pose significant health risks. In this study, vancomycin-loaded KG cryogels are prepared in order to mitigate the intraoperative and postoperative complications related to dental surgeries such as tooth removal. KG cryogels were characterized in terms of physicochemical and biological properties to investigate the suitability of the cryogels as dental biomaterials.

For this purpose,

- preparation of KG cryogels with different concentrations of KG and investigation of the effect of polymer concentration on the swelling properties of cryogels,
- loading of vancomycin into KG cryogels to obtain multifunctional dental biomaterials,
- conducting physicochemical analysis to ensure crosslinking between KG and divinyl sulfone (DVS),
- investigation of the effectiveness of vancomycin-loaded KG cryogels against common Gram-positive bacteria found in oral microflora (*Streptococcus pyogenes* and *Streptococcus mutans*),
- evaluation of the biocompatibility of KG cryogels against L929 mouse fibroblast cell line,

are the primary objectives of this study.

1.3 Hypothesis

Dental sponges play a crucial role in assisting hemostasis after dental surgeries due to their porous structure, which allows the filtration of blood. Cryogels are macroporous polymeric matrices that can permit the free flow of blood throughout their pores, and act as a physical plug in the swollen state. It is hypothesized that KG cryogels offer an alternative treatment option for postoperative complications due to their biocompatibility, safety, inexpensiveness, and swelling properties. It is also suggested that vancomycin-loaded KG cryogels will exhibit excellent antibacterial properties against oral microflora bacteria S. pyogenes and S. mutans.

2
GENERAL INFORMATION

2.1 Periodontium

Periodontium, also referred to as the periodontal tissue, is a complex and dynamic structure that plays a crucial role in supporting and anchoring the tooth. It consists of a distinctive vasculature, an integrated lymphatic system, and a highly specialized network of nerves, serving to protect the tooth from mechanical insults and microbial invasions [29]. These protective and supportive functions are facilitated by the four main components including the gingiva, periodontal ligament, cementum, and alveolar bone as shown in Figure 2.1.

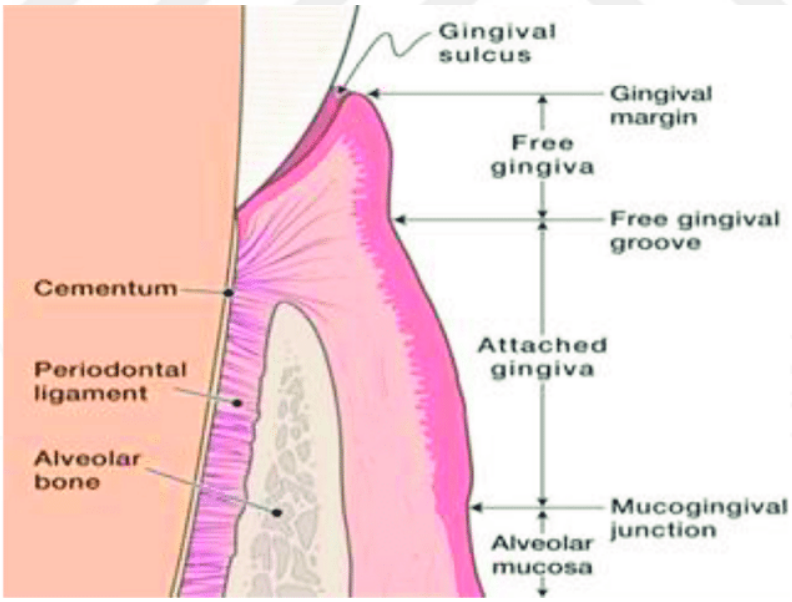


Figure 2.1 Schematic representation of the periodontium and its components [30]

In adults, the gingiva surrounds the alveolar bone and is divided into free, attached, and interdental units. The free gingiva, also known as the unattached or marginal gingiva, forms the terminal edge of the gingiva, positioned adjacent to the gingival sulcus. The attached gingiva is continuous with the free gingiva and it is firmly bound to the periosteum of the underlying alveolar bone, providing a stable support.

The interdental gingiva occupies the space between neighboring teeth, filling the gap beneath the contact site.

The gingiva consists of a core of connective tissue and a surrounding stratified squamous epithelium [31]. The gingival epithelium is primarily cellular, and the predominant cell type of the stratified squamous epithelium is the keratinocytes along with Langerhans cells, Merkel cells, and melanocytes. Although the gingival connective tissue is less cellular, the predominant cell type is the fibroblasts of mesenchymal origin. Fibroblasts are responsible for the synthesis of collagen and elastin fibers which constitute the majority of the gingival connective tissue along with glycoproteins and glycosaminoglycans. The gingival connective tissue is considered to have an excellent healing and regenerative capacity, largely due to the high cell turnover rate [32].

Periodontal ligament is a highly vascular and cellular connective tissue that surrounds the tooth root and anchors the tooth to the alveolar bone. It consists of a fibrous tissue that primarily contains type I collagen and hydrophilic proteoglycans. Fibers embedded in cementum and the alveolar bone on each side are referred to as the Sharpey's fibers. Due to the high fiber content, the periodontal ligament acts as a viscoelastic system that absorbs the mechanical forces, protecting the tooth from occlusal loads.

Another component of the periodontium is the cementum, which is a calcified and avascular tissue that covers the tooth root. Cementum consists of a calcified interfibrillar matrix as well as collagen fibrils and unlike the other components, it does not typically undergo remodeling [33]. The cementum is formed by cementoblasts, and the fibroblast in the periodontal ligament contributes to its formation by secreting extrinsic fibers. The primary role of the cementum is to attach the tooth to the alveolar bone by anchoring the periodontal ligament as mentioned previously. Alveolar bone forms the tooth socket (alveoli dentales), and it is a tooth-dependent structure that appears when the tooth erupts and disappears after the tooth is lost. It is a specialized part of the maxilla and mandible that serves as the attachment site for the fibers of periodontal ligament.

Although all four components of the periodontium possess distinct biochemical compositions and architecture, recent research demonstrates that the extracellular matrix (ECM) molecules of each component may affect the cellular activities of neighboring structures [34]. Therefore, pathological alterations that occur within any compartment of the periodontium can adversely impact the regenerative and reparative processes of the remaining components.

2.2 Tooth Extraction: Complications and Treatment

Tooth extraction is a commonly performed procedure in dental surgery, often necessitated by several conditions such as periodontal diseases, dental trauma, and severe tooth decay. Despite advances in preventative measures, tooth extractions are still associated with a range of intraoperative and postoperative complications (Table 2.1), posing significant challenges to patients.

Table 2.1 Intraoperative and postoperative complications of tooth extraction

Intraoperative Complications	Postoperative Complications
• Injury of the molars • Excessive bleeding • Dislocation of the temporomandibular joint • Fracture • Nerve injury to the inferior alveolar nerve or the lingual nerve	• Alveolitis (Dry Socket) • Osteomyelitis • Pain • Secondary bleeding • Trismus • Delayed-onset infections

Excessive bleeding is an intraoperative complication that can occur in both healthy and medically compromised patients. Certain patients who use anticoagulants and individuals with underlying conditions such as diabetes are at an elevated risk of excessive bleeding due to impaired clotting and wound healing mechanisms. In addition to excessive bleeding. alveolitis is a frequently occurring condition that causes pain and discomfort in patients post-operation. Alveolitis, commonly known as dry socket, arises when a blood clot fails to form or becomes dislodged from the tooth socket following an extraction, exposing the underlying bone structure. Although the main cause of alveolitis is unknown, it is hypothesized that the activity of bacterial enzymes in the tooth socket prevent the formation of blood clots. Therefore, the management of bacterial infections after the operation is of high importance. While external factors such as smoking and poor oral hygiene increase the incidence of alveolitis, the treatment is focused on the production of a durable layer of epithelium to cover the exposed bone. In instances where the socket is infected by pathogens, the resulting inflammation of the bone structures may lead to osteomyelitis. MRSA is the most prominent pathogen causing osteomyelitis; however, other pathogens such as streptococci, enterococci, and pseudomonas can also lead to serious infections after dental operations.

These conditions not only raise significant health risks to the patients but also contribute to severe pain and discomfort. While preventative care is focused on sterile conditions, the treatment of the complications varies depending on the severity of the condition. Usually, the treatment plan consists of the use of a dental sponge along with antibiotics and pain medication. Most commonly used antibiotics in this case include amoxicillin, ciprofloxacin, clindamycin, doxycycline, and erythromycin [35–37]. While these antibiotics target infections, dental sponges are primarily used to control excessive bleeding. Combination of antibiotics and dental sponges address the underlying infection and effectively manage postoperative bleeding, resulting in a smoother recovery.

2.3 Dental Sponges

Dental sponges are practical and effective biomaterials used in case of excessive bleeding after tooth extraction. They are often produced using natural polymers such as gelatin and collagen in order to assist hemostasis. A summary of dental sponges currently on the market is displayed in Table 2.2. While most of the products in the market are focused on rapid hemostasis, novel products bear multiple functions such as antibacterial activity.

Table 2.2 Summary of dental sponges currently on the market

Product	**Polymer**	**Property**	**Absorption Time**	**Manufacturer**
Gelfoam	Gelatin	Hemostasis	4-6 weeks	Pfizer
CollaPlug	Collagen	Hemostasis, wound healing	2-4 weeks	Zimmer Biomet
Hemcon	Chitosan	Hemostasis, antibacterial	A few days	HemCon Medical
Halistat	Collagen	Hemostasis, wound healing	1-3 weeks	Integra Lifesciences
Avitene	Collagen	Hemostasis	2-4 weeks	Davol
ActCel	Oxidized regenerated cellulose	Hemostasis, bactericidal	1-2 weeks	Coreva Health Sciences
Spongostan	Gelatin	Hemostasis	4-6 weeks	Ferrosan Medical Devices

Dental sponges are designed as porous structures that allow the aggregation of platelets and eventually the formation of a stable blood clot, thereby serving as effective hemostatic agents [38][10]. Additionally, they act as physical barriers to stop the excessive blood flow within and around the tooth socket. As seen in Table

2.2, the majority of commercially available dental sponges are based on gelatin and collagen. However, it is crucial to note that dental sponges derived from gelatin and collagen may pose significant health risks for patients who are allergic to porcine or bovine products [39, 40]. Therefore, it is important to consider the potential allergic reactions when producing novel dental sponges.

In addition to their hemostatic efficacy, dental sponges with antibacterial activity have been a critical factor in preventing and treating infectious diseases caused by the oral microflora or cross-contamination following dental surgery. Although prescription of antibiotics is a conventional approach to address such infections, the outgrowing antibiotic resistance and the potential side effects associated with systemic antibiotic use underscore the necessity for intrinsically antibacterial dental sponges. Local administration of antibiotics has been shown to reduce the risk of side effects in previous studies [41]. Currently, commercially available dental sponges contain silver nanoparticles or oxidized regenerated cellulose for antibacterial efficacy. While silver nanoparticles (AgNPs) can cause bacterial cell death through various pathways, oxidized regenerated cellulose creates a low pH-environment to inhibit bacterial growth [42, 43].

Recent research on dental sponges are focused on producing a multifunctional biomaterial that enhances wound healing and exerts antibacterial effect as well as providing assistance in hemostasis. This can be achieved by using bioactive agents or functionalization of the polymers. Several natural polymer-based sponges were evaluated as hemostatic agents to prevent dry socket. Alginate, kappa carrageenan, and gelatin were previously used to produce novel hemostatic sponges [44–46]; however, studies on multifunctional dental sponges are still quite limited.

2.4 Hydrogels and Cryogels

Hydrogels are physically or chemically crosslinked biomaterials with high hydrophilicity and porosity. They are predominantly used in tissue engineering and wound healing due to their resemblance with the native tissue microenvironment. While the first report of hydrogels dates back to the 1960s, hydrogels have been functionalized and synthesized using various methods for years to produce a wide range of materials (Figure 2.2) [47]. While the porous structure of hydrogels creates a favorable environment for cell migration, attachment, and proliferation; their microporous architecture and poor mechanical integrity remain as crucial drawbacks.

Various preparation methods have been previously employed to overcome these

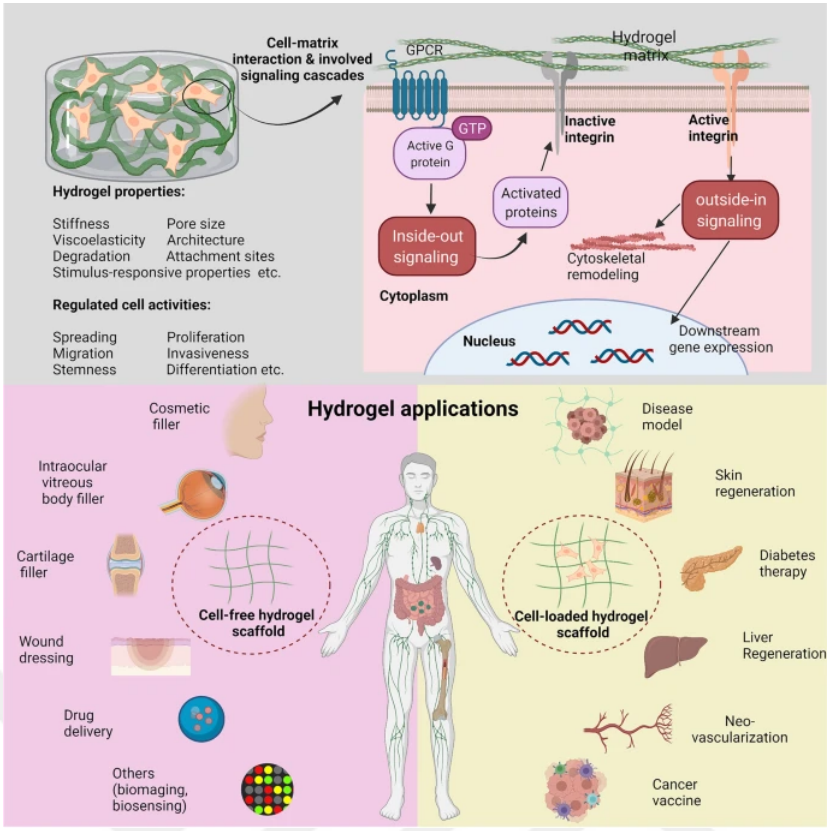


Figure 2.2 Application areas of hydrogels [48]

limitations and produce macroporous hydrogels [49]. These methods include porogen templating, gas foaming, electrospinning, 3D printing, and cryogelation. While having distinct advantages and disadvantages, all methods aim to tackle the challenges associated with the micro-sized pores structure in hydrogels. Among the preparation methods of macroporous hydrogels, cryogelation stands out due to its ability to create highly interconnected macroporous architecture. Cryogelation is a technique involving the freezing and thawing of a monomer or polymer solution, typically in subzero temperatures [50]. The ice crystals formed during the freezing process melt away when the gel is thawed, acting as a porogen and resulting in the formation of cryogels (Figure 2.3).

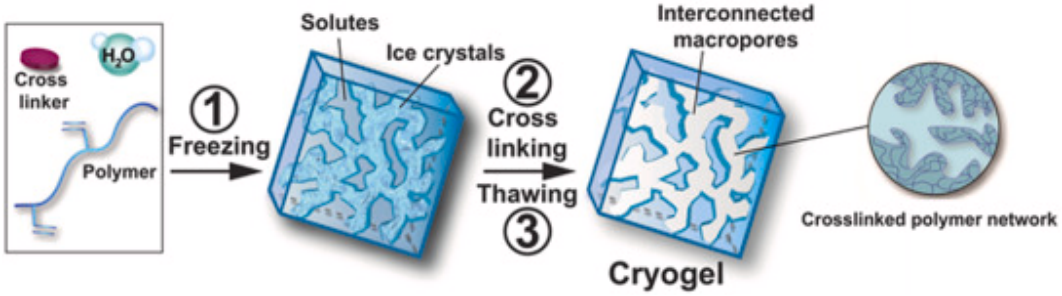


Figure 2.3 Schematic representation of cryogelation process [51]

Having a macroporous structure, cryogels have advantages such as rapid swelling

and deswelling, indicating that they can absorb large volumes of body fluid promptly. Moreover, the pore size and distribution can be controlled by altering the freezing conditions and gelation parameters [52, 53]. Due to the dense polymer walls and interconnected morphology, cryogels have outstanding elasticity as well as shape memory. These properties make cryogels excellent candidates as dental biomaterials, as their inner morphology allows the unhindered flow of blood while their mechanical strength permits them to function effectively as a physical barrier. Moreover, cryogels can be easily functionalized to impart additional properties such as antibacterial activity.

2.5 Polymers Used in Cryogel Production

Polymers play a crucial role in shaping the properties of cryogels such as porosity, biodegradability, and biocompatibility; and therefore, choosing an appropriate polymer for the intended application area is of high importance. While cryogels can be prepared from both natural and synthetic polymers (Figure 2.4), dental sponges are predominantly produced using natural polymers like gelatin and collagen [54]. Biopolymers are natural polymers derived from biological sources, typically exhibiting high biocompatibility and, in some cases, bioactivity. Various studies have reported that collagen assists in wound healing [54, 55]; however; gels that consist of only collagen demonstrate low mechanical integrity [56], limiting the use of collagen-based cryogels in biomedical applications.

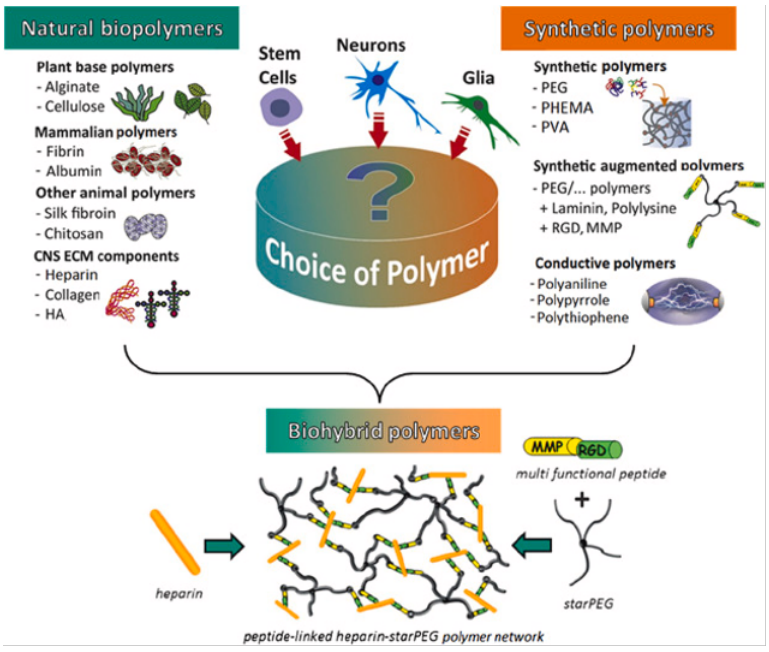


Figure 2.4 Natural and synthetic polymers used for the production of cryogels [51]

Gelatin is the predominant natural polymer in the literature for the production of

dental sponges. While it is generally considered safe, low mechanical strength and rapid, uncontrolled degradation of gelatin remain as important drawbacks. In order to overcome these drawbacks, gelatin is frequently blended with other natural polymers to produce cryogels [57]. Another polymer commonly used to prepare cryogels is cellulose, a rigid biopolymer often modified for increased biodegradability and chemical reactivity. An example is the oxidized regenerated cellulose, which is a modified form of cellulose obtained by the conversion of hydroxyl groups to carboxyl groups [58]. It is widely used in surgical settings due to its hemostatic and antibacterial properties.

Synthetic polymer-based hydrogels offer several advantages such as tunable mechanical properties, precise chemistry, and ease of modification [59]. Polymers such as polyethylene glycol (PEG), polyvinyl alcohol (PVA), and polyurethane are frequently used to produce hydrogels for biomedical applications [60]. While having attractive properties, synthetic polymers lack bioactivity, which is a crucial feature for dental applications. In order to mitigate this issue, synthetic polymers are often coupled with bioactive molecules and stem cells [61, 62], enhancing the functionality and suitability of synthetic polymer-based hydrogels.

In conclusion, it is important to choose the polymer according to the desired properties of the cryogel. For the production of dental sponges, high biocompatibility and bioactivity are crucial parameters to be considered. Therefore, in most cases, the use of natural polymers is preferred over synthetic polymers. While aforementioned natural polymers are commonly used and highly advantageous, novel options for the production of cryogels continue to be explored to meet the evolving demands of postoperative complications in dental surgery.

2.5.1 Karaya Gum

Natural gums are complex polysaccharides widely used in the food industry as thickening agents due to their ability to form viscous solutions. Owing to their attractive properties such as high biocompatibility, non-toxicity, accessibility, and inexpensiveness, many types of natural gums have been recently utilized in biomedical applications as well [63, 64]. Among these, KG excels as an anionic gum obtained from the dried exudates of Sterculia urens tree. It is constituted of α-L-rhamnopyranose, β-D-galactopyranose, α-D-galacturonic acid, and β-D-glucopyranuronic acid units as seen in Figure 2.5. The unique structure of KG allows easy modification for further functionalization. As an acylated polysaccharide, KG is soluble in aqueous solution and retains its solubility in altered pH values. Dissolving KG in alkaline or acidic solutions decreases the viscosity of

the solution, with KG solutions resembling mucilage at pH levels over 7.

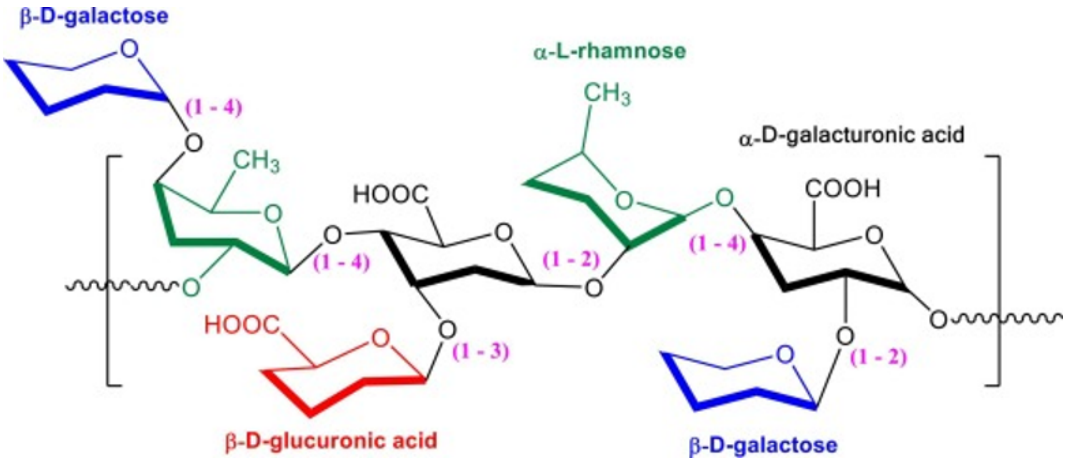


Figure 2.5 Chemical structure of KG [65]

KG is not only highly biocompatible but also non-immunogenic, non-mutagenic, and non-toxic [66]. It was previously reported that people who consume KG over 100 mg/kg for 4 weeks did not experience any adverse effects while some individuals suffered from abdominal discomfort – which is considered as undesirable rather than adverse [67]. Therefore, KG is generally considered safe to use and has been studied extensively in the field of tissue engineering.

While there are no chemically crosslinked KG cryogels reported in the literature to the best of our knowledge, KG-based hydrogels are predominantly produced via grafting initiated through free radical polymerization of acrylic acid [66]. This method consists of grafting of acrylic acid to KG and the subsequent cross-linking between the grafted polymer moieties, resulting in pH-sensitive and hydrophilic hydrogels [68]. Polymerization usually takes place using N,N'–methylenebisacrylamide as crosslinker and ammonium persulphate as the initiator. KG-based hydrogels obtained through free radical polymerization have been previously used for a variety of application areas including oral drug delivery and removal of cationic dyes [69, 70]. KG was blended with other natural polymers as well in order to impart antibacterial activity to hydrogels. Drápalová et al. introduced ionically crosslinked transparent wound dressings from KG and chitosan with antimicrobial effect against both Gram-positive and Gram-negative bacteria [71].

While KG is a natural polymer with high accessibility, safety, and inexpensiveness, studies on KG-based hydrogels are still very limited. To our knowledge, there have been no reports of KG cryogels. The ease of modification is an attractive property of KG that allows the production of multifunctional dental biomaterials. Given these promising attributes, further research on KG is required to fully explore the potential

of KG-based cryogels particularly in dental applications. Expanding research in this area is highly crucial in order to develop innovative and environmentally friendly materials that meet the evolving demands of dental wound healing.

2.6 Antibacterial Cryogel Production

Antibacterial agents are commonly utilized in order to functionalize cryogels for the treatment of various types of infections (Figure 2.6). Inorganic antibacterial agents such as metallic nanoparticles are widely preferred due to their ability to enhance and maintain antibacterial activity for a long period of time [72]. While AgNPs and gold nanoparticles (AuNPs) are the most prominent in the literature; zinc and titanium nanoparticles are also excellent antibacterial agents that are incorporated into cryogels [73–75]. Moreover, the antibacterial mechanisms of metallic nanoparticles vary significantly. For instance, AgNPs act upon bacteria by disrupting the bacterial cell wall whereas AuNPs inhibit the activity of ATPase [76]. Despite demonstrating superior antibacterial activity, numerous studies have reported that metallic nanoparticles exhibit notable cytotoxicity [77].

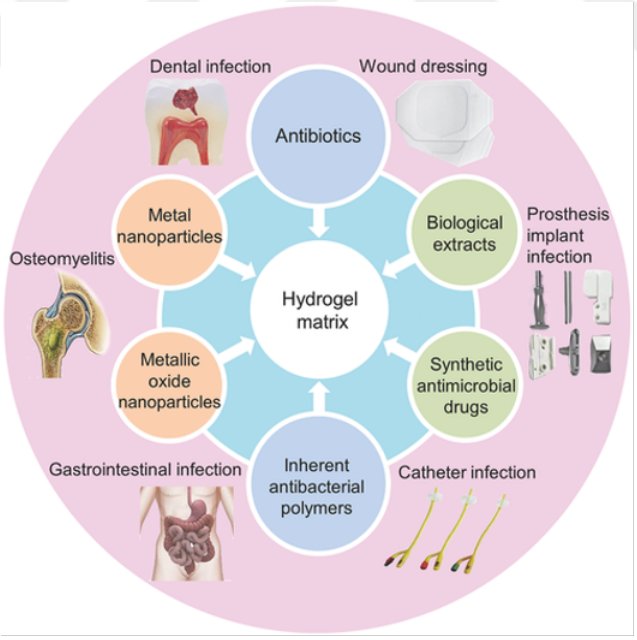


Figure 2.6 Antibacterial agents used to functionalize hydrogels for various infection types [72]

Biological extracts such as alkaloids, tannins, flavonoids, and terpenoids are usually non-toxic alternatives that exhibit remarkable antibacterial activity against common pathogenic bacteria [78]. However, variability in the efficacy depending on the source and extraction method, poor solubility of some extracts, and low solubility in aqueous solutions as well as limited spectrum of activity compared to

synthetic activity remain as important challenges for the widespread use of natural compounds as antibacterial agents.

Inherently antibacterial cryogels can be produced using intrinsically antibacterial polymers such as chitosan or antimicrobial peptides [79]. These are typically cationic polymers [80], and their mechanism of action is due to the electrostatic interaction between the positively-charged groups and the negatively-charged bacterial cell wall [81]. The antibacterial efficacy of cationic polymers is dependent on various parameters like molecular weight and charge density, which are parameters that also influence the cytotoxicity.

As mentioned previously, antibiotics are antimicrobial agents frequently used for the prevention and treatment of postoperative complications. However, systemic antibiotics remain as major contributors to the side effects and development of antibiotic resistance. Local administration of antibiotics has proven to be more effective than systemic applications in previous studies [82], most likely due to the availability of higher drug concentrations at the wound site. Integrating antibiotics into cryogels is a novel approach that permits the sustained release of the antibiotics, thus enhancing the antibacterial effect [83]. Therefore, antibiotic-loaded cryogels can act as multifunctional wound dressings due to their ability to mitigate bacterial infections, assisting hemostasis, and potentially enhancing wound healing.

2.6.1 Vancomycin

Vancomycin is a glycopeptide antibiotic used for the treatment of a wide range of infections caused by Gram-positive bacteria, and its chemical structure is displayed in Figure 2.7. Vancomycin exhibits bactericidal effects by preventing the polymerization of N-acetylmuramic acid and N-acetylglucosamine in the peptidoglycan layer of the bacterial cell wall [84]. It binds to D-alanyl D-alanine dipeptide, thus inhibiting the activity of glucosyltransferase enzymes necessary for the polymerization reaction.

Vancomycin is not absorbed by the intestines due to its high molecular weight; and therefore, it is usually administered intravenously rather than the enteral route [86]. Consequently, it is important to enhance the bioavailability of vancomycin for the effective treatment of conditions such as osteomyelitis. This can be achieved by integrating vancomycin into cryogels and the direct application of the cryogels to the infected area, allowing the controlled release of the antibiotic. Vancomycin was previously integrated into cryogels for the treatment of osteomyelitis [8]. It was also previously reported that poly(β-cyclodextrin)-based cryogels exhibit sustained

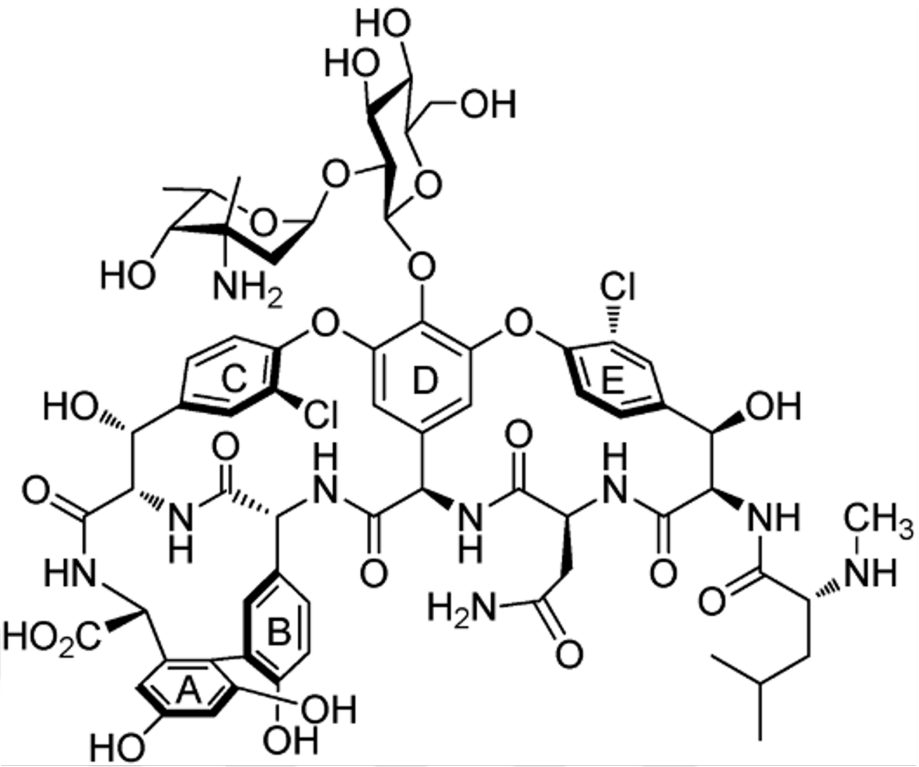


Figure 2.7 Chemical structure of vancomycin [85]

release profiles, with 89.9 ± 10.5% of the loaded vancomycin being released within 6h [87].

Vancomycin has been used as the "last-resort" drug due to its ability to treat a wide range of infections caused by Gram-positive bacteria effectively. A drawback of vancomycin is its ineffectiveness against Gram-negative bacteria [88]. Although the oral microflora consists of both types of bacteria, over 60% of the osteomyelitis cases involve MRSA [89]. Considering that the majority of infections are caused by Gram-positive bacteria, using vancomycin-loaded cryogels present an attractive approach for the prevention and treatment of intraoperative and postoperative complications.

Most of the commercially available dental sponges lack antibacterial activity, a crucial function for the prevention of infectious diseases such as osteomyelitis. Postoperative infections can arise from bacteria that reside in the oral microflora such as *S. pyogenes* and *S. mutans*. It was previously reported that oral infections stemming from *S. pyogenes*, an opportunistic bacteria, have the risk of advancing to systemic infections in immunologically compromised patients [90]. Moreover, uncontrolled infections of *S. pyogenes* can lead to severe infections such as necrotizing fasciitis and streptococcal toxic shock syndrome, which is a rare but [91]. In addition, *S. mutans* is often associated with oral infections such

as periodontitis and endocarditis that can jeopardize the wound healing process. Therefore, it is highly important to impart antibacterial activity to dental sponges and eliminate the risk of infection associated with dental operations.

In this thesis, KG cryogels were prepared in different concentrations and functionalized with vancomycin to prevent infections stemming from the bacteria in the oral microflora. The antibacterial activity of vancomycin-loaded cryogels with varying polymer concentrations was evaluated against *S. pyogenes* and *S. mutans* strains, and the results were compared to assess the influence of polymer concentration on the properties such as porosity, swelling, mechanical performance, and antibacterial activity.

3
MATERIALS AND METHODS

3.1 Materials

KG, DVS, vancomycin hydrochloride, and sodium hydroxide (Sigma-Aldrich) were used as received. Milli-Q was obtained from Yildiz Technical University, Department of Bioengineering. Brain-Heart Infusion Agar (BHI-A) and Brain-Heart Infusion Broth (BHI-B) as well as *Streptococcus pyogenes* and *Streptococcus mutans* were obtained from Yildiz Technical University, Department of Molecular Biology and Genetics for antibacterial activity tests. Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), dimethyl sulfoxide (DMSO), and penicillin/streptomycin were also obtained from Yildiz Technical University, Department of Molecular Biology and Genetics for cell viability assay.

3.2 Equipment

Equipment used during this study are listed below.

Table 3.1 Equipment Details

Equipment	**Brand/Model**
FTIR	Bruker Tensor 27
Magnetic stirrer	Heidolph MR300
Freeze dryer	Heto / Drywinner DW 1.0-60e
FTIR	Bruker Tensor 27
SEM	Thermoscientific Apreo 2S
Cryostat	Kerman
Compression device	Zwick Roell Z0.5 TH
UV-Vis Spectrophotometer	PG Instruments T60

3.3 Preparation of KG Cryogels

KG cryogels were prepared using three different polymer concentrations (0.5% w/v, 1.0% w/v, 1.5% w/v) and the cryogels were labeled as KG-C1, KG-C2, and KG-C3, respectively. As seen in Figure 3.1, KG powder was dissolved in 10 mL of 0.2M NaOH solution overnight. Next, the polymer solution was allowed to rest in an ice bath until it reached to a final temperature of 0°C. 200 µL of DVS was added into the polymer solution dropwise, followed by mixing for 1 min. After achieving a homogeneous mixture, the pregel solutions were loaded into syringes and placed in a cryostat at -18°C for 48 h.

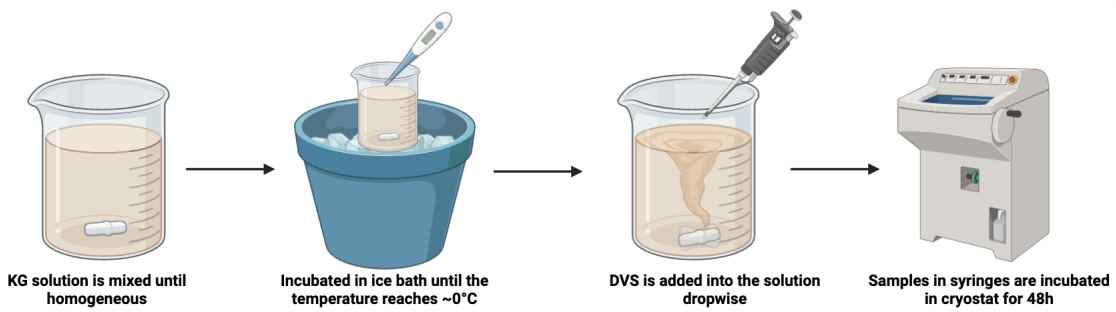


Figure 3.1 Schematic representation of the preparation of KG cryogels

Upon the completion of 48 h, the obtained cryogels were cut into smaller pieces and washed in distilled water. All samples were lyophilized for 48 h and stored at room temperature until further use.

3.4 Characterization of KG Cryogels

3.4.1 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectra of the KG cryogels were obtained from Bruker Tenson 27 FTIR spectrometer with attenuated total reflectance (ATR) attachment within the wave number range of 4000-400 cm^{-1}. Dried samples of the cryogels were used to investigate the cross-linking between KG and DVS.

3.4.2 Field Emission Scanning Electron Microscopy (FESEM)

The internal morphology of the cryogels were investigated using FESEM analysis (Thermoscientific Apreo 2S) at 200X, 500X and 2000X magnification. Dried samples of KG cryogels were coated with gold-palladium prior to analysis.

3.4.3 Swelling Tests

Swelling tests were conducted by soaking lyophilized samples in distilled water. The weight and diameters of the samples were then measured in their swollen state. Weight swelling ratio of the dental cryogels were calculated using the following equation:

$$q_w = \frac{m_{\text{s}}}{m_{\text{dry}}} \tag{3.1}$$

where q_w is the swelling ratio, mdry is the dry weight of the sample and ms is the weight of the sponge in the swollen state. For the measurement of swelling kinetics, variations in the samples were recorded gravimetrically at predetermined time intervals.

3.4.4 Mechanical Tests

The mechanical performance of KG cryogels were evaluated at room temperature by uniaxial compression tests using Zwick Roell Z0.5 TH, equipped with a 500 N load cell. Cylindirical cryogels in dried and swollen states with approximately 5 mm diameter were used for mechanical tests. The analysis yielded nominal stress (σ_{nom}), strain (ε), fracture strength (σ_f), fracture strain (ε_f), Young's modulus (E), and toughness (W).

3.4.5 Antibacterial Activity Tests

Test bacteria (*Streptococcus mutans* and *Streptococcus pyogenes*) were grown in Brain-Heart Infusion Broth (BHI-B, Merck, Germany) at 37 °C for 16–18 h. The bacteria cultures were diluted to an OD_{600} of 0.2 $\pm$ 0.02 (corresponding to $1.5 - 3.0 \times 10^8$ CFU/mL, approximately) in 3 mL BHI-B for use in this study.

The antibacterial activity of gel samples (KG-C2 and KG-C3) was evaluated against *S. mutans* and *S. pyogenes* test bacteria as described in a previous study, with minor modifications [78] as seen in Figure 3.2. Briefly, the entire surface of the gels was sterilized using ultraviolet radiation. In the study, two conditions were tested: vancomycin-loaded and antibiotic-free cryogels. Based on the MIC data from the EUCAST standard, vancomycin was loaded into the gels to achieve a final concentration of 1 µg/mL [92].

For all four gel samples, 10 µL of a bacterial suspension ($\sim$ 10^8 CFU/mL) was inoculated onto the gel surfaces. KG cryogels, inoculated with bacteria,

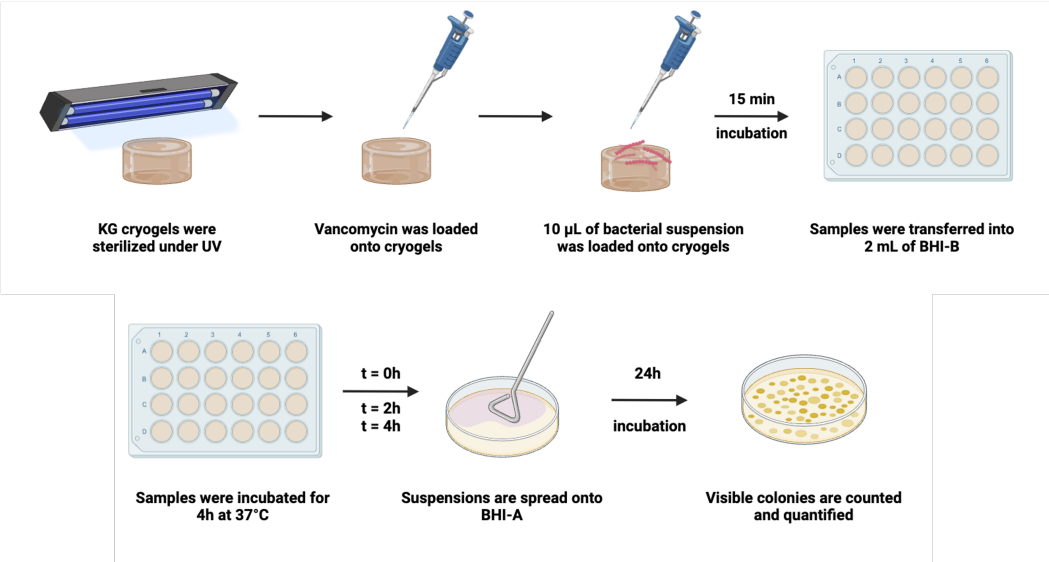


Figure 3.2 Schematic representation of the antibacterial activity test conducted on KG-C2 and KG-C3 cryogels.

were incubated for 15 minutes at room temperature. Subsequently, cryogels were transferred into tubes containing 2 mL of BHI-B using sterile forceps. Simultaneously, only test bacteria were inoculated into separate tubes to assess bacterial viability. The samples were then incubated at 37 °C for 4 hours. At specific time intervals, suspensions were taken from the tubes, spread onto Brain-Heart Infusion agar (BHI-A, Merck, Germany), and incubated overnight at 37 °C. The following day, visible colonies were enumerated to determine the number of surviving bacteria. The antibacterial activity of the gels was quantified by calculating the logarithmic reduction in viable bacteria as per ASTM standard E2149 [93]:

$$\text{Logarithmic reduction: } \log(B) - \log(A) \tag{3.2}$$

where, A refers to CFU per milliliter for the vial containing bacteria treated sample after the specified contact time, and B refers to CFU per milliliter for the "control group" vial after the specified contact time. All experiments were independently conducted in triplicate.

3.4.6 Cell Viability

Cell viability of KG cryogels was determined using MTT colorimetric assay. Cryogel samples were sterilized using UV radiation prior to analysis.

L929 mouse fibroblast cells were cultured in DMEM growth medium supplemented

with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin/streptomycin. Cells were seeded in 96-well plates at a density of 5×10^3 cells per well in 100 µL medium and incubated at 37°C and 5% CO_2 for 24 h. Prior to cell treatment, sterilized cryogel samples (KG-C1, KG-C2, and KG-C3) were incubated in Dulbecco's modified Eagle medium (DMEM) for 72 h at 37°C to obtain extracts. Following incubation, extracts were filtered to avoid contamination using a 0.22 µm filter. Subsequently, the medium in each well was aspirated and replaced with 100 µL of filtered extracts. For the control group, the medium was replaced with fresh DMEM. The plates were incubated at 37°C and 5% CO_2 for 24 and 48 h.

MTT (Neofroxx) was dissolved in 1X PBS to achieve a final concentration of 5 mg/mL and sterilized using a 0.22 µm filter. Then, 10 µL of MTT solution was added into wells containing the extracts of cryogels and to the control group. Subsequently, the well plate was incubated at 37°C for 4 h. After incubation, the MTT-containing medium was aspirated, and 100 µL dimethyl sulfoxide (DMSO) was added to each well to dissolve formazan crystals. Plates were gently shaken for 5 min to ensure complete solubilization. The optical density (OD) was measured at 570 nm using a microplate reader. All experiments were performed in triplicate (n = 3), and the standard deviations were calculated.

Cell viability (%) was calculated using the following equation:

$$\text{Cell Viability (\%)} = \frac{\text{OD}_{570\,(\text{sample})}}{\text{OD}_{570\,(\text{control})}} \times 100 \tag{3.3}$$

4
RESULTS AND DISCUSSION

In this study, KG cryogels were prepared to assist hemostasis during or after dental operations and imparted with vancomycin for the prevention of bacterial infections. For this purpose, cryogels with three different polymer concentrations (0.5% w/v, 1.0% w/v, 1.5% w/v) were produced and characterized. Resulting KG cryogels were cut into smaller pieces after the reaction was completed, and the cryogels bear a yellowish color owing to the color of KG (Figure 4.1). KG cryogels were further characterized to determine the crosslinking mechanism, evaluate the mechanical integrity, and assess the antibacterial activity.

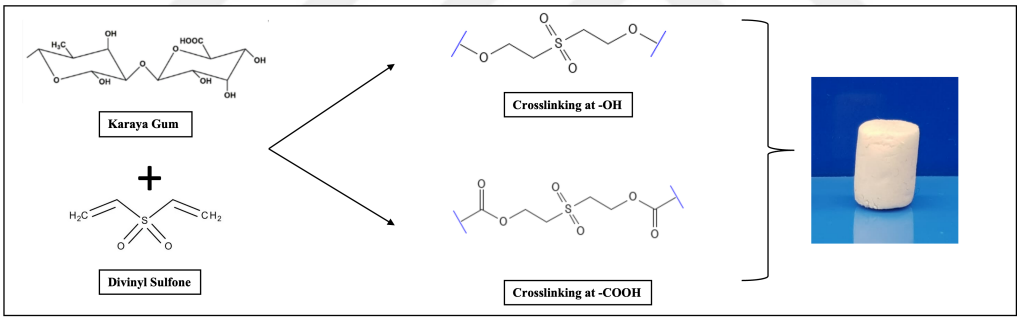


Figure 4.1 Schematic representation of the crosslinking between KG and DVS, and the obtained KG cryogel

4.1 FT-IR Spectroscopy Results

Dried samples of KG cryogels were analyzed using FT-IR spectroscopy to ensure the crosslinking between KG and DVS as well as to determine the crosslinking mechanism. FT-IR spectra of KG, DVS, and KG-C2 sample are displayed in Figure 4.2. KG shows characteristic peaks at 3319 cm^{-1} belonging to hydroxyl groups, which did not undergo a significant change after crosslinking. KG also exhibits peaks at 1719 cm^{-1} (C=O stretch) and 1601 cm^{-1} indicating the presence of acetyl and carboxyl groups, respectively [94]. Following crosslinking with DVS, the carboxyl group peak disappears and the peak of acetyl groups overlap with the peaks of freshly formed ester bond peaks at 1632 cm^{-1}. In addition, the peak at 1200

cm^{-1}, corresponding to the glycosidic bonds (C–O–C) within the structure of KG, exhibits a significant reduction in intensity following crosslinking.

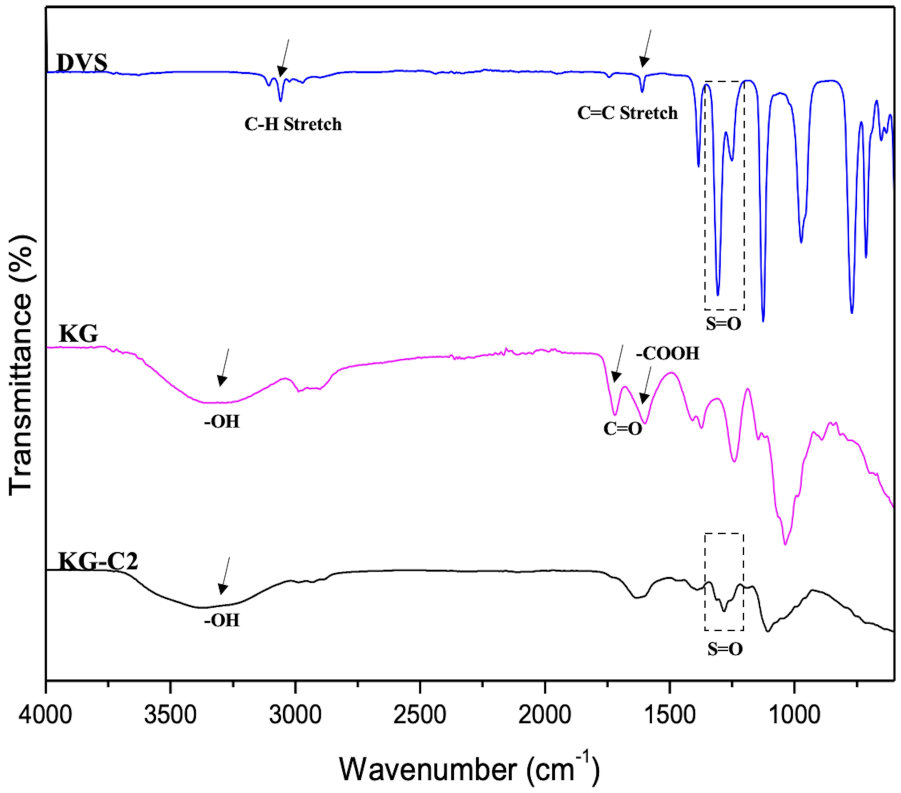


Figure 4.2 FT-IR spectrum of KG, DVS, and KG-C2 cryogel in the range of 4000-400 cm^{-1}.

Vinyl groups of DVS exhibit peaks at 3056 cm^{-1} due to C-C stretch and at 1607 cm^{-1} due to C=C stretch [95]. Disappearance of these peaks and emergence of S=O bands in the spectrum of KG-C2 further indicate crosslinking between KG and DVS. The peaks on 1306-1250 cm^{-1} belong to the S=O stretch of DVS and are also visible on the FT-IR spectrum of KG-C2 cryogel. Overall, these spectral alterations confirm the successful crosslinking of KG with DVS.

4.2 Inner Morphology and Porosity

The inner morphology and porosity are undoubtedly one of the most important parameters of dental biomaterials. Porous structure of cryogels can be tailored by changing the polymer concentration, freezing temperature, and freezing rate [53, 96]. In this study, KG cryogels were prepared in various polymer concentrations to investigate the effect of polymer concentration on porosity and determine the cryogel with optimal pore structure.

SEM images in Figure 4.3 reveal that all samples bear an interconnected macroporous structure with varying pore sizes. Although the majority of the current studies in the literature suggest that the pore size decreases with increasing polymer concentration [79], only a slight change in the pore diameter was detected in this study with varying polymer concentrations. According to the pore size distribution and average pore size calculated using the ImageJ software, KG-C1 sample bearing the lowest polymer concentration has a relatively lower average pore size (38.92±13.63 µm), with a narrower pore size distribution centered around 20-50 µm while the average pore size of KG-C2 sample was determined as 44.4-15.7 µm. KG-C3 sample reveals a higher average pore size (62.27±25.23 µm) as well as a broader distribution of large pore sizes predominantly ranging between 30-80 µm and reaching up to 120 µm.

While previous studies have reported similar trends [97, 98], the observed differences in average pore sizes across KG cryogels with varying polymer concentrations are relatively small. This indicates that the change in polymer concentration did not lead to a significant alteration in the overall porosity of the cryogels, with the average pore size varying only by a few micrometers. Given the consistency of the data within each group and the small magnitude of change observed, it can be inferred that the polymer concentration did not substantially affect the structural porosity of the cryogels within the range tested in the scope of this thesis.

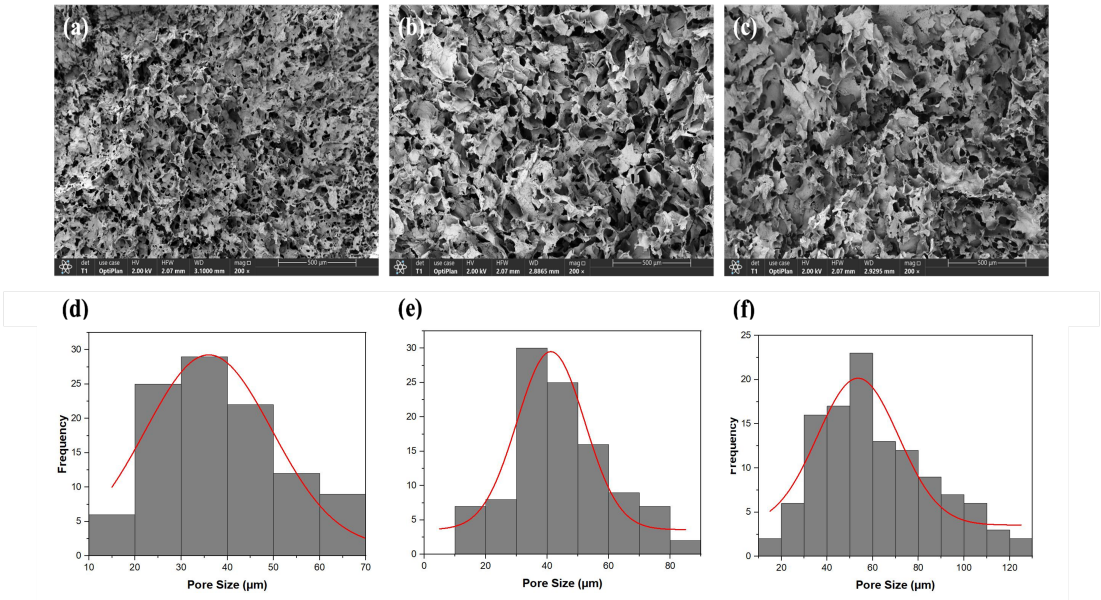


Figure 4.3 SEM images of (a) KG-C1, (b) KG-C2, and (c) KG-C3 at x200 magnification and pore size distribution of (d) KG-C1, (e) KG-C2, and (f) KG-C3 samples.

In a previous study, Sahiner et al. developed gum arabic-based cryogels with

pore sizes ranging from 50 to 150 μm, using DVS as the crosslinking agent [95]. These observations collectively support the current SEM findings, indicating strong alignment with prior research on natural gum-based cryogels.

Pore size and interconnectivity of cryogels are crucial determinants of the swelling properties that drive the absorption of blood and thus, the filtration of platelets from the blood. Higher porosity allows the rapid absorption of fluids and subsequent swelling of the structure. These findings suggest that the KG cryogels can serve as an ideal structure for the absorption and filtration of blood.

4.3 Swelling Behavior of KG Cryogels

Rapid swelling and high swelling ratio are arguably the most favorable characteristics of cryogels, especially in dental applications to assist hemostasis. Dental operations often demand biomaterials with superior swelling capabilities to treat excessive bleeding during and/or after the operation. Swelling property of cryogels is driven by the pore architecture and can be affected by various factors such as polymer concentration, interconnectivity, and porosity.

Swelling ratios of KG cryogels displayed in Figure 4.4 reveal that the KG-C1 sample has the highest swelling ratio (42.1 ± 4.9). The swelling ratio gradually declines with increasing polymer concentration – down to 39.9 ± 1.8 for KG-C2 and 38.2 ± 2.3 for the KG-C3 sample. A likely explanation is that higher crosslinking density promotes thicker pore walls, thereby constraining the overall capacity of the network for water absorption despite bearing larger pore size [79]. Similar results have been reported by several studies where the swelling ratio decreased with increasing polymer content due to the denser network structure [97, 99, 100].

Figure 4.5 displays the swelling kinetics of each sample. As shown, all samples reach the equilibrium swelling ratio within 1 min. While the highly porous structure of cryogels are factors that facilitate rapid swelling, another consideration influencing the swelling behavior is the mode of mass transfer through the pores. Within large pores, convection is a more favorable and faster process than diffusion for the mobility of water [101]. This characteristic is especially significant for dental applications, where materials often need to equilibrate swiftly to function effectively. A biomaterial that can swell rapidly while maintaining a robust, interconnected network can provide both mechanical support and controlled fluid absorption. Thus, the interplay between pore size, convective flow, and crosslink architecture in cryogels helps not only to achieve fast and efficient swelling but also underscores their promise for dental applications.

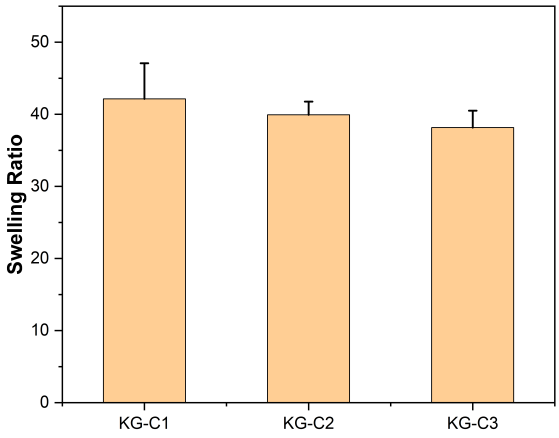


Figure 4.4 Swelling ratios of KG-C1, KG-C2, and KG-C3 samples.

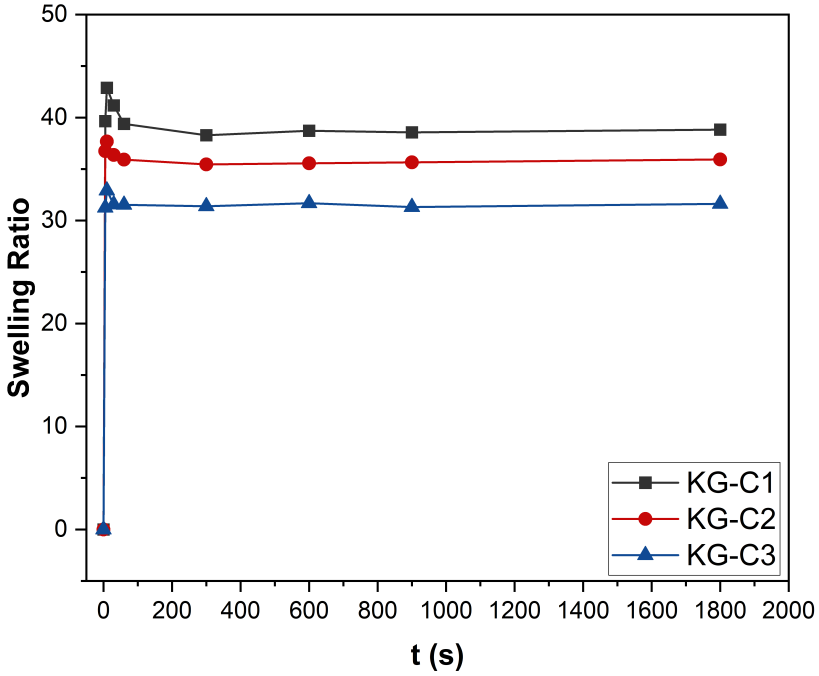


Figure 4.5 Swelling kinetics of KG cryogels between 0-1800 s.

During dental operations, the immediate absorption of blood is of high importance due to several reasons including assisting the hemostasis, concentrating the platelets, and consequently speeding up the coagulation process. This allows the formation of a stable clot and helps prevent the incidence of postoperative complications such as dry socket [55]. In this context, findings regarding the swelling behavior of the samples suggest that KG cryogels possess superior

swelling properties, a crucial feature for the treatment and prevention of excessive bleeding in dental operations.

4.4 Mechanical Performance of KG Cryogels

In order to evaluate the mechanical properties of KG cryogels, a series of uniaxial compression tests were carried out using cylindrical cryogels in a dried and swollen state. Figure 4.6 showcases the stress-strain curves of KG cryogels with different concentrations in both dried and swollen states. The strain, ε, is defined as the ratio of the change in length to the original length of the specimen. The stress is represented by its nominal value, σ_{nom}, which is calculated as the force per unit cross-sectional area of the undeformed specimen. Cryogels initially exhibit a linear stress response, referring to the Young's Modulus values. Following the linear trend, there is a limited increase in stress where the porous structure of KG cryogels is easily compressed almost up to 80% in dried state and ~90% in the swollen state before mechanical failure. Above these strain values, the network structure that is non-porous is compressed, showcasing a rapid increase in stress against strain [78]. It is noteworthy to mention that the sample with 0.5% KG concentration (KG-C1) was mechanically unstable for compression testing after swelling. In the dried state, KG-C3 sample with 1.5% polymer concentration presents slightly enhanced mechanical integrity compared to other samples with lower polymer concentrations. KG-C2 and KG-C3 samples can withstand a higher strain rate in the swollen state; however, the fracture stress of the samples differ greatly. In the swollen state, water acts as a plasticizer and the ability of the samples to deform before breaking is enhanced. However, the cross-sectional area bearing the compression load decreases when the pores are filled with water, decreasing the fracture stress in the swollen state [102].

The fracture stress of KG-C2 and KG-C3 samples are displayed in Figure 4.7(a). The fracture stress σ_f was calculated from the maxima of the corresponding true stress-strain curves [103–105]. According to the results, the force tolerable by the cryogel with 1.0% KG concentration (KG-C2) sharply declined from 4.1 MPa to 1.9 MPa in the swollen state. In contrast, the cryogel sample with 1.5% KG concentration (KG-C3) exhibited a unique trend where the nominal stress the sample could withstand before complete breakdown increased to over 5 MPa. These results suggest that the mechanical integrity of the cryogels is highly influenced by the polymer concentration. Polymers tend to form a denser network and thicker pore walls at high concentrations, minimizing the effects of water absorption during swelling. This feature enables cryogels to preserve their mechanical integrity and,

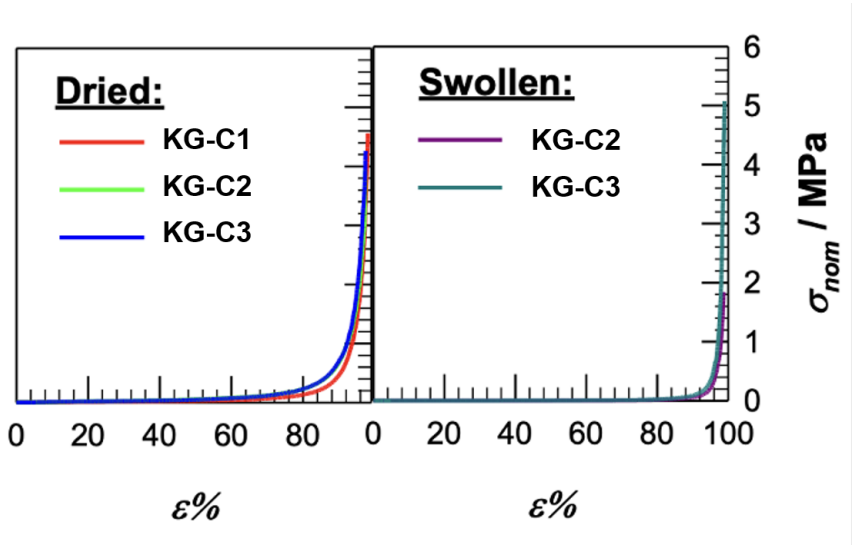


Figure 4.6 Stress-strain curves of KG cryogels in dry and swollen states.

in this case, improve their stability even after swelling [99]. Young's modulus of each sample was determined as 47.7±3.6 kPa for KG-C1, 53.7±5.1 kPa for KG-C2, and 122.1±2.1 kPa for KG-C3. Young's modulus reflects the stiffness of a material, and cryogels tend to be more rigid in the dried state and typically have a higher Young's modulus. After swelling, the water molecules inside the cryogel plasticize the structure, reducing the stiffness as a result. In alignment with this, Young's moduli of the samples in the swollen state were determined to be 6.7±0.9 kPa for KG-C2 and 11.9±0.8 kPa for KG-C3. It was previously reported that Young's modulus of cryogels can decrease significantly in the swollen state [106]. Overall, KG-C3 was determined to be the stiffest sample of all, most likely due to high polymer concentration. Similarly, KG-C3 exhibits the highest toughness (207.7±3.8) compared to KG-C2 (186.8±7.9) and KG-C1 (171.1±5.9), meaning that it can absorb the highest amount of energy before mechanical failure. After swelling, the toughness of KG-C2 and KG-C3 samples undergo a drastic shift to 35.5±1.4 KPa and 74.3±2.2 KPa, respectively. A different trend is observed in fracture stress where KG-C1 exhibited a higher fracture stress (4446.5±111.5 KPa) compared to samples with higher polymer concentration. This indicates that KG-C1 can withstand a higher amount of stress before its breakdown, likely due to its flexibility caused by lower crosslinking density.

Cryogels with robust mechanical integrity can effectively serve as physical barriers against blood flow, assisting hemostasis during or after dental procedures. The results of the mechanical tests demonstrate that KG cryogels maintain their structural stability not only in the dry state but also after swelling. KG-C3 excels with its strong mechanical properties, suggesting its suitability for the

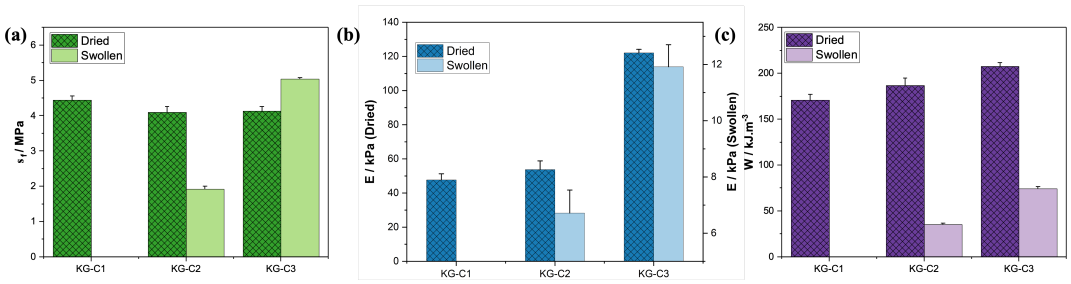


Figure 4.7 (a) Fracture stress, (b) Young's modulus, and (c) toughness of KG cryogels in dried and swollen states.

management of excessive bleeding. Moreover, the ability to swell rapidly and maintain mechanical integrity under physiological conditions enhances the potential of KG cryogels as reliable alternatives to traditional hemostatic agents in dental and surgical applications.

4.5 Antibacterial Activity of KG Cryogels

To evaluate the antimicrobial activity, KG cryogels were exposed to *S. pyogenes* and *S. mutans* at an initial cell concentration of 1.5-3.0 $\times$ 10^8 CFU/mL for 15 minutes. The viable cell counts were measured on agar plates over a 4-hour period (Figure 4.8-4.9).

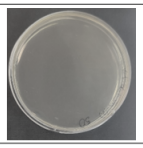
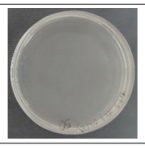
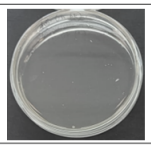
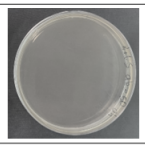
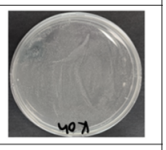
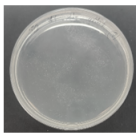
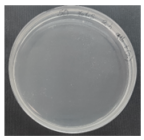
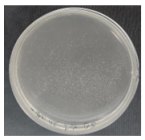
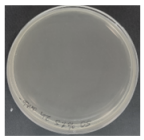
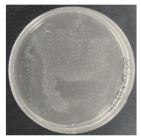
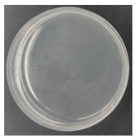
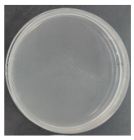
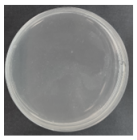
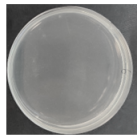
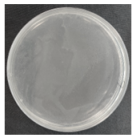
S. mutans	Samples				
Time (h)	KG-C2 with vancomycin	KG-C3 with vancomycin	KG-C2	KG-C3	Bacteria Control
0					
2					
4					

Figure 4.8 Resulting plate images of KG cryogel samples treated with *S. mutans* at 0h, 2h, and 4h

The log reductions for each sample were calculated by comparing the results of the control and test samples (Figure 4.10). Vancomycin-loaded KG-C2

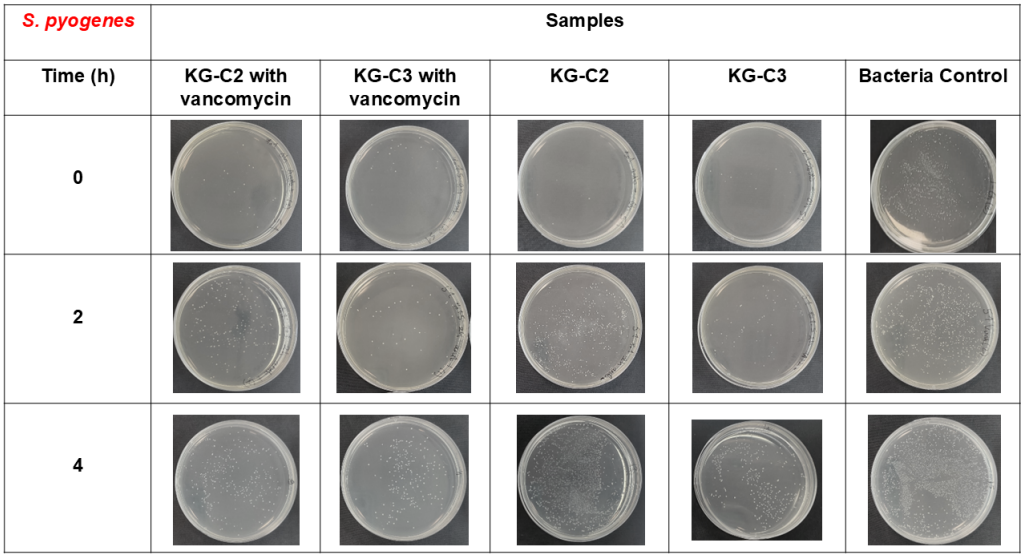


Figure 4.9 Resulting plate images of KG cryogel samples treated with *S. pyogenes* at 0h, 2h, and 4h

cryogel exhibited higher antibacterial activity against *S. pyogenes* compared to *S. mutans*, with approximately 0.5 and 1 log reduction, respectively, after 4 h. For vancomycin-free KG-C2 sample, no significant difference in activity was observed between two bacterial species; however, after 4 h, the vancomycin-loaded KG-C2 sample showed a 0.8-log reduction in *S. pyogenes* (Figure 4.10(b)). The vancomycin-loaded KG-C3 cryogel demonstrated an initial 1-log reduction in both *S. mutans* and *S. pyogenes*, sustaining this reduction over a 4-hour period. Without vancomycin, KG-C3 sample achieved a 1.7-log reduction against *S. mutans* and an average 1-log reduction against *S. pyogenes*. Although the inclusion of the antibiotic did not result in a statistically significant enhancement of antibacterial activity, KG-C3 exhibited a markedly superior ability to entrap bacteria.

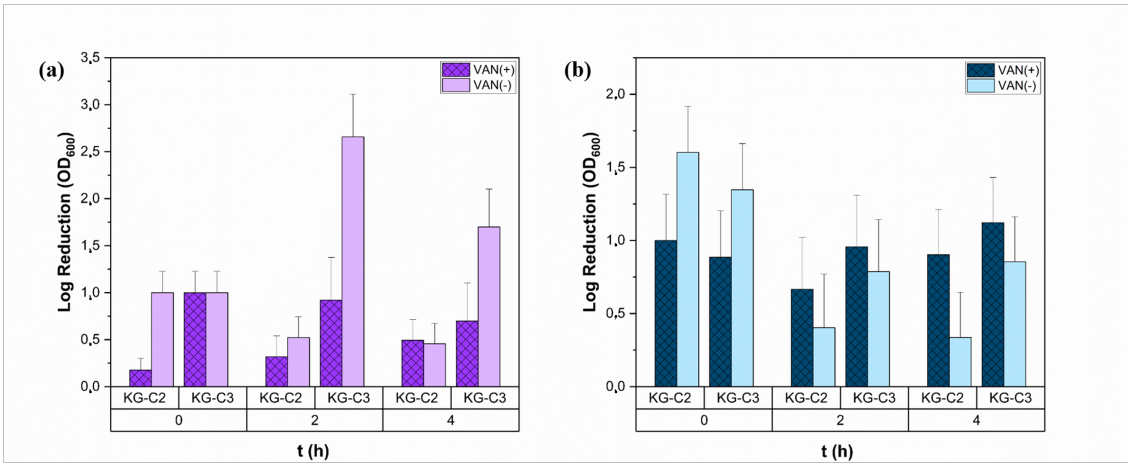


Figure 4.10 Antibacterial activity of KG cryogels on (a) *S. mutans* and (b) *S. pyogenes* at designated time intervals (t = 0h, 2h, and 4h)

KG cryogels exhibit antibacterial activity due to their ability to retain bacteria within the network, a mechanism that may result from the bacterial preference for the polysaccharide components of the gels as a carbon source, as reported by previous studies [107]. Figure 4.10 reveals that both KG-C2 and KG-C3 cryogels exhibit a more consistent effect on *S. pyogenes*, while the log reduction of vancomycin-loaded KG-C3 cryogels spiked at 2h and 4h against *S. mutans*. Given that the antibacterial activity primarily results from bacterial retention, it can be hypothesized that *S. mutans* is more effectively retained within the cryogel network than *S. pyogenes*. While the size and morphology of *S. mutans* may contribute to its strong entrapment, facilitating deeper penetration into the gel matrix, the small difference in size between the two bacterial strains suggests that this might not be the primary factor. Instead, the enhanced antibacterial performance of KG cryogels against *S. mutans* is likely driven by its preference for sugar-rich environments, which may promote adhesion and retention within the cryogel through potential utilization of KG as a carbon source [107]. In conclusion, KG-C3 demonstrates strong potential as an antibacterial biomaterial, particularly for infections caused by *S. mutans* and *S. pyogenes*. Its ability to attract and retain bacteria for extended periods further underscores its suitability for such applications.

Postoperative infection is a frequent complication following dental procedures, and managing it effectively without relying on systemic antibiotics is crucial to prevent adverse side effects and combat antibiotic resistance. In a clinical setting, the cryogels could be designed for single-use applications, with disposal recommended every four hours to maximize antibacterial efficacy and minimize potential bacterial accumulation. This suggested usage aligns with the observed antibacterial activity trends, ensuring sustained protection during application. The antibacterial activity tests of KG cryogels reveal promising results, highlighting the potential of cryogels as effective alternatives to commercially available dental sponges. These results indicate that KG cryogels could offer a targeted, localized solution for preventing infections, reducing the need for systemic antibiotics, and providing a more sustainable approach in dental applications.

4.6 Biocompatibility of KG Cryogels

The biocompatibility of KG cryogels was investigated via MTT cell viability assay on L929 mouse fibroblast cells, and the results of the MTT assay are displayed in 4.11. All groups are normalized with respect to the control group. All experiments were performed in triplicate (n = 3), and the resulting standard deviations were quite minimal (less than 0.3%). Therefore, error bars are omitted from the graphical

representation for clarity. According to the results, all KG cryogels exhibit high cell viability (above 70%), indicating notable biocompatibility. Among all samples, KG-C3 excels with the highest cell viability at 24 h (92 ± 0.06%) and at 48 h (88 ± 0.15%) followed by KG-C1 sample with 88.8 ± 0.18% at 24 h and 84.3 ± 0.27%. KG-C2 shows relatively lower cell viability, with 74 ± 0.13% and 71 ± 0.02% at 48 h. All cryogel samples exhibit a slight decrease in cell viability after 24 h.

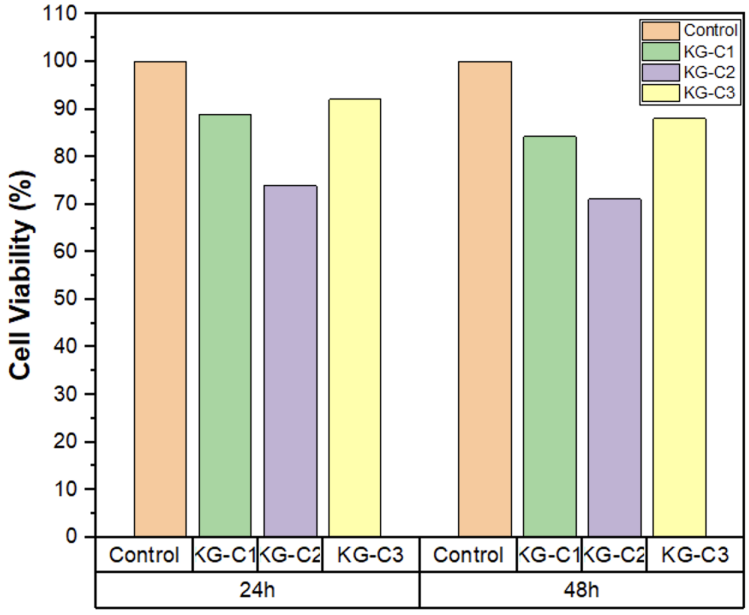


Figure 4.11 Cell viability of KG cryogels KG-C1, KG-C2, and KG-C3 in 24 and 48 h, normalized to control.

The biocompatibility of KG cryogels is dependent on two main components: KG and DVS. Previous studies on KG-based hydrogels consistently report excellent biocompatibility and high cell viability [108, 109], as KG is a non-toxic biopolymer. For instance, recent studies demonstrated that KG-based gels exhibited favorable cell viability profiles using various cell types, including NIH/3T3 fibroblasts and human skin fibroblasts [94, 109], which aligns closely with the findings reported in this thesis.

In contrast to KG, DVS is an organic compound that was previously reported as cytotoxic in numerous studies. Lai et al. demonstrated that hyaluronic acid scaffolds crosslinked with increasing concentrations of DVS resulted in reduced cytocompatibility and increased cytotoxicity [110]. Similarly, Hinsenkamp et al. reported that using DVS in optimal concentration mitigates any possible cytotoxic effects [111]. Therefore, a high cell viability rate can be maintained using DVS at an optimal concentration. In this case, the lower cell viability of KG-C2 can be associated with experimental conditions, particularly inadequate washing steps

since the concentration of DVS was constant for all cryogel samples. Insufficient removal of residual (unreacted) DVS might have caused a mild decrease in the cell viability, considering that the polymer concentration did not cause a drastic difference between KG-C1 and KG-C2. Additionally, prolonged exposure to DVS can also cause a reduction in cell viability, which explains the decrease in cell viability after 24 h.

Overall, KG cryogels exhibit remarkable biocompatibility against L929 mouse fibroblast cells, suggesting that they can be utilized in dental applications for up to 4h. Further investigations must be performed in order to determine the optimal DVS concentration for cell viability, and the residual DVS should be effectively cleared to eliminate undesired effects.

5
CONCLUSION

In this study, KG cryogels were prepared in varying polymer concentration followed by the addition of vancomycin, a potent antibiotic effective on Gram-positive bacteria. To the best of our knowledge, KG cryogels were fabricated for the first time in the literature. Based on the results of characterization and analysis, following points have been reached:

- KG cryogels were successfully prepared in three different concentrations (0.5%, 1.0%, 1.5%), and the crosslinking between KG and DVS was confirmed by the FT-IR results.
- KG cryogels have a macroporous and interconnective pore structure as confirmed by the SEM images and pore size distribution analysis, allowing the rapid absorption of fluids.
- Having a macroporous structure, all samples exhibited rapid swelling and high swelling ratio.
- Mechanical tests suggest that KG cryogels can undergo high levels of stress (up to 80%) before breaking down.
- KG-C3 sample shows excellent mechanical properties with the highest Young's modulus and toughness values, proving that the KG cryogels are mechanically stable at the swollen state as well.
- Antibacterial activity test results suggest that KG cryogels exhibit antibacterial activity against both *S. pyogenes* and *S. mutans*.
- KG cryogels exhibit remarkable biocompatibility against L929 mouse fibroblast cells.

Findings of this study indicate that KG cryogels possess a pore structure and swelling profile that allows the rapid absorption of bodily fluids upon swelling.

Moreover, mechanical test results imply that KG cryogels remain mechanically integrated even after swelling, allowing them to withstand the mechanical insults in the oral cavity. With the addition of vancomycin, the cryogels displayed strong antibacterial activity needed for the prevention of postoperative bacterial infections. Collectively, these attributes suggest that the KG cryogels developed in this study could serve as effective and reliable alternatives to existing dental sponges in the market.

Further studies are planned to evaluate the biodegradation and in vitro wound healing performance of the cryogels, along with detailed drug release analyses. These efforts aim to determine the feasibility and potential applications of the cryogels before and after dental operations.

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PUBLICATIONS FROM THE THESIS

Papers

1. "Novel Karaya Gum Cryogels for Preventing Postoperative Infections in Dental Care" Akin B., Tasdurmazli S., Findikli B., Su E., Ozbek T., Ozmen M.M. (in preparation).

Conference Papers

1. Akın B., Özmen MM., "Synthesis and Characterization of Karaya Gum-Based Cryogels as Dental Biomaterials", 3. Bilsel International Turabdin Scientific Researches and Innovation Congress, September 14-15, 2024.

Projects

1. "Super Elastisite ve Emme Kabiliyetli Antibakteriyel ve Hemostatik Dental Kriyojel Üretimi", Yildiz Technical University Scientific Project Coordination Unit, Project Number: FBA-2023-5560, Project Supervisor: Prof. Dr. Mehmet Murat Özmen.