T.R.
GEBZE TECHNICAL UNIVERSITY
INSTITUTE OF NANOTECHNOLOGY

DEVELOPMENT OF INJECTABLE CHITOSAN-BASED HYDROGELS WITH ANTIBACTERIAL PROPERTIES FOR WOUND HEALING

SÜMEYYE DANIŞ
A THESIS SUBMITTED FOR THE DEGREE OF
MASTER OF SCIENCE IN
DEPARTMENT OF NANOSCIENCE AND NANOENGINEERING

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YARA İYİLEŞTİRMESİ İÇİN ANTİBAKTERİYEL ÖZELLİKLERİ OLAN ENJEKTE KİTOSAN ESASLI HİDROJELLERİN GELİŞTİRİLMESİ

SÜMEYYE DANIŞ
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NANOBİLİM VE NANOMÜHENDİSLİK

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GEBZE TEKNİK ÜNİVERSİTESİ	**YÜKSEK LİSANS JÜRİ ONAY FORMU**

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SUMMARY

Hydrogels are three-dimensional structures with high water absorption capacity, and similar structure to the extracellular matrix. Due to the unique properties, hydrogels have become promising material for wound dressing applications in recent years. However, the current hydrogel structures are not sufficiently effective in treating irregularly shaped wounds. Injectable hydrogels with antibacterial properties have gained increasing importance as they can fill irregular wound gaps and promote cell growth. Chitosan is one of the most suitable natural materials for hydrogels because of its hemostatic properties, antimicrobial activity, biocompatibility, biodegradability. Injectable chitosan-based hydrogels offer greater insertable tissue penetration, reducing the risk of infection, scarring, and pain. To improve the properties of hydrogels for wound healing applications, there is a need to enhance production methods by optimizing hydrogels properties. Due to a number of significant benefits, microfluidic systems are an excellent choice for the manufacturing of hydrogels. This thesis study aims to produce low-cost, efficient and easy-to-apply injectable chitosan-based hydrogel and to apply it in wound healing applications with minimal toxicity. The microfluidic method is utilized to take advantage of its benefits and create injectable chitosan hydrogels. The properties of these hydrogels figured out in terms of various aspects such as morphology, chemical composition, and thermal properties by using SEM, FTIR, DSC, and Optical Microscopy. Furthermore, the antibacterial activity of hydrogels produced through both microfluidic and manual methods is evaluated against gram-positive and gram-negative bacteria using the disk diffusion test. The cytotoxicity of the hydrogels is evaluated on L929 cells using a resazurin cell viability assay. The overall results indicated the produced injectable chitosan-based hydrogels with their decent features like biocompatibility in this thesis study shows great promise for wound healing applications.

Keywords: Hydrogel, Wound Healing, Antibacterial, Injectable, Chitosan.

ÖZET

Hidrojeller, yüksek su emme kapasitesine sahip üç boyutlu yapılar olup, hücre dışı matrikse benzer üç boyutlu bir yapısı vardır. Hidrojellerin benzersiz özellikleri nedeniyle, son yıllarda yara iyileştirme uygulamalarında kullanılmak üzere bilim insanları için umut verici bir malzeme olmuştur. Mevcut hidrojel yapıları, düzensiz şekilli yaraları tedavi etmede yeterince iyi değillerdir. Yara boşluklarını çok iyi şekilde doldurabilen ve hücre büyümesini destekleyen antibakteriyel özelliklere sahip enjekte edilebilir hidrojeller giderek daha önemli hale gelmektedir. Kitosan, hemostatik özellikleri, antimikrobiyal aktivitesi, biyouyumluluğu, biyobozunurluğu nedeniyle hidrojeller için en uygun doğal malzemelerden biridir. Kitosan temelli enjekte edilebilir hidrojeller, daha fazla doku penetrasyonu sağlar, enfeksiyon riskini, yara izini ve ağrıyı azaltır. Yara iyileştirme uygulamaları için hidrojellerin özelliklerini iyileştirmek ve hidrojellerin özelliklerini optimize ederek üretim yöntemlerini geliştirmeye ihtiyaç vardır. Sahip oldukları bir dizi önemli fayda nedeniyle, mikroakışkan sistemler hidrojellerin üretimi için mükemmel bir seçimdir. Bu tez çalışması, düşük maliyetli, verimli ve kolay uygulanabilir enjekte edilebilir kitosan bazlı hidrojel üretilmesi ve yara iyileştirme uygulamalarında minimum toksisite ile uygulanmasını amaçlamaktadır. Bu amaç doğrultusunda hem mikroakışkan yöntem hem de manuel geleneksel yöntem ile enjekte edilebilir kitosan hidrojeli üretilerek morfolojik, kimyasal ve termal özellikler açısından SEM, FTIR, DSC, Optik Mikroskop ile değerlendirilmiştir. Ayrıca, mikroakışkan ve manuel yöntemlerle üretilen hidrojeller, disk difüzyon testi ile gram-pozitif ve gram-negatif bakterilere karşı antibakteriyel aktivite performansı açısından değerlendirilirken, sitotoksisite açısından ise L929 hücrelerinde resazurin hücre canlılığı testi kullanılarak değerlendirilmiştir. Genel sonuçlar, bu tez çalışmasında biyouyumluluk gibi iyi özellikleri ile üretilen enjekte edilebilir kitosan bazlı hidrojellerin yara iyileştirme uygulamaları için büyük umut vaat ettiğini göstermiştir.

Anahtar Kelimeler: Hidrojel, Yara İyileşmesi, Antibakteriyel, Enjekte edilebilir, Kitosan.

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LIST of ABBREVIATIONS and ACRONYMS

Abbreviations and Acronyms		**Explanations**
CS	:	Pure Chitosan
CS 1	:	1% ionically crosslinked chitosan hydrogel prepared manually
M-CS1	:	1% ionically crosslinked chitosan hydrogel prepared by microfluidics
CS/β-GP	:	1% chitosan hydrogel ionically crosslinked with β-glycerophosphate disodium salt
A	:	Absorbance
AA	:	Acetic Acide
C	:	Concentration
DSC	:	Differential scanning calorimetry
E. coli	:	Escherichia coli
ECM	:	Extracellular Matrix
FTIR	:	Fourier Transform Infrared Spectroscopy
LB	:	Luria-Bertani
MF	:	Microfluidic
SEM	:	Scanning Electron Microscopy
S. aureus	:	Staphylococcus aureus
3D	:	Three Dimensional
v/v	:	Volume per volume
w/v	:	Weight per volume
w/w	:	Weight per weight
β-GP	:	β-Glycerophosphate disodium salt

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1. INTRODUCTION

1.1. Motivation

The skin, which is the largest organ in the body, synthesizes vitamin D by protecting it from dangerous and harmful environmental conditions such as dust and microorganisms in the body, facilitates water transfer, provides CO2 exchange for homeostasis, and has an important barrier function such as feedback transmission. From nerve endings to the brain [1], [2]. Skin composed of three main layers (outermost covering, epidermis, dermis, and hypodermis) composed primarily of keratinocytes; dermis, connective tissue rich in collagen; and the hypodermis, or subcutaneous layer, consisting of adipose tissue that provides thermal insulation and mechanical protection to the body [3]. If a microorganism attack or side effect that causes skin damage occurs, the patient suffers pain, which reduces the patient's quality of life [4].

When the damage is not complex, the skin knows how to deal with this lesion, but if it is a serious condition, it is difficult to complete multielement combination of tissue regeneration processes without external factors [5]. The wound healing process usually includes the following steps: Haemostasis, Inflammation, Proliferation, Remodelling (Figure 1.1) [6]. The homeostasis stage is the most important stage of the wound healing step. The first action is taken to stop blood loss with temporary barriers through fibrin glands. If the wound cannot be closed, pathogenic microorganisms and foreign bodies can directly invade the body circulation. In the inflammation stage (24 hours to 4-6 days) after wound closure, invading microorganisms, neutrophils and macrophages are recruited, which sweep the wound bed of foreign particles, tissue debris increase the permeability of blood vessels, and redness and swelling accompany pain [7]. A sterile environment contributes to the wound healing process in order to save the tissue and prevent secondary wound healing and tissue necrosis. During the healing process, the body releases molecules such as cytokines and enzymes to promote the growth of new tissue [8]. The wound exudate provides necessary moisture, and in the proliferation phase, new tissue starts to fill the wound, producing a new extracellular matrix. In the remodelling phase, the formation of a strong collagen network increases the strength of the new tissue [3].

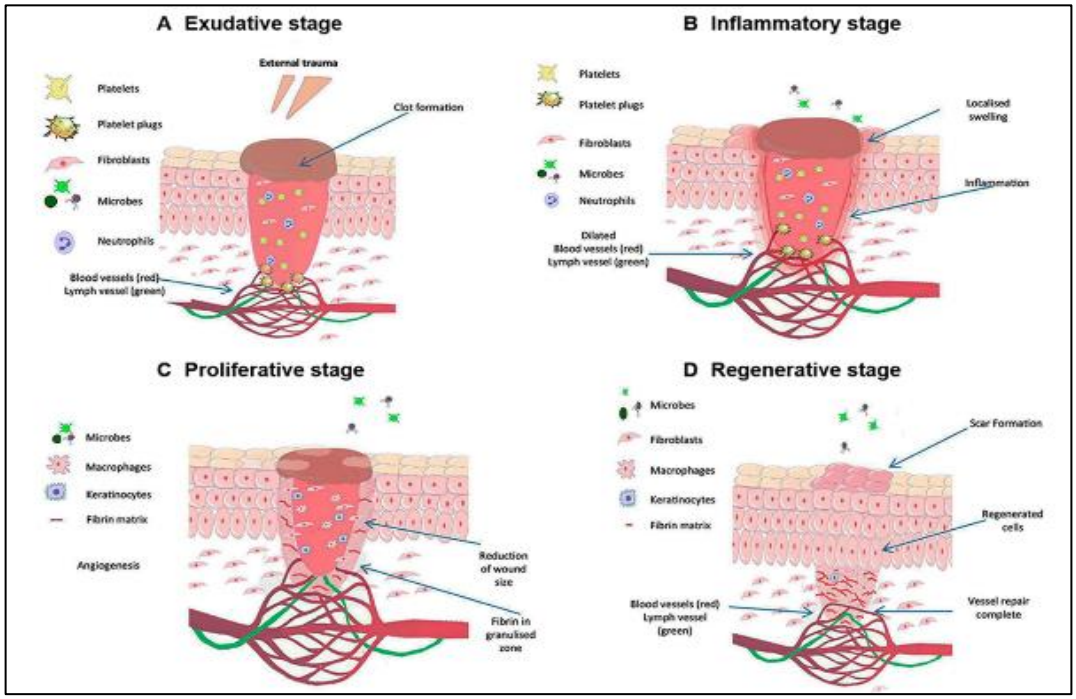


Figure 1.1. Schematic diagram for the four stages of wound healing: A) hemostasis, B) inflammation, C) proliferation, D) remodeling [4].

The effects of external factors should be evaluated in order to successfully and quickly complete the multiple mechanisms required for the healing of skin wounds. It is important to build a system with materials that will support the healing process and provide the most harmony with the skin, and do not create toxic effects [3]. Current treatment approaches include cleaning the wound bed, removing infection and tissue debris, and dressing the injured area. Treatment methods of injured wound can be classified as autograft, allograft, tissue engineering [9]. Autograft, allograft, methods reduce the quality of life of the patient who suffers for various reasons such as long surgical time, complications after the surgical procedure, and high costs. For this reason, it is possible to obtain a cost effective and efficient treatment method by developing the most preferred traditional method and microfluidic production treatment method, which is suitable for development and successful results. Dressing, which is another treatment method, should provide moisture in the wound, adequate oxygen and water vapor permeability, be sticky and flexible to provide mechanical adaptation to the body [10]. Dressings such as gauze or bandages should be made to protect the wound from external supports, however many external supports provide a low hydration state and adhere to the wound easily, thus causing re-injury when

removed and needing more time for healing. To achieve rapid and successful wound healing, there is an urgent need to maintain a moist environment and accelerate closure, promote tissue growth and reduce scar formation [2]. After the wound is formed, local skin functions such as protection, substance exchange and information transmission, which are the functions of the skin, are severely damaged and sometimes even destroyed [4]. Plasma exposure in the wound creates a nutrient-rich and moist microenvironment in local tissue. Bacteria are attracted and collected in the wound, triggering a severe inflammatory response for the immune system to fight infections and eliminate pathogens that delay healing. To solve the problem, the application of the extra hydrogel masker in the early stages of wound healing has been shown to be an effective method in the clinic [11]. If the hydrogel can kill the bacteria after contact with the wound, prevent the wound nutrients from being released, and if it can cut the contact of the bacteria, it can eliminate the most dangerous stages that delay healing [6].

1.2. Objective of the Thesis

Developing an efficient and biocompatible material for improved cutaneous tissue regeneration is a challenge in healthcare [12]. Currently, various wound dressing materials including nano-fiber, foam membrane, hydrogel, etc. have been designed and fabricated, with hydrogel being considered a promising material due to its high-water content and biocompatibility [13]. However, current hydrogels have limitations in matching irregularly shaped wounds and being applied to special areas like joints and oral wounds. Injectable properties can address this issue by quickly filling large, irregular wounds, leading to self-healing and a single, ideally shaped hydrogel dressing [14]. The polymers used in hydrogel materials can be classified into several categories based on their composition and origin for example synthetic polymer such as polyacrylamide, polyethylene glycol, and polyvinyl alcohol and natural polymer derived from biological sources such as alginate, chitosan, hyaluronic acid, gelatine, collagen, and fibrin. Chitosan, a biological material, has received attention for its excellent biocompatibility and potential use as a wound dressing [15]. It is biodegradable, non-toxic, antimicrobial, biologically adhesive, biologically active, and haemostatic, with high chemical versatility due to easily reachable amino and

hydroxyl groups. However, its poor solubility and mechanical properties in neutral water hinder its medical application [16]. Injectable hydrogels are gaining importance as they can reach deep tissue defects with minimum invasiveness and provide better adaptation to wound margins. They also result in reduced risk of infection, scarring, and pain, and lead to neovascularization, the development of new blood vessels in impaired tissues [17]. Injectable hydrogels can be produced using various methods, each offering different advantages in terms of fabrication, tunability, and application such as chemical crosslinking, physical gelation, photo-polymerization, enzymatic crosslinking and ionic crosslinking. Injectable hydrogels can be produced with microfluidic systems, providing a free diffusion medium for small biomolecules and easy observation of molecular diffusion and cell behaviour under a microscope [6]. They are also easily shaped and commercially available, and most hydrogels are biocompatible with a Young's modulus comparable to that of cells [18].

1.3. Scope of the Thesis

The scope of this thesis study is to produce chitosan-based injectable hydrogel by figured out its critical features for wound healing application by comparing microfluidic production and manual mixing production method. Defining the best production method for injectable chitosan-based hydrogel which is low-cost, efficient, and easy application method that minimizes toxicity while maximizing the material's advantages for wound healing. The current limitations of wound dressing materials, including hydrogels, are addressed by the potential use of injectable hydrogels. The aim is to create an injectable hydrogel that can quickly fill large, irregular wounds, with improved biocompatibility, reduced risk of infection, scarring and pain, and better adaptation to wound margins. This study also evaluates at the production of hydrogels using microfluidic systems for easy observation of molecular diffusion and cell behaviour.

2. LITERATURE REVIEW

2.1. Treatment Strategies for Wound Healing

Wound can be divided into two main parts as acute and chronic. Acute is the healing of the skin within 8-12 weeks after damage such as burning, chemical damage or cutting. Chronic wounds are requiring for a long time to healing such as weeks, moths to years caused by diseases such as cancer, venous or arterial vascular insufficiency, diabetes. These wounds complicated than acute wounds often fail to reach normal healthy state persisting in a pathological condition of inflammation [19]. Due to the delayed and inadequate treatment processes in the recovering of both chronic and acute wounds, treatment costs and waste are increasing every year in the world [10].

There has been published reports that show that the annual cost of care for non-healing wounds, also called chronic wounds, is over $25 billion in the United States for the 21st century [20]. Especially chronic wounds struggle with many factors that prevent wound healing, such as increased bacterial load, high pH, excessive wound drainage, oxidation and increased tissue enzyme activity [21]. Bacterial infections in the wound environment can lead to the release of endotoxins that cause elevation of proinflammatory cytokines or exotoxins that can cause tissue necrosis [22]. An ideal wound dressing should provide a locally moist environment, stimulate growth factors, absorb wound fluids and exudates, protect the site from infection, and be biocompatible [23]. Treating difficult wounds with current treatment methods is painful and takes a long time. There is a need for a combined treatment method that will provide the support needed by the skin to reduce bacterial growth and increase cellular activity by supporting healing, especially in injuries [24]. Current treatment approaches fall short of meeting this demand because they primarily address the outward signs of chronic wounds rather than the underlying pathophysiological conditions that prevent tissue reorganization and wound healing [25]. Additionally, because bandages must be changed many times per day, necrotic tissue must be removed from the wound site, and other necessary maintenance operations, existing treatment modalities are costly, uncomfortable, and time-consuming. The need for a

low-cost, low-maintenance, multifunctional treatment alternative has grown as a result of the high cost of care, low patient compliance, and lengthy wound care procedures [26].

Acute skin injuries, such as cuts, burns, or other trauma, can also be painful. It is important to prevent and treat many bacterial infections and pathogens that are constantly gaining resistance in a short time and efficiently [27]. In burn trauma, both the immune system is impaired, and the physical barrier is damaged, leaving the body more vulnerable to bacterial colonization and infections. If treatment is not started immediately and bacterial growth is not prevented, skin and soft tissue infections begin and are one of the most common bacterial infections in the human population [28]. Microbial eradication and applications that support the wound healing process are needed both in burn treatment and other acute skin injuries. Combining nanotechnology and biomaterial technologies and designing a treatment method with materials that exhibit antimicrobial properties as well as internal wound healing is important to meet this need [29].

The body's mechanism for replacing injured tissue is wound healing. There are many methods used to heal wounds [30]. These methods vary according to the size, depth and cause of the wound. Some general wound healing methods are as follows; Cleaning the wound: Benefits include helping to prevent infection and removing debris that can help speed up the healing process. Disadvantages include that it can be painful, especially if the wound is deep or has a lot of debris. Applying a dressing: Benefits include helping keep the wound clean and moist, which can help speed healing. Disadvantages include that it takes time to change the dressing and that the wound can dry out or become irritated if the dressing is not changed often enough. Using over-the-counter ointments: Advantages include being easy to apply and can help keep the wound moist and protect it from infection [31]. The downsides are that they may not be as effective as prescription drugs and can cause allergic reactions in some people. Taking antibiotics: Their advantage is that they can help clear up an infection and prevent it from spreading. The disadvantages are that they can cause side effects such as stomach upset or allergic reactions and can contribute to the development of antibiotic-resistant bacteria [32]. Closing the wound with stitches: Benefits include helping to bring the edges of the wound together and promoting faster healing [33]. The disadvantages include that it can be painful and there is a risk of infection if the wound is not properly cared for. Using topical growth factors: The benefits are that

they can help stimulate the growth of new skin cells and blood vessels, which can help speed up the healing process. The disadvantages are that they may not be effective in all cases and may cause side effects such as skin irritation or allergic reactions. Using negative pressure wound therapy: The advantages are that it can help remove excess fluid and speed healing. The disadvantages include that it can be time consuming and inconvenient, and the risk of infection if the device is not properly maintained [34]. Using laser therapy: Its benefits include being able to stimulate the growth of new skin cells and blood vessels, which can help speed up the healing process. The downsides are that it can be expensive and there is a risk of side effects such as skin irritation or scarring. Having surgery: Its advantages are that it can be effective in repairing damaged tissue or removing dead tissue. The downsides are that it can be painful and there is a risk of complications such as infection or scarring [26].

2.2. Hydrogels for Wound Healing Applications

Hydrogels are gels that are composed primarily of water. They have a soft, spongy texture and are often used in medical and healthcare applications due to their ability to retain moisture and provide a moist environment for cells to grow. In the context of wound healing, hydrogels can be used as dressings to help keep the wound moist and promote the growth of new skin cells.

Hydrogels can be made from a variety of materials, including natural polymers such as collagen and chitosan, and synthetic polymers such as polyacrylamide and polyethylene glycol. They can be used as standalone dressings or as a delivery system for medications and other substances that are intended to promote healing. Hydrogels offer several benefits in wound care. They maintain moisture, aiding in the growth of new skin cells and expediting the healing process. Additionally, they alleviate pain and discomfort during dressing changes. Hydrogels are user-friendly, allowing easy application and customization to fit the wound’s dimensions. They are also safe and non-toxic. Moreover, hydrogels do not adhere to the wound, facilitating painless removal and minimizing discomfort. While hydrogels have numerous advantages in wound healing, there are potential drawbacks to consider. These include a short shelf life due to contamination risk, limited absorbency of exudate necessitating additional dressings, and the higher cost compared to other wound dressings. Injectable

properties can address this issue by quickly filling large/irregular wounds, leading to self-healing and a single, ideally shaped hydrogel dressing [14].

2.3. Injectable Hydrogels in Wound Healing

Injectable hydrogels are a type of hydrogel that can be injected directly into the body. They are often used in medical and healthcare applications, including in wound healing. In the context of wound healing, injectable hydrogels can be used to deliver medications or other substances that are intended to promote healing directly to the site of the wound. They can also be used to fill in gaps or defects in tissue and to stimulate the growth of new cells.

Injectable hydrogels offer numerous advantages in wound healing, including precise delivery, non-invasiveness, controllable release, and ease of use. However, there are potential disadvantages to consider, such as limited availability, higher cost, the risk of infection if not properly sterilized or cared for, and the possibility of allergic reactions [35]. Some people may be allergic to the materials used to make the injectable hydrogel, which can cause side effects such as skin irritation or allergic reactions.

There have been a number of studies conducted on the use of injectable chitosan hydrogels for wound healing [6]. Injectable hydrogels can be made from a variety of materials, including natural polymers such as collagen and chitosan, and synthetic polymers such as polyacrylamide and polyethylene glycol [36]. In general, these studies have found that injectable chitosan hydrogels can be effective at promoting the growth of new cells and the healing of wounds. One study published found that injectable chitosan hydrogels can be used to deliver medications directly to the site of a wound and can help to promote the growth of new cells [37]. Another study published found that injectable chitosan hydrogels can be used to fill in gaps or defects in tissue and can stimulate the growth of new cells [38]. Other studies have examined the use of injectable chitosan hydrogels in combination with other substances, such as growth factors or medications, to enhance their effectiveness in promoting wound healing [15]. Overall, the literature suggests that injectable chitosan hydrogels can be a useful tool in the treatment of wounds, although more research is needed to fully understand their potential and to determine the optimal conditions for their use [39].

2.4. Enhanced Chitosan-based Hydrogel's Antibacterial Properties

Chitin, a form of polysaccharide found in the shells of crustaceans like shrimp and crabs, is the source of the natural polymer chitosan[16]. Chitosan has a variety of possible uses in medicine and healthcare, including as a hydrogel for healing wounds. Due to its biocompatibility, biodegradability, inherent antibacterial, and wound-healing capabilities, chitosan natural, cationic polymer generated from the deacetylation of chitin has been investigated as a biomaterial for wound care as shown in Figure 2.1. Because of their possible interactions with the structure of the skin and capacity to solubilize drugs with poor solubility, lipid-based delivery methods like liposomes are frequently of interest for topical skin therapy[39]. Chitosan can be infused with liposomes to form chitosomes, which have been shown to have improved antimicrobial properties and have been used in vaginal Candida infections. Through synergistic effects on the bacterial membrane, combining chitosomes with antimicrobials that target membranes, like chlorhexidine, has the potential to further boost the antimicrobial capacity [26]. In this study, chitosan was chosen as a hydrogel base for its bio-adhesive and biocompatible properties and its antibacterial activity was also investigated as a means of rapid and efficient microbial prevention and eradication [26]. The mechanism by which chitosan exhibits antimicrobial activity is believed to be due to its ability to disrupt the cell membrane of bacteria and fungi, leading to cell death [40]. Additionally, chitosan has been found to have a positive charge, which can attract and trap negatively charged bacteria and other microorganisms [41]. One of the properties of chitosan that has attracted interest in the medical field is its antimicrobial activity [42]. Chitosan has been shown to have antimicrobial activity against a number of different types of bacteria's such as Escherichia coli, Pseudomonas aeruginosa and Staphylococcus aureus [43]. This antimicrobial activity is thought to be due to the ability of chitosan to interact with the cell walls of bacteria and to disrupt their structure, which can prevent the bacteria from growing and multiplying [44].

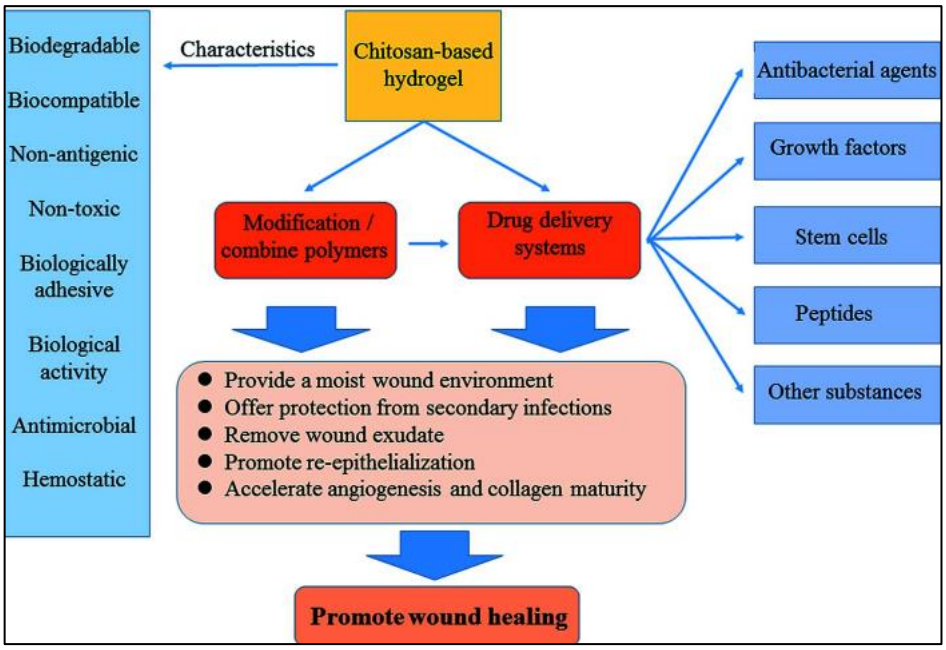


Figure 2.1. Application of chitosan-based hydrogel dressings [15].

Chitosan is a natural substance that has been studied for its potential antibacterial and cytotoxic properties [45]. The results of some studies have been showed that chitason showing strong antibacterial activity against a wide range of bacteria, while other studies have found little or no antibacterial activity. Some studies have reported that chitosan is effective against both gram-positive and gram-negative bacteria, and has been found to be particularly effective against methicillin-resistant Staphylococcus aureus (MRSA) [26]. Chitosan has been found to disrupt the cell membrane of bacteria, leading to cell death. Additionally, chitosan has been found to have a positive charge, which can attract and trap negatively charged bacteria. In terms of cytotoxicity, some studies have reported that chitosan has low cytotoxicity against a range of cell lines, including human skin fibroblasts and blood cells, making it a promising material for use in biomedical applications [39]. However, other studies have reported that chitosan can be toxic to certain cell lines, particularly when used at high concentrations [46]. Overall, the literature results on chitosan antibacterial activity and cytotoxicity are mixed. More research is needed to determine the full potential of chitosan as an antibacterial and cytotoxic agent [27].

In addition to its antimicrobial properties, chitosan-based hydrogels have also been found to have other beneficial properties such as biocompatibility, biodegradability, and non-toxicity [47]. These properties make chitosan-based hydrogels a promising material for use in biomedical applications such as wound

healing, tissue engineering, and drug delivery [4]. For example, chitosan-based hydrogels have been used as wound dressings to promote wound healing by providing a moist environment for cells to migrate and multiply [11]. They have also been used as scaffolds for tissue engineering, providing a supportive environment for cells to grow and differentiate [35]. In conclusion, because of their antibacterial qualities, biocompatibility, biodegradability, and non-toxicity, chitosan-based hydrogels offer a wide range of potential uses in the medical and various industries [48].

In the context of wound healing, chitosan-based hydrogels are useful in helping to prevent infections and to promote the healing of wounds. Chitosan-based hydrogels may also be useful in preventing the spread of infection in cases where a wound becomes infected.

2.5. Production Strategies to form Injectable Hydrogels

There are numerous production methods that can be used to make injectable hydrogels, each of which has benefits and is suitable for a range of applications. Chemical crosslinking is one of the simplest processes used to crosslink the components of the hydrogel. This can be accomplished either by using a crosslinking agent or by introducing reactive groups to the hydrogel precursors that can react with one another to produce stable crosslinks. For instance, in the case of synthetic hydrogels, free-radical polymerization can be utilized to polymerize multifunctional monomers or macromers into crosslinked hydrogels [6]. Crosslinking physically, chemical processes are not involved in physical crosslinking techniques. To create the hydrogel network, they rely on non-covalent interactions. Physical crosslinking mechanisms that are often used include ionic interactions, hydrogen bonds, and hydrophobic interactions. Polyelectrolyte hydrogels, for instance, can be produced by mixing oppositely charged polymer components that interact via electrostatic interactions [49]. Biological crosslinking; enzymes that catalyse particular interactions between the hydrogel precursors can be used to crosslink some hydrogels. Gelation that is both cell- and biocompatible is possible with this technique. Transglutaminase enzymes, for instance, can be used to crosslink hydrogels made of gelatine [50]. Thermoresponsive gelation; changes in temperature can cause temperature-responsive hydrogels to gelate or shift from a sol-gel state. The hydrogel remains liquid for easy

insertion below a crucial temperature and solidifies into a gel at physiological levels. Light responsive gelation, when exposed to particular light wavelengths, light-responsive hydrogels are prompted to crosslink [20]. The hydrogel precursors contain photo initiators or photoreactive groups, and light is utilized to start the crosslinking reaction. Visible light and ultraviolet (UV) light are frequently used in light-responsive gelation techniques. Pre-formed injectable hydrogel particles, This method involves the synthesis of hydrogel precursors, which are then converted into injectable particles like microgels or nanoparticles [51]. These particles can self-assemble and form the hydrogel network when they are directly injected into the desired location.

The application for which the hydrogel is to be used, its biocompatibility, the rate at which it gels, and its required mechanical qualities are only a few of the variables that influence the production approach [52]. The most appropriate production method will also depend on the hydrogel's intended function (such as drug delivery, wound healing and tissue engineering).

2.6. Microfluidic Systems

Microfluidics and micro-processing methods such as photolithography, electron beam lithography, chemical vapor deposition etc. have enabled micro-scale-controlled studies of difficult-to-control studies such as in-vivo and in-vitro performed in cell biology studies. Microfluidic systems are highly advantageous for artificial tissue engineering and disease treatment in biomedical research and healthcare. These systems enable precise control and manipulation of fluids at the microscale, essential for creating tissue structures with specific gradients of nutrients and signalling molecules. They also support high-throughput screening of experimental conditions, making them ideal for testing various cell types, biomaterials, and drugs in tissue engineering and disease models as shown in Figure 2.1. The small size of microfluidic devices reduces the sample volumes required, improving efficiency and cost-effectiveness in tissue engineering. Additionally, by replicating native tissue microenvironments, such as perfusion and oxygen gradients, microfluidic systems create more physiologically relevant tissue models, enhancing the accuracy of disease studies and drug testing [53].

These systems facilitate the generation of 3D tissue models that better mimic native tissue complexity, allowing researchers to study tissue behaviour and interactions realistically. Microfluidic platforms can also be designed to promote controlled cell-cell interactions, leading to more functional and organized tissues. By integrating sensors and imaging components, microfluidic devices enable real-time analysis and monitoring of tissue growth, cellular responses, and disease progression, providing valuable insights into tissue engineering processes and disease treatment dynamics. Microfluidic systems are adaptable for personalized medicine, enabling the creation of patient-specific tissue models to study disease mechanisms and evaluate drug responses, potentially leading to more tailored and effective treatments [54].

Moreover, microfluidic systems offer an ethical alternative to traditional animal testing, as they allow relevant in vitro studies, thereby reducing the need for animal experiments and refining preclinical research practices. Overall, the advantages of microfluidic systems contribute significantly to advancing tissue engineering and disease treatment methodologies.

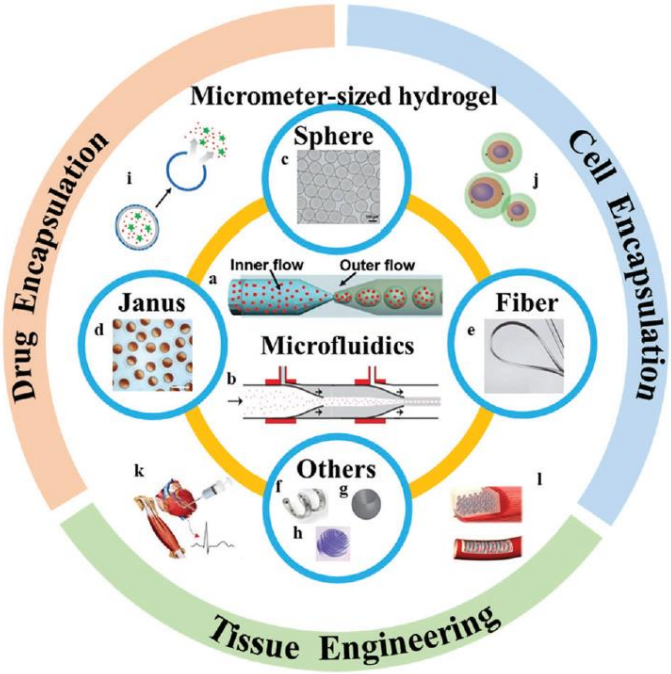


Figure 2.2. Overview of micrometer-sized hydrogels with diverse shapes produced by microfluidics and their biological applications [53].

Microfluidics is a multidisciplinary field that includes disciplines such as engineering, physics, chemistry, and nanotechnology as shown in Figure 2.2. This area

is flow systems that require precise control and manipulation on systems created with various geometric channels such as Y-junctions, T-junctions, flow-focusing and co-flow as shown in Figure 2.3 [53]. In the initial design phase, microfluidics was primarily created for miscible flows. Droplet-based microfluidic technology was discovered with further studies and attracted great interest [55]. With this system, microgels with high monodispersed and reproducibility can be produced based on precise flow control, and shapes that would be difficult for large volume mixtures can be optionally adjusted. There are two different methods called [56] dispersion phase and continuous phase [57], these involve injecting one liquid into another immiscible or partially miscible liquid with the droplet based microfluidic system and the droplets are formed at the meeting point due to shear force [53]. One of the main advantages of injectable hydrogels when fabricated with microfluidic is that they can form scaffolds more quickly in situ after being injected into targeted areas for tissue regeneration and regrowth[58]. After injection, a microporous interconnected hydrogel network can be bonded with surrounding tissues for rapid cell migration, cell adhesion and integration. In addition, studies have shown that microporous annealed hydrogel, especially for in vitro cell culture, is superior to other hydrogel structures in cell migration rate and cellular level connectivity[59].

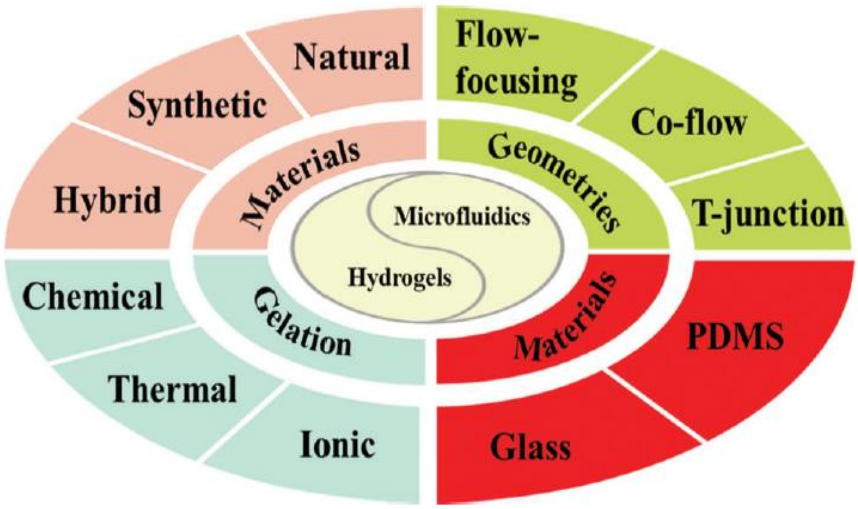


Figure 2.3. Hydrogels and microfluidic device classification[53].

3. MATERIALS AND METHOD

In this section, details about the materials and devices used in this thesis is presented. The production methodology of hydrogel platforms by conventional and microfluidic production is described in detail. The procedures, preparation and characterization methods examined followed during both microfluidic production and conventional production methods, cytotoxicity and antibacterial activity tests of resultant chitosan hydrogels achieved chitosan polymeric solution is described. Statistical analysis evaluations of the experimental results are presented.

3.1. Materials

The main materials used to produce injectable chitosan hydrogel in the experimental studies are shown in Table 3.1. Chitosan polymer was used for the production of injectable hydrogels. Since chitosan, which has a 75% diacylation degree, is not soluble in water, it is provided to dissolve chitosan more easily by using acetic acid and distilled water. B-Glycerophosphate disodium salt powder was used as for formation of injectable chitosan hydrogels.

99%-100% pure acetic acid (AA, glacial) was purchased from Tekkim company. Shrimp-derived chitosan (CS) (75% deacetylated) and β-Glycerophosphate disodium salt (β-GP) were purchased from Sigma Aldrich. Distilled water (DW) collected from the Sartorius device in Gebze Technical University (GTU) Nanotechnology Institute was used.

Table 3.1. Materials used in polymeric solution preparation of this research.

Material	Brand	City/Country
Shrimp-derived chitosan (CS) (%75≥ deacetylated)	Sigma-Aldrich	Shnelldorf/Germany
β-Glycerophosphate disodium salt	Biosynth Carbosynth	Compton/UK
Acetic Acid (glacial)	TEKKİM	Bursa/Turkey

3.2. Methods

This thesis study focused on development of missing point of injectable chitosan hydrogels materials with the using of T-shaped microfluidic junction, which would contribute to improve antibacterial treatment and enhance the long-term quality of life for patients by eliminating the need for hydrogel change routine when they need. This thesis aims to compare two production techniques which are microfluidic and manual production of injectable chitosan-based hydrogel and define a way which is a low-cost, efficient, and easy application method that minimizes toxicity while maximizing the chitosan material's advantages. The microfluidic method is utilized to take advantage of its benefits and create injectable chitosan hydrogels. The properties of these hydrogels are evaluated in terms of morphology, chemical composition, and thermal properties using scanning electron microscopy (SEM), Fourier-transform infrared spectroscopy (FTIR), differential scanning calorimetry (DSC), and optical microscopy. Furthermore, the antibacterial activity of hydrogels produced through both microfluidic and manual methods is assessed against gram-positive and gram-negative bacteria using the disk diffusion test and following the procedure ASTM F895-11. The results achieved was assessed compared the reference material, with a reference of amoxicillin. The cytotoxicity of the hydrogels is evaluated on L929 fibroblast cell lines using a resazurin cell viability assay. The overall findings suggest that injectable chitosan-based hydrogels hold tremendous promise for wound healing applications.

3.2.1. Manual Preparation of Chitosan-Based Hydrogels

The procedure followed by Melda Elmas in her thesis study was followed in the manual production and microfluidic hydrogel production method. She has proven in her optimization studies that the best feature is achieved with 1% (w/v) chitosan. In this study, experimental studies were carried out with only 1% (w/v) chitosan concentration, making use of her experience[60].

Chitosan powder was mixed in distilled water for roughly 2 hours at a moderate stir (1000–1550 rpm) at 37 °C temperature to create a chitosan solution (1%(w/v)). 1% acetic acid was gradually added to the chitosan powder using a 2.50 micropipette at 37

°C and 1500 rpm for 1 hour while the solution was stirred magnetically. As a crosslinker solution, distilled water was used to generate an 80% (v/v) β-glycerophosphate disodium salt-water solution was prepared to obtain chitosan injectable hydrogels. For gelation, this β-glycerophosphate solution was refrigerated for 1 hour at 4 °C. The chitosan solution was likewise put in the fridge after one hour of stirring. Cold glycerophosphate solution was gradually added to the chitosan solution in an ice bath at a volume ratio of 1:1 (v/v) in order to achieve ionic gelation.

Table 3.2: Polymer solution and salt solution concentrations for chitosan injectable hydrogels.

Solution	**Acetic Acid**	**Temperature**	**Time**	**Velocity of Stirring**
1% w/v CS	1% v/v	37˚C	roughly 2 hours	1000-1500 ± 20 rpm
Solution	**β-GP**	**Temperature**	**Time**	**Velocity of Stirring**
Cross-linker solution	7.5% w/v	4°C	10 min	1000± 20rpm

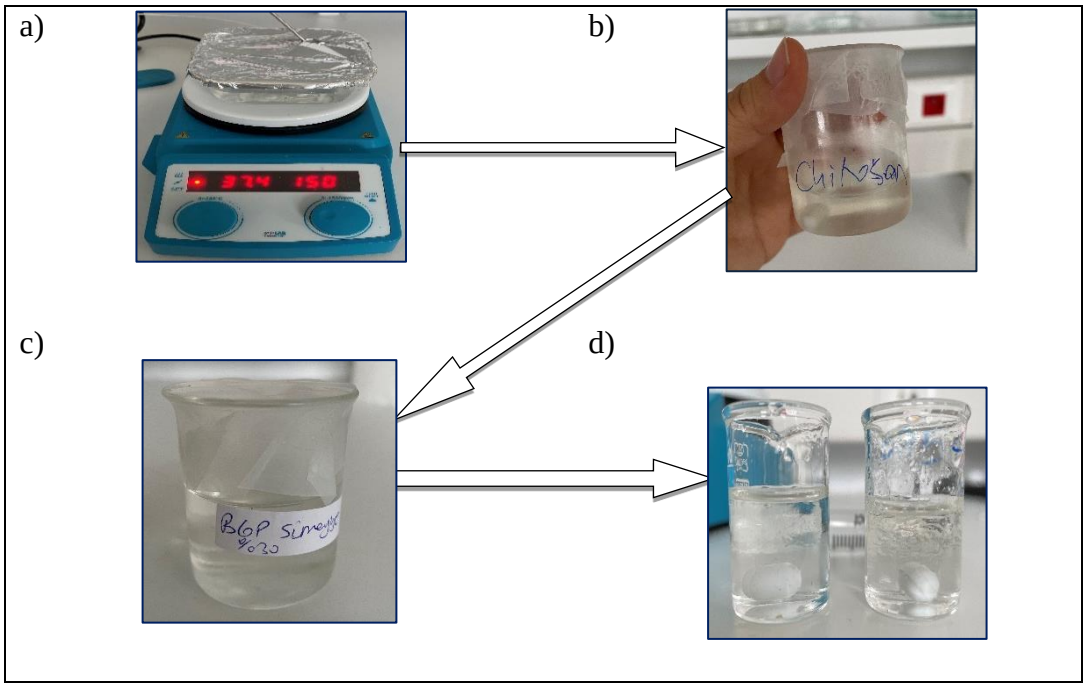


Figure 3.1: a) Chitosan solution is prepared by dissolving chitosan granules in DI water and acetic acid solution, b) chitosan solution ready for ionic crosslinking, c) 80% (v/v) β-glycerophosphate solution, d) the ionically crosslinked chitosan gel produced using a standard approach.

The positively charged chitosan solution and the negatively charged groups of the β-glycerophosphate solution interacted ionically. After gelation, hydrogels were put in a vacuum oven (model CLRC-17, CLS Scientific, Turkey) for 1 hour at 30 °C under 70 mPa of pressure. They were removed from the vacuum oven and put in a refrigerator set to 4 °C until further while they awaited characterisations, antibacterial activity analysis, and cell culture tests.

3.2.1.1. Characterization of the Chitosan-Based Polymer Solution

There is only one concentration of the chitosan polymer solution, which is 1% (w/v) is evaluated for this thesis study due to suggestion of successful thesis study which conducted by Melda Elmas et al., 2022. The chitosan polymer solution's viscosity, density, and surface tension were all measured. The adjustment of the chitosan polymer solution's microbubble size was not the main goal of this experimental work. Therefore, for examining the antibacterial activity of hydrogel, a single formula and a single temperature were chosen.

At least three times, the device's plates were submerged in a 50-mL dispersion cuvette (model SV-10, Vibro Viscometer, A&D, Japan) to measure the viscosity of the polymer solution at 23 °C. 40 mL of a (1% (w/v)) chitosan solution were produced before the measurements in order to ensure accuracy. The dispersion cuvette was then filled with the chitosan polymer solution (1% (w/v)) (Figure 3.2). All data were recorded, and the viscosity of the solution was calculated in accordance with temperature readings.

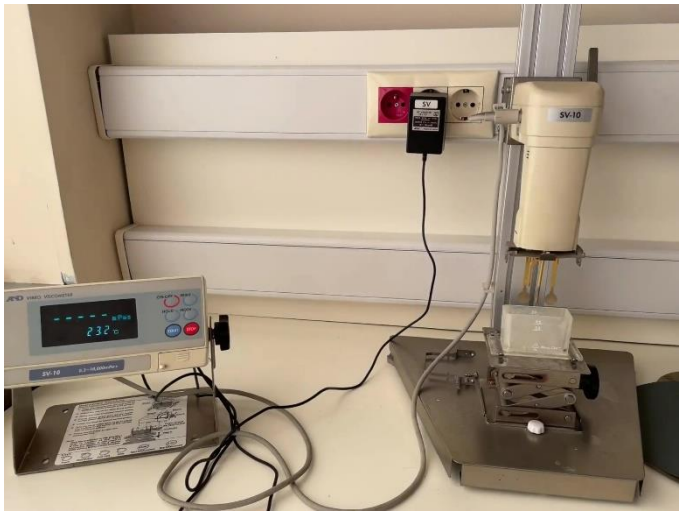


Figure 3.2: Viscosity measurement set-up for identifying viscosity of chitosan solutions used in the experimental work.

A pycnometer (Pycnometer Density Bottle Meter, ISOLAB, Germany) was used to measure the density of the chitosan polymer solution (25 mL, 1% (w/v)) (Figure 3.3). After adding 25 mL of the 1% chitosan solution to the pycnometer's empty weight of 29.813 g, the combined weight of the pycnometer plus the solution was 56.747 g. Eq. 3.1 was used to determine the density of the polymer solution, and the difference shows the weight of the polymer:

$$d = \frac{m}{V} \tag{3.1}$$

Here, m is the mass of the sample (g), d is density (g/cm^3), and v is volume (cm^3). Using Eq. 3.1, we calculated the density of the 1% chitosan polymer solution as shown in Eq. 3.2:

$$D = \frac{m}{V} = \frac{(56.747 - 29.813)g}{25\ mL = 25cm^3} = 1.07\frac{g}{cm^3} \quad (3.2)$$

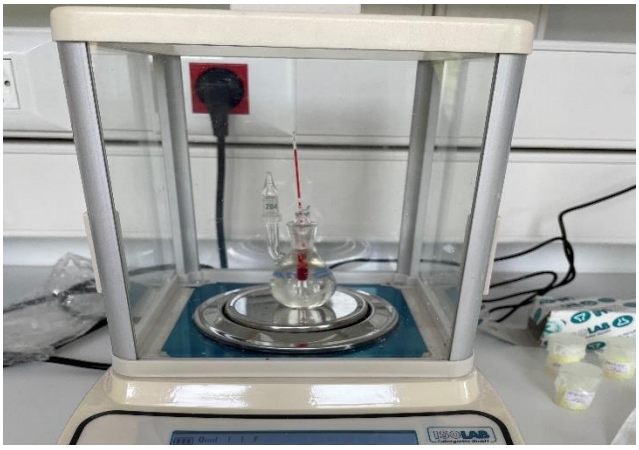


Figure 3.3: Chitosan polymer solution density measurement using a pycnometer.

KSV Cam 200 Tensiometer was used to measure the surface tension of the 1% (w/v) chitosan polymer solution as shown in Figure 3.4. The measurement sensitivity of this instrument was 0.1 mN/m. The surface tension of the solution was measured using the instrument's needle. Calculating the gap at the break allowed for the evaluation of the surface tension as the drop dropping from the needle's tip broke, creating a cross-sectional area. Pendant Drop analysis technique used during surface tension analysis.

Figure 3.4: Chitosan polymer solution surface tension analysis.

3.2.2. Microfluidic Preparation of Chitosan-Based Hydrogel

Microfluidic preparation of chitosan-based hydrogels involves the use of microfluidic devices to control the mixing and flow of chitosan or other polymer solutions. Microfluidic devices allow for precise control over the size, shape, and distribution of the hydrogel particles, which can be tailored to specific applications. To prepare chitosan-based hydrogels, chitosan is dissolved in an aqueous solution with a pH value of around 6-7. The solution is then passed through a microfluidic device, where it is mixed and flowed through channels. The mixing of the solutions leads to the formation of hydrogel particles, which can be controlled by varying the flow rates and mixing conditions. The resulting hydrogels have a variety of properties, including mechanical strength, biocompatibility and biodegradability, making them useful for various applications such as wound healing, tissue engineering and drug delivery. Additionally, chitosan-based hydrogels can also be used in food preservation to prevent the growth of microorganisms that cause food spoilage. In summary, microfluidic preparation of chitosan-based hydrogels allows for the precise control over the size, shape, and distribution of the hydrogel particles.

As stated in Chapter 3.2.1, chitosan (1% (w/v)) and β-glycerophosphate crosslinker solutions were prepared following the procedure. A 0.45-m syringe filter (Cellulose Acetate Syringe, GVS Filter Technology, UK) was used to filter the chitosan solution after it had been made in order to remove any undissolved particles and avoid clogging the microchannel while feeding the polymer solution into it. A 10-mL BD plastic syringe and digitally piston-controlled pumps (IPS-14-RS, Inovenso, USA) were used to add the polymer solution. The microfluidic device design was like a T shaped. T-shaped microfluidic junction system used for experimental studies. Two immiscible phases were utilized in this microfluidic device to create microbubbles at the junction of the microchannels. The continuous phase was the chitosan solution, while the dispersed phase was a gas flow which is nitrogen as shown in Figure 3.4.

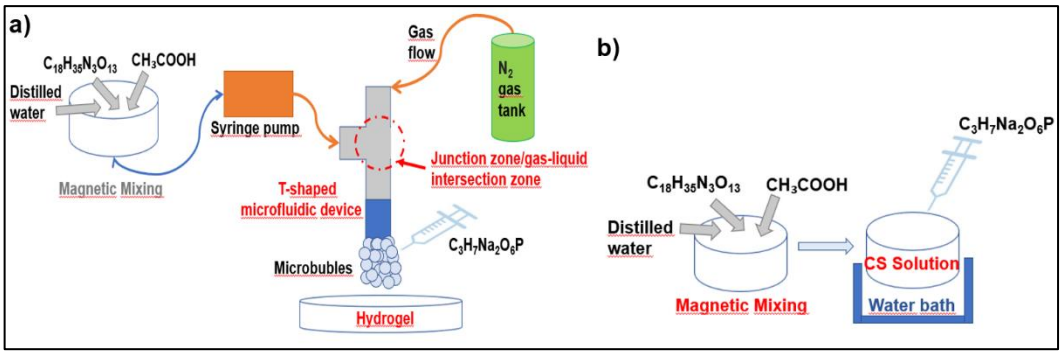


Figure 3.5: Schematic representation of experimental of a) T-shaped microfluidic chitosan-based hydrogel production and, b) traditional manual preparation of chitosan-based hydrogel.

The pressure used during bubble creation was between 10-15 bar at a temperature of 25 °C, and the liquid flow rate was at 200-250 L/min while inert gas was flowing. The system successfully allowed to bubbles of a consistent, continuous size to form in the system. Following their formation, microbubbles are gathered in a beaker in an ice-filled container. A volume ratio of 1:1 (v/v) between the crosslinker and polymer solutions was maintained for maximum gelling. The crosslinker solution is simultaneously introduced dropwise gently to the bubbles gathered in the ice bath as microbubbles form. After gelation, the hydrogels are maintained for an hour at 30 °C and 70 mPa pressure in a vacuum oven (CLRC-17, CLS Scientific, Turkey). In order to prepare the hydrogels for characterisation, antibacterial activity tests, and cell viability investigations, the air inside the hydrogels was evacuated under vacuum and the hydrogels were then stored at 4 °C in a refrigerator.

3.2.2.1.Microbubble Characterization of Microfluidic Produced Chitosan Hydrogel

An optical microscope (AxioCam MRc 5, Zeiss, Germany) was used to assess the shape and size of the microbubbles created by the T-junction microfluidic system (Figure 3.5). A portion of chitosan polymeric microbubbles was gathered at the outlet channel of the T-junction microfluidic device used in this thesis study. The size of the emerged bubbles was measured and particle size distribution was calculated using the data collected after at least 10 measurements delivered. The magnification of the micrographs was adjusted to achieve accurate view of bubbles allocated. The photos

were captured and the microbubble sizes were calculated. The ImageJ software was used to determine microbubble sizes. Equation 3.4 was used to identify the polydispersity index (PDI) of the microbubbles allocated.

$$PDI(\%) = \frac{\sigma}{\text{davg}} \text{x}100 \qquad 3.3$$

Here, “davg” represents the mean value of the bubble diameter allocated and represents the standard deviation of the bubbles.

Figure 3.6: Optical microscope used for microbubble characterization.

3.2.3. Chemical Analysis of Produced Hydrogels by Using Fourier Transforms Infrared Spectroscopy Technique

Fourier transform infrared spectroscopy (FTIR) is a powerful analytical tool that can be used to study the chemical composition of chitosan-based hydrogels. The IR spectrum of chitosan shows characteristic peaks corresponding to the amine, amide, and hydroxyl groups that are present in the polymer. In addition, FTIR can also be used to determine the degree of deacetylation of the chitosan, which is an important factor that can affect the properties of the hydrogel and hydration state of the hydrogel, and can determine the degree of cross-linking that has occurred between the chitosan and any other polymers that have been used in the hydrogel. This information can be used to optimize the properties of the hydrogel for specific applications[33].

Chitason, β-glycerophosphate, hydrogels which were produced by using two different methods (microfluidic and traditional manually) were subjected to FTIR

analysis to evaluate whether the microfluidic method has an effect on the chemical structure of the chitosan polymer and to examine how the chitosan structure changes after hydrogel formation by comparing the interactions of the compounds after incorporation by observing the shifts in wavenumbers. Wavenumber measurements were evaluated between 600-4000 cm^{-1} while each sample was placed in the spectroscopic chamber (Spectrum 100, PerkinElmer, USA) at a concentration of about 3 mg.

3.2.4. Thermal Analysis of Produced Hydrogels by Using Differential Scanning Calorimetry

The glass transition temperature (Tg) and melting temperature (Tm) are two key features of the thermal properties of materials that could be determined using the differential scanning calorimetry (DSC) technology. DSC can be used to describe the changes in the thermal behaviour of hydrogels brought on by the addition of other polymers, the degree of crosslinking, and the degree of hydration. It can also be used to analyse the thermal properties of chitosan-based hydrogels. DSC may be used to look at how the inclusion of other polymers, the level of crosslinking, and the level of hydration affect hydrogels' thermal behaviour. The properties of the hydrogel can be improved using this knowledge for particular purposes.

A differential scanning calorimeter (DSC 404 C Pegasus, Netzsch-Gerätebau GmbH, Germany) was used to analyse the samples' thermal properties. Chitason, β-glycerophosphate, hydrogels which were produced with two different methods (microfluidic and traditional manually) were analysed. Phase changes and temperatures were measured with a heating/cooling rate of 10 °C/min in the temperature range from -25 °C to 400 °C.

3.2.5. Morphological Investigation of the Produced Hydrogels by Using Scanning Electron Microscopy

Scanning electron microscopy (SEM) is a powerful technique to analyse chitosan-based hydrogels. By scanning a focused beam of electrons over the sample's

surface and detecting emitted electrons, SEM generates detailed images known as SEM images. These images provide valuable insights into the structural properties of the hydrogels. SEM enables the examination of the hydrogel bubble size, shape, and distribution, shedding light on the morphology and surface topography of the particles. It also allows the study of the hydrogel's microstructure, including pore distribution and hydration level. This information is crucial for optimizing hydrogel properties in various applications like wound healing and tissue engineering.

The purpose of using SEM (Win10 FEI (Philips) XL30 microscope, FEI Company, USA) was to evaluate the architectures of hydrogels. For injectable chitason-based hydrogels, the porosity and pore size of the hydrogels are crucial characteristics. Electrical current applied was 150 μA at a voltage of 5–15 kV. Before scanning, a gold-coating process was applied for samples, and gold sputtering was done for non-conducting and poorly conducting specimens (SC7620 Mini Sputter Coater Operating Manual, Quorum Technologies, UK).

3.2.6. Antibacterial Activity of Produced Hydrogels with Antibiotic Disk Diffusion Assay

The surface antibacterial activity of manually prepared chitosan-based hydrogel and microfluidic produced chitosan-based hydrogel were evaluated using antibiotic disk diffusion assay on gram-positive and gram-negative bacteria. The antibiotic disk diffusion assay, also called the Kirby-Bauer test, is a useful tool for determining how susceptible bacteria are to antibiotics. It enables the testing of various bacteria and medications and is easy, affordable, and broadly applicable. Rapid results, standardized procedures, and the ability to analyse susceptibility both qualitatively and quantitatively are key features of the assay. Additionally, it makes it possible to test several antibiotics at once on a single plate.

The hydrogels which were produced by T-junction microfluidic device and traditional manual mixing methods evaluated in terms of antibacterial activity performance against to the gram-positive and gram-negative bacteria’s using a disk diffusion test according to the procedure ASTM F895-11. Amoxicillin is used as a control reference material and assessment done successfully.

The reason using amoxicillin as a reference is amoxicillin is an antibiotic that can be used to treat bacterial infections. It cannot treat the wound alone, but it aids in the prevention or treatment of a bacterial infection that hinders wound healing. The primary objective with the developed gel in this thesis study is to prevent the infection through the antibacterial effect by using the injectable chitosan hydrogels achieved by T-shaped microfluidic junction device technique and provide a support the healing process of wounding area.

Under sterile conditions, a suspension of bacteria *Escherichia coli (E. coli)* and *Staphylococcus aureus (S. aureus)* adjusted at $1x10^6$ concentration was evenly spread on Luria-Bertani (LB) agar plates. 3 plates were used for one bacterium. Sterile discs with a diameter of 6.2 mm were placed in an empty sterile petri dish and the hydrogel samples to be tested were dripped onto 20 µl discs. Two control samples were made for each hydrogel. Sterile discs impregnated with test material were used in experiments after drying. PBS was used for negative control and amoxicillin was used for positive control. The medium-impregnated discs were carefully placed on the surface of the petri dishes containing the medium on which the microorganisms were cultivated, in such a way that they did not come into contact with each other. After placing the discs, they were kept for 10-15 minutes and then incubated for 24 hours in an incubator at 37°C. The circular region where bacteria could not grow around the samples was accepted as the inhibition zone. At the end of the incubation, the growth inhibition zones formed in the petri dishes were measured using a millimetric ruler.

3.2.7. Cytotoxicity Analysis of Produced Hydrogels by Using Resazurin Cell Viability Assay

Cytotoxicity of hydrogels was evaluated on L929 cells with resazurin cell viability assay [61]. The reason using L929 cells is that L929 cells are an important fibroblast cell line responsible for a healing of the damaged area on a tissue repairing damaged tissue and L929 cells are widely used in wound healing studies [62]. Being easy to maintain, L929 cells are fast and relatively inexpensive to grow in culture [63].

The response of human cells to injury and the response of L929 cells to injury are very similar. In the event of injury, growth factors and other signals that stimulate the repair process are released and the production of collagen, an important component

of scar tissue, begins [64]. L929 cells are frequently used to investigate various wound healing treatments such as effects of antibiotics/drugs, growth factors and biomaterials [65].

Prior to adsorption, the conventionally produced hydrogel and the microfluidic produced hydrogel were sterilized under UV light for 30 minutes. Hydrogels were diluted with complete medium (DMEM with 10% (v/v) FBS and 1% (v/v) penicillin) to the concentrations of 0.1, 0.25, 0.5, 1, 2.5, 5 mg/mL, respectively. Briefly, 1.0×10^4 L929 cells in 100 μL of culture medium was added to 96-well plates and incubated overnight for cell attachment. The following day, cells were incubated with the above diluted concentrations of both injectable chitosan-based hydrogels, separately. After 24h, resazurin reagent was added directly to the wells at a ratio of 1:10 (v/v). As controls, wells with entire medium were employed. After 24 hours, cell viability was evaluated. AlamarBlue reagent was applied to each well in a quantity of 20 μl. A plate reader was used to measure the absorbance at 590 nm after 24 hours of incubation [46].

4. RESULTS AND DISCUSSION

4.1. Characterization Studies of the Produced Chitosan Polymer Solution

Solution characterization was carried out to determine whether the solution is suitable for the microfluidic system and to understand whether there is a relationship between the properties of the solution we obtained and the properties of the samples produced. Future research on microfluidic devices will be guided by the identification of the physicochemical characteristics of the chitosan solution. The result of the viscosity, density, and surface tension of the polymer solution all have an impact on droplet size, which in turn has an impact on microbubble sizes [54]. The result of the viscosity, density, and surface tension measurement of the chitosan polymer solution at room temperature were shown in Table 4.1.

The following experimental procedure carried out by the 1% (w/v) chitosan solution and the 80% (w/w) salt solution. With appropriate heating and careful acid addition during preparation, the contaminants from the undissolved chitosan powders were reduced.

The viscosity, surface tension, and density values of the chitosan polymer solution were measured for microfluidic production, as shown in Table 4.1. Measured parameters of a 1% (w/v) chitosan polymer solution made (at 23°C, n=3) were shown in Table 4.1. The polymer solution was successfully transmitted via the T-shaped microfluidic device's microchannels thanks to the chosen characteristics of the polymer solution.

Table 4.1. Measured properties of 1% (w/v) chitosan polymer solution prepared (at 23°C, n=3).

Viscosity (mPa.s)	**Density (g/cm^3)**	**Surface tension (mN/m)**
89.4 ±0.05	1.07±0.01	62.89±0.5

Melda Elmas et al., 2022 in their thesis study showed the 1% (w/v) chitosan polymer solution is best concentration for successfully passed through the microchannels of the T-shaped microfluidic device. When we compare our %1 (w/v) chitosan polymer solution physicochemical properties with her thesis study it is clearly show that results are very similar for both thesis study [60].

4.2. Result of Fourier transform infrared spectroscopy Analysis of produced hydrogels

4.2.1. FTIR Results for Chitosan and β -Glycerophosphate

In the literature [66], it is reported that chitosan shows characteristic peak at 3370 cm^{-1} [67] was due to symmetrical stretching vibrations of OH and NH groups of the -OH asymmetrical stretching and of the vibrations of C-H stretching at 3200-3500 cm^{-1} [68]. Peaks are also seen Figure 4.4 very similar with the results achieved in literature [69] for C-H stretching at 2900-2800 cm^{-1}, for the amide I band at 1630-1660 cm^{-1} and for the amide II band at 1510-1570 cm^{-1} . A band at 1565 cm-1 corresponds to the N-H bending of the primary amin. A range of 1200–1500 cm^{-1} was recorded for the C–O stretching of primary alcohol groups. The bands at 1045 cm^{-1} correspond to C-O stretching [70]. In the literature [71], it is reported peaks at 1597-1459 cm-1were attributed to N-H bending in amide II, while those at 1685-1657 cm^{-1} were linked to C=O stretching in amide I. The peak at 1645 cm^{-1} (C=O stretching in amide I) and 1560 cm^{-1} (N–H bending in amide II) appeared in the result for chitosan [72]. C-O stretching was reported for C-O-H at ~1150 $cm^{-1}$and for C-O-C at 1026 cm^{-1} and 1064 cm^{-1}, the wavenumbers seen, similar with the results achieved in the study conducted by (Porntipa et al., 2018). The peak at 800-1200 cm^{-1} was associated with -O- stretching of glucosamine residues (894, 1026, 1064, 1150), very similar result like the achieved in the study conducted by (Agata et al., 2018) [73]. Relevant peaks were observed with the results in the range of wavenumber of 3600-3100 cm^{-1} achieved in the study conducted by (Agata et al., 2018)[73] (Figure 4.4) at 3285 cm^{-1} and 3357 cm^{-1} as peaks of -OH (asymmetrical) stretching. In the case of strong hydroxyl bonds and amine N-H groups in the structure, the asymmetrical shape of these peaks is observed in the lower wavenumber range.

Peaks between 3600-3100 cm^{-1} were associated with oscillation of O-H according to (Porntipa et al., 2018), peaks of β-glycerophosphate 3217, 3361, 3534 cm^{-1} might be associated this result. Peaks at 1677 cm^{-1} is associated with the C=O (amide I) group, the peaks observed similar with results achieved in the study conducted by (De Souza Costa et al., 2009). Peaks of β-glycerophosphate is very similar with the results achieved in the study conducted by (Xiao et al., 2021) [74]. The study conducted by (Han et al., 2018) also ensures the peaks of chitosan and β-glycerophosphate are very similar with the literatures [75]. The peak at 800-1000 cm^{-1} is characteristic of β-glycerophosphate (aliphatic stretching of P-O-C) [73]. The peaks at 1076 and 1055 cm^{-1} were attributed to the characteristic PO_4^{3-} group with the presence of β-glycerophosphate, those peaks observed were similar with the results obtained in the study conducted by (Porntipa et al., 2018) [71]. For β-glycerophosphate, the peak present at 1076 cm^{-1} is characteristic for C–O bond whereas peak at 978 cm^{-1} represents P–O bonds were similar with the results obtained in the study conducted by (Noha et al., 2017) [69]. The peak present at 1652 and 1564 cm^{-1} corresponded to amide I and amide II whereas strong peak at 3285 due to hydrogen-bonded O-H stretch [76].

The characteristic peaks of both chitosan and β-glycerophosphate were observed in the spectra of the gel, similar with the results achieved in the study conducted by (Nga et al., 2022) [77], [78].

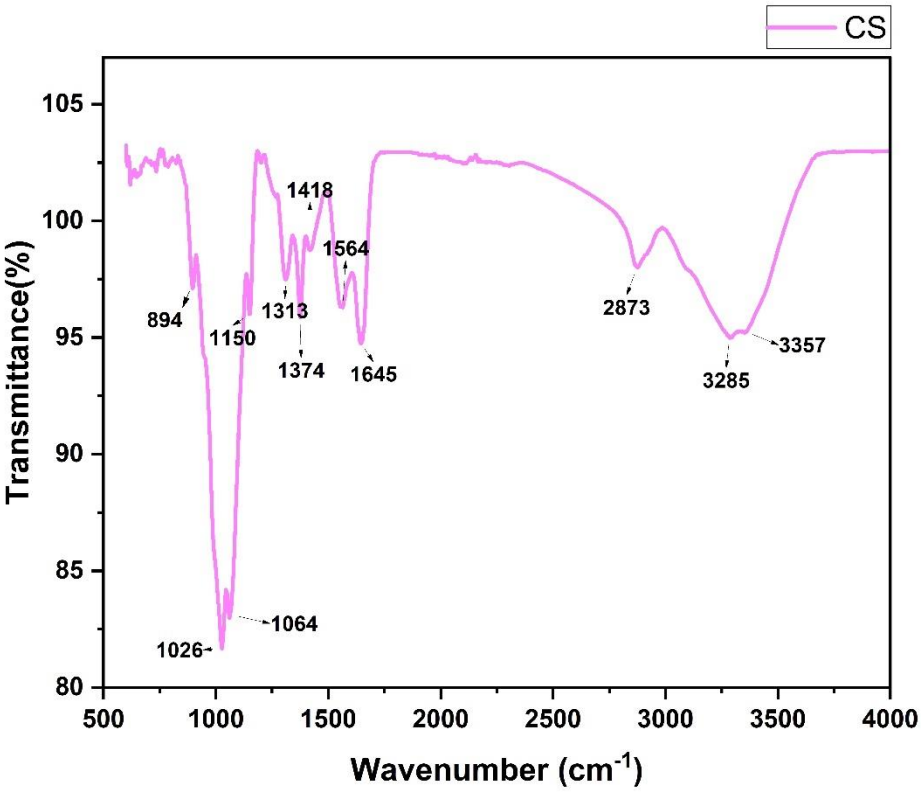


Figure 4.1: FTIR spectra of chitosan powder.

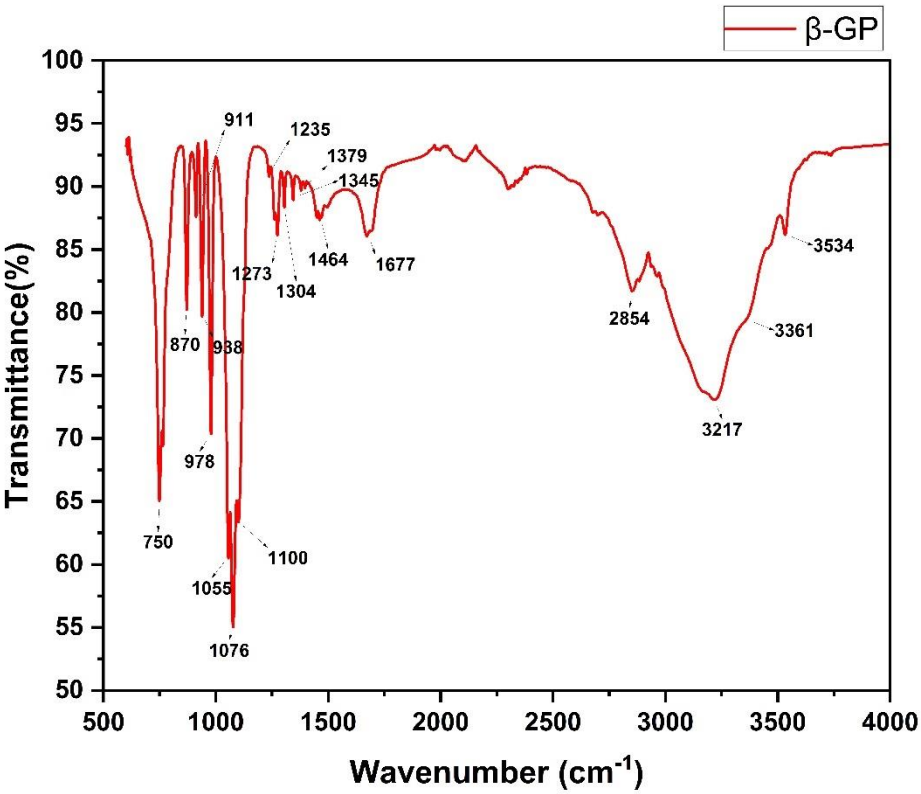


Figure 4.2: FTIR spectra of β-glycerophosphate disodium salt powder.

4.2.2. FTIR Results for Manually and Microfluidically Prepared 1% Chitosan/ β-Glycerophosphate Disodium Salt Hydrogel

The literature provides several unique peaks for chitosan solution that has been ionically crosslinked with β-glycerophosphate disodium salt (hydrogel) because of the phosphate groups of the gelling agent as shown in (Figure 4.3) [79] and (Figure 4.4). Peaks at the 3262 cm^{-1} of manually prepared hydrogel and at 3228 cm^{-1} of microfluidically prepared hydrogel, in the range between 3000–3500 cm^{-1} represents the hydroxyl groups and the stretching vibration of the N-H bonds in the structure of chitosan/β-glycerophosphate disodium salt hydrogel. In the range of 2850-2960 cm^{-1} were attributed of characteristic of the pyranose ring of chitosan and the stretching vibrations of aliphatic groups (-CH_2 and -CH_3) according the study which is conducted by (Agata et al., 2018). The peaks at 2888, 2945 cm^{-1} of manually prepared hydrogel and 2880, 2941 cm^{-1} of microfluidically prepared hydrogel might be represent the stretching and bending vibrations of the CH_2 groups in chitosan chains. Peaks at 1656 cm^{-1} of CS1 (manually prepared hydrogel) and 1655 cm^{-1} of M-CS (microfluidically prepared hydrogel) represent the C=O (amide I) and NH_2 (amide II) groups of chitosan. The spectra of CS/GP hydrogel show the characteristic peaks of β-glycerophosphate disodium salt at 974 cm^{-1} (P-OH bond) and 1055 cm^{-1} of M-CS and 1045 cm^{-1} of CS1 (P-O bond). The hydroxyl group of β-glycerophosphate disodium salt enhances the increase in peak intensity in the 3000-3500 cm^{-1} area of the 1% chitosan/β-glycerophosphate disodium salt hydrogel [80]. In the range of 1200-1500 cm^{-1} the peaks are at 1410 cm^{-1} of CS1 and 1412 cm^{-1} of M-CS can be considered as characteristic of OH and C-H bending of CH_2 groups and representing the C-O stretching of the primary alcoholic group -CH_2 -OH in this frequency range. Peaks are at 796, 974, 1045 cm^{-1} of CS1 and 750, 764, 929, 974, 1055 cm^{-1} of M-CS representing the bridge -O- stretch of the glucosamine residues which are very similar with the results achieved in the study conducted by (M. Boido et al., 2018) [81]. The peak at 1045 cm^{-1} of CS1 and 1055 cm^{-1} of M-CS are associated with the characteristic of the -PO_4^{2-} group, whereas the band at 929, 974 cm^{-1} of M-CS and CS1 associated with the presence of the -HPO_4^- group. The vibration peak of 654, 796 cm^{-1} of CS1 and 650, 750, 764 cm^{-1} of M-CS considered as the aliphatic P-O-C stretching of β-glycerophosphate. The result of FTIR of 1% chitosan/β-glycerophosphate disodium

salt hydrogel shows that after gelation mechanism CS and glycerol phosphate disodium salt characteristic bands were represented. Those peaks observed similar with the results achieved in the study conducted by (Agata et al., 2018).

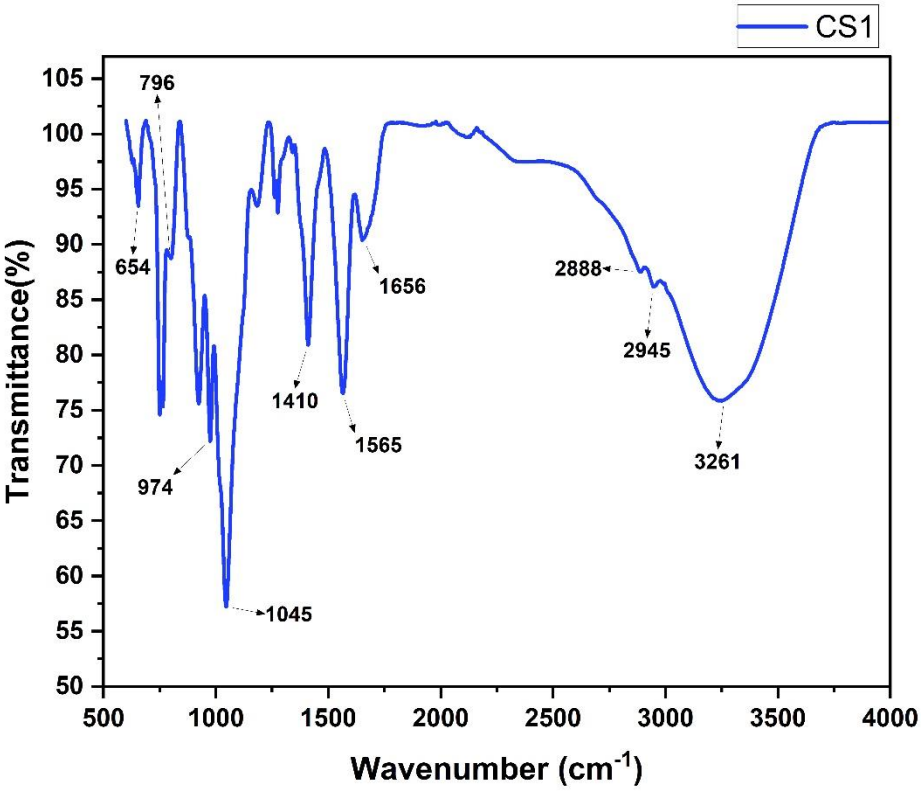


Figure 4.3: FTIR results for manually prepared 1% chitosan/β-glycerophosphate disodium salt hydrogel.

FTIR spectra of hydrogel produced using microfluidics (M-CS) is shown in Figure 4.4. Relevant almost all chitosan wavenumbers were seen for microfluidically prepared 1% chitosan/β-glycerophosphate disodium salt hydrogel, as explained above. In the range of 3000-3600 cm^{-1} characteristic peaks in CS spectrum were at O-H and N–H stretching bands overlapped. The ionically crosslinked microfluidically prepared hydrogel (CS/β-GP) has dominant peaks. The peak at 3228 cm^{-1} might be associated with the -OH (asymmetrical) group and 2880 cm^{-1} and 2941 cm^{-1} might be related -C-H stretching group according the study which is conducted by (Porntipa et al., 2018). The peak at 1655 cm^{-1} is related to the presence of the C=O (amide I) group, and at 1412 cm^{-1} is related with the NH_2 (amide II) group. The crosslinker β-GP) might be associated with other peaks that were seen; specifically, that at 1055 cm^{-1} is associated with the P-O and -P=O groups, that at 974 cm^{-1} corresponds to the P-OH group, and that at 872-750 cm^{-1} is

related to the aliphatic stretching of the P-O-C group. Those peaks are very similar with the study which is conducted by (Porntipa et al., 2018).

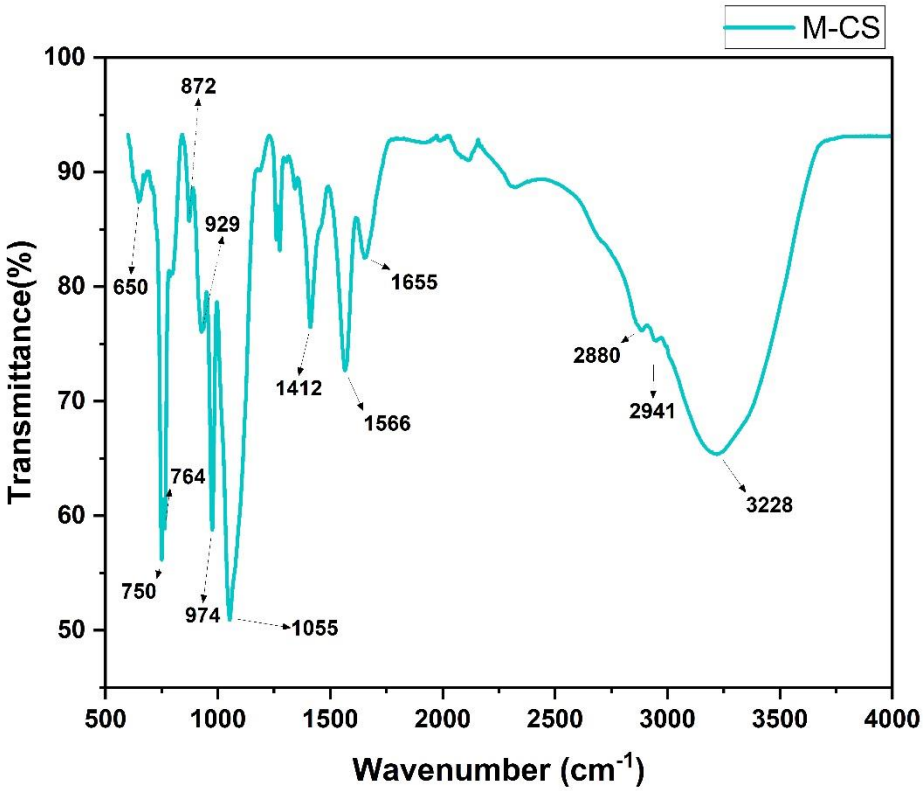


Figure 4.4: FTIR results for microfluidically prepared 1% chitosan/β-glycerophosphate disodium salt hydrogel.

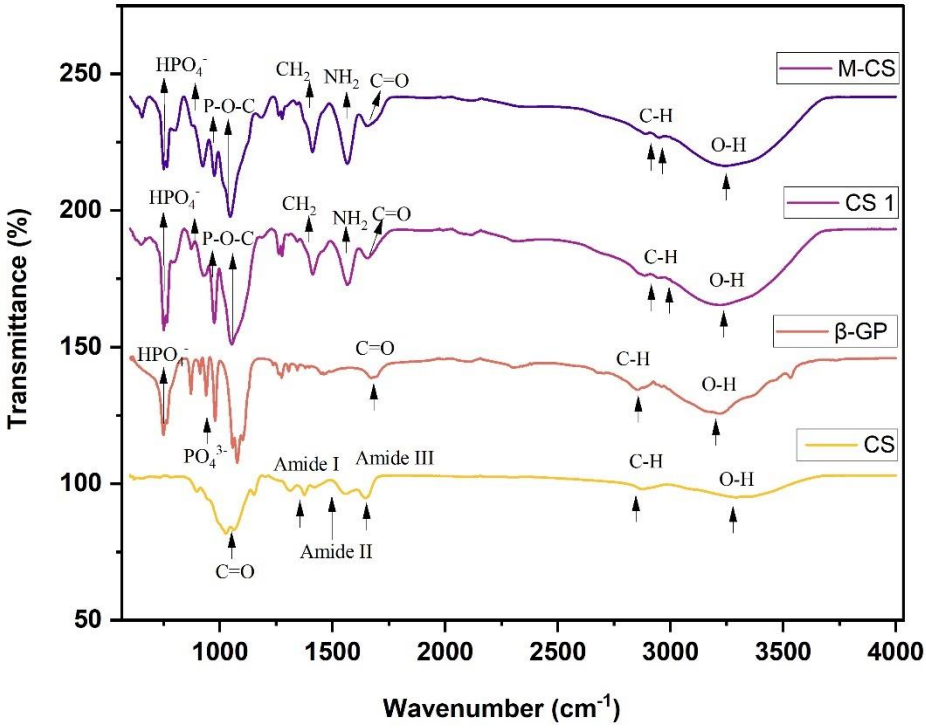


Figure 4.5: FTIR spectra of CS, pure chitosan powder; β-GP, β -glycerophosphate disodium salt; CS1, manually prepared CS/β-GP hydrogel; M-CS, microfluidically prepared CS/β-GP hydrogel.

The characteristic peaks which are achieved for CS, pure chitosan powder; β-GP, β-glycerophosphate disodium salt; CS1, manually prepared CS/β-GP hydrogel; M-CS, microfluidically prepared CS/β-GP hydrogel had perfect match with the results achieved in the study conducted by (Agata et al., 2018 and M. Boido et al., 2018). Peaks confirmed that there no significant difference between traditional manual produced hydrogel and microfluidic produced hydrogel. Peaks confirmed that chitosan (CS) and β-glycerophosphate (β-GP) successfully crosslinked.

4.3. Structural Analysis of Produced Hydrogels

4.3.1. Optical Microscopy Results for Manually Prepared Chitosan Hydrogel and Microfluidically Prepared Chitosan Hydrogel

Uniform, chitosan microbubbles were visible under the optical microscope photographs. The needed uniformity was obtained successfully. Initially monodisperse and stable obtained microbubbles kept their shapes for around one hour, however they lost their stability in air with time. The bubble structure and size distribution of the chitosan microbubbles is shown in Figure 4.6. The bubble size of the microfluidic hydrogel changes from 80 µm to 240 µm. The mean microbubble diameter is 135.6 µm and the PDI value was 17%.

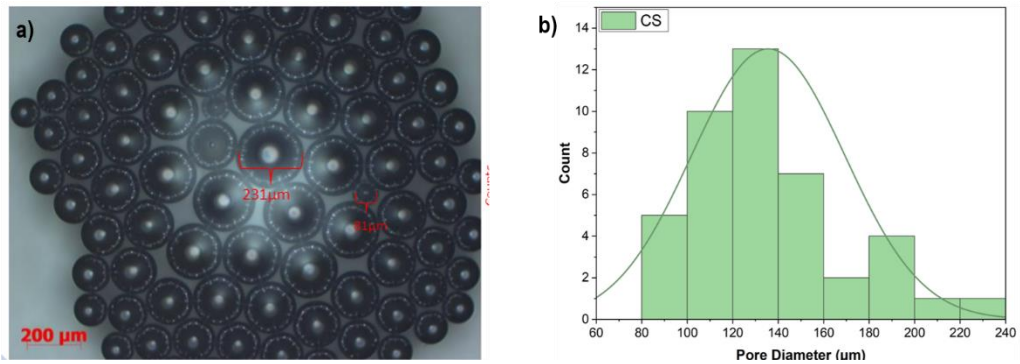


Figure 4.6: a) Optical microscopy images of chitosan microbubbles via T-junction microfluidic device, b) Mean bubble size distributions of microbubbles via T-junction microfluidic device.

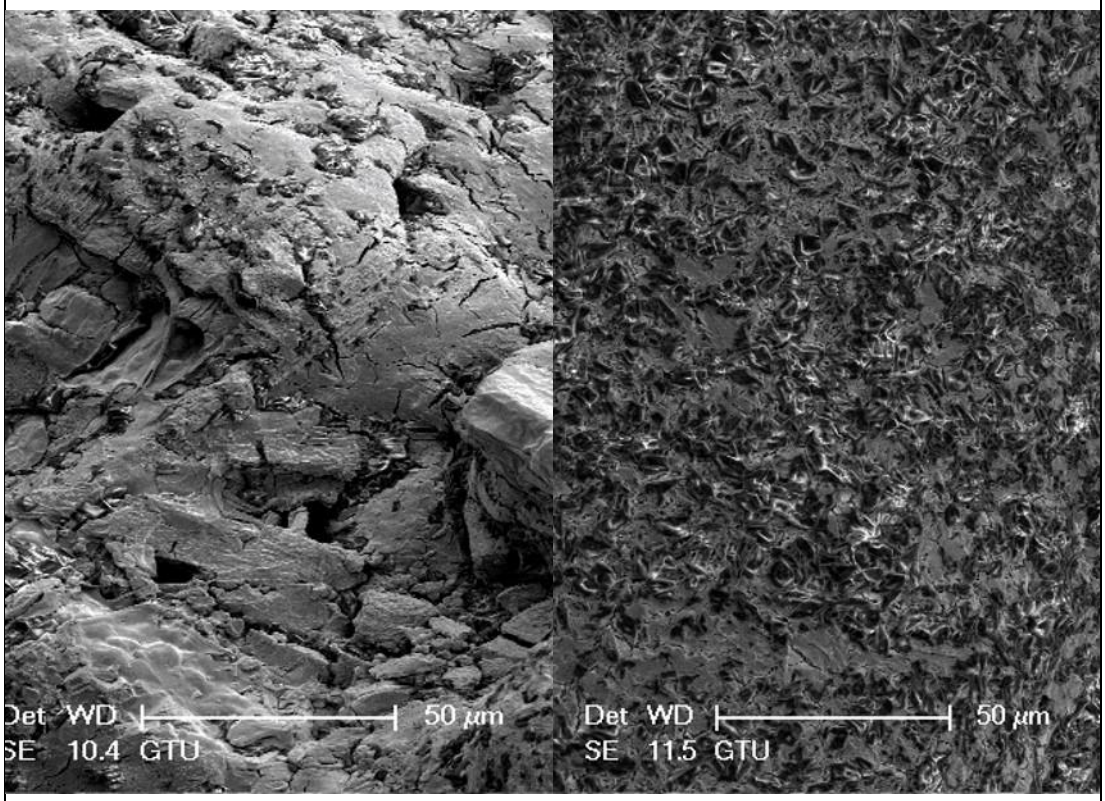
4.3.2. SEM Results for Manually Prepared Chitosan Hydrogel and Microfluidically Prepared Chitosan Hydrogel

SEM images also revealed the morphology of the for manually prepared chitosan hydrogel and microfluidically prepared chitosan hydrogel (Figure 4.7). The porous nature of the freeze-dried samples of 1% chitosan/β-glycerophosphate disodium salt hydrogel is shown. The morphological appearance of the hydrogel prepared by the manual method showed an irregular pore. The morphological appearance of the

microfluidic fabricated hydrogel provided a porous structure with micron-sized pores, while the size of these pores was significantly smaller compared to conventionally prepared hydrogels. The cavities of the manually prepared hydrogel were not as regular as the microfluidic produced hydrogel.

Manually prepared hydrogels SEM morphology is very similar with the study conducted by (Nga et al.,2022). In this study they claim that increasing concentration of β-GP decreasing the pore diameters. Hydrogels might become more compact and irregular by increasing concentration of CS according the study conducted by (Stephanie et al., 2013) [82]. Hydrogel matrix structure strongly depend on the type of acid used in CS solution and ionic strength. Rafael et al., 2017 observed the sheet-shaped particles CS/GP Hydrogel [83]. Porntipa et al., 2018 observed an irregular pore structure of CS/β -GP hydrogel [1], claim that irregular porosity surfaces can facilitate the diffusion of medicines out of hydrogel structures and the entry of water or biological fluids into the hydrogel structures. Noha et al., 2017, Han et al., 2018 and M.Boido et al., 2019 observed chitosan/β-glycerophosphate thermogel has a uniform microporous network which is indicating good interaction between the components [69], [75], [81]. Sheila et al., 2020 observed that physically crosslinked chitosan had smaller pore sizes than chemically crosslinked chitosan, which produced open networks with the highest pore sizes. This observation can be explained by the stronger hydrophobic interaction than chemical crosslinking. Additionally, the covalent hydrogels' chitosan/crosslinker molar ratio was lower than it was for physical hydrogels, which led to a lower crosslink density (larger pore diameters) and more open networks. The same observation done for also increasing the B-GP content, leads to smaller pore sizes and more uniform morphology of the networks. The physical crosslinking caused by a higher salt content results in fewer pores and more homogeneous mesh morphology [84].

Manually prepared hydrogel generally reduced the pore size, a more condensed structure could be seen and the cavities were observed (Figure 4.7.b.), while microfluidically generated hydrogels had a sponge-like morphology and more distinct pore structure (Figure 4.7.a.). As seen in Figure 4.7, the manually prepared hydrogels have higher pore size, compared to hydrogels prepared by the microfluidic method. Parhizkar et al., 2014 explained that the cumulation of the microbubbles will, create pressure on bubbles located on the lower layers while collecting them, and will lead to a broad range of pore size distribution [85].



a) **b)**

Figure 4.7: Scanning electron micrographs of a) of microfluidically prepared 1% chitosan/β-glycerophosphate disodium salt hydrogel and b) manually prepared 1% chitosan/β-glycerophosphate disodium salt hydrogel.

4.4. Thermal Property Analysis of Produced Hydrogels with Differential Scanning Calorimetry

4.4.1. DSC Results of Raw Materials which are Used for a Preparation of Injectable Chitosan Hydrogels

Differential Scanning Calorimetry (DSC) is a thermal analysis technique that measures the change in heat capacity of a sample as it is heated or cooled. It is intended to identify the critical temperature ranges to evaluate the stability and performance of the hydrogels with the body temperature they are exposed to after being injected into the body to support wound healing. It aids in understanding how these hydrogels respond to temperature changes and how their structure and properties may affect their

performance at the wound site. This will be done by performing DSC analysis on the samples and raw materials obtained within the scope of this thesis.

DSC analysis is an important technique for understanding the thermal behaviour, gelation process, stability and formulation optimization of injectable hydrogels for wound healing. In this experimental study, chitosan powder, β-GP powder, microfluidically and manually prepared 1% chitosan/β-glycerophosphate disodium salt hydrogels were used for DSC analysis. As shown in Figure 4.8 chitosan powder has two peaks at 92 °C (endothermic) and 303 °C (exothermic) and β-glycerophosphate showed a sharp endothermic peak at 70°C as shown in Figure 4.9. Sharp endothermic peaks between 60-95 °C were seen in both CS and β-GP, which can be associated water loss as a result of polymer hygroscopicity and polymer degradation, which includes saccharide ring dehydration, depolymerization, and acetylated decomposition of the chitosan unit which similar results also seen in the study conducted by (Naho et al.,2017) [69].

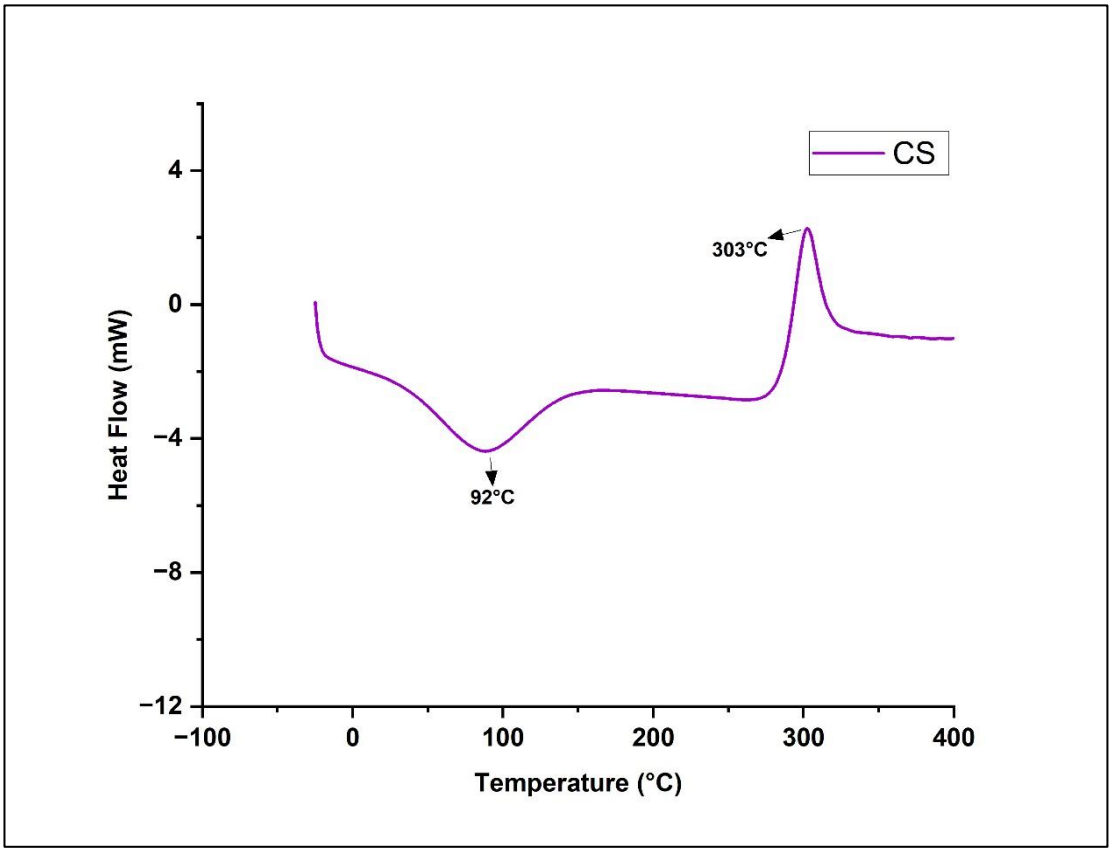


Figure 4.8: DSC analysis of chitosan powder.

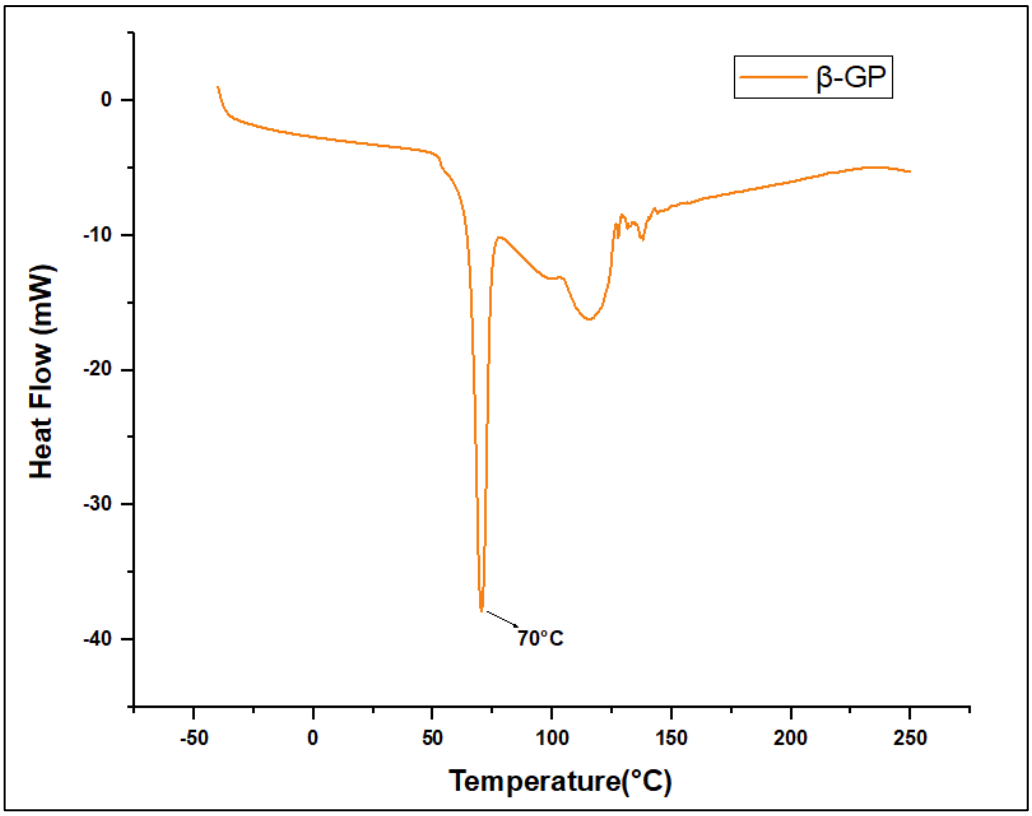


Figure 4.9: DSC analysis pure beta glycerophosphate (β-GP).

4.4.2. DSC Results for Manually and Microfluidically Prepared Chitosan Hydrogel

As shown in Figure 4.13, for manually prepared chitosan hydrogels (CS1) demonstrated three endothermic peaks at 65 ℃, 120℃, and 260℃. Due to the interaction between the crosslinker and powder chitosan in the DSC graph of the CS/BP hydrogel, changes were observed on the endothermic and exothermic peaks compared to pure chitosan. The endothermic peak disappeared whereas additional peaks were observed.

Thermal behaviour of manually prepared chitosan hydrogels (CS1) shown in Figure 4.10 and microfluidically prepared chitosan hydrogel (M-CS1) shown in Figure 4.11 and Figure 4.12. Both the hydrogel CS1 and M-CS1 demonstrated three endothermic peaks. While the peaks of the hydrogel produced by the manual method are 65 ℃, 120 ℃, 260 ℃, the peaks of the hydrogel produced with the microfluidic method are at 64 ℃, 145 ℃ and 188 ℃. Compared to CS 1, M-CS 1's endotherm peak values are lower [68].

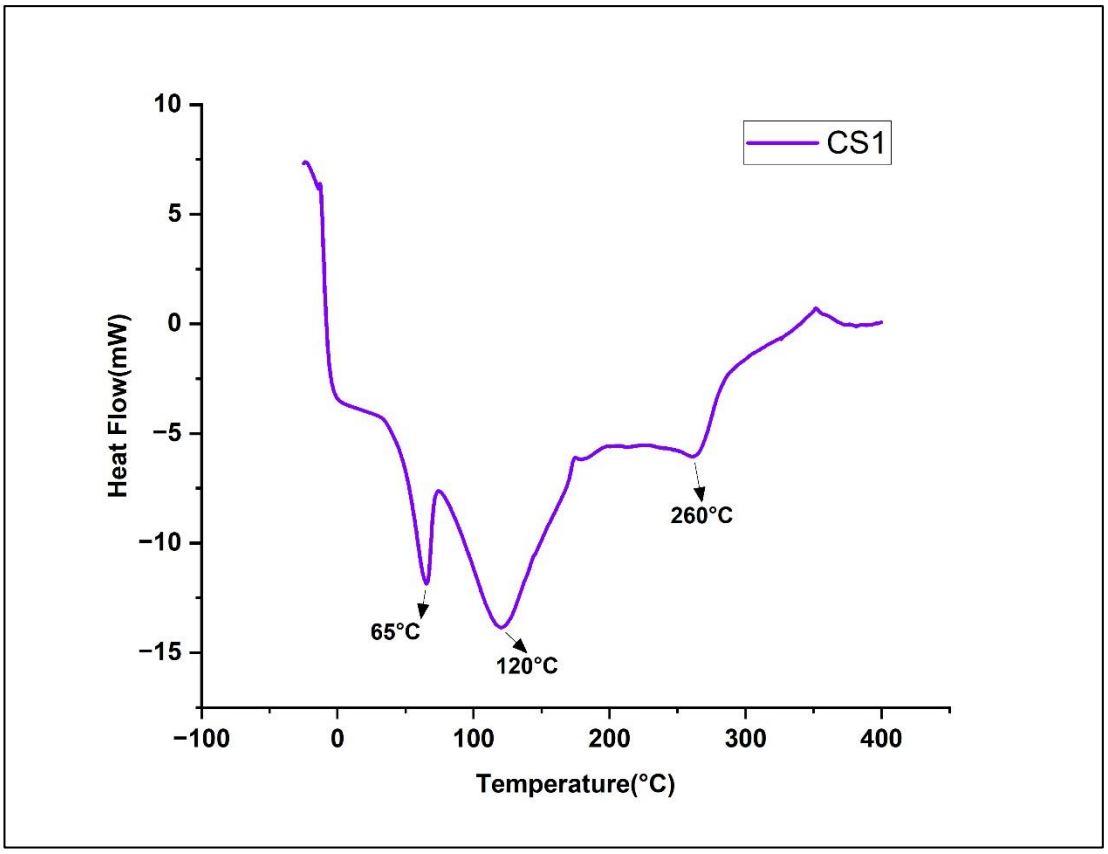


Figure 4.10: DSC analysis of manually prepared CS/β-GP hydrogel (CS 1).

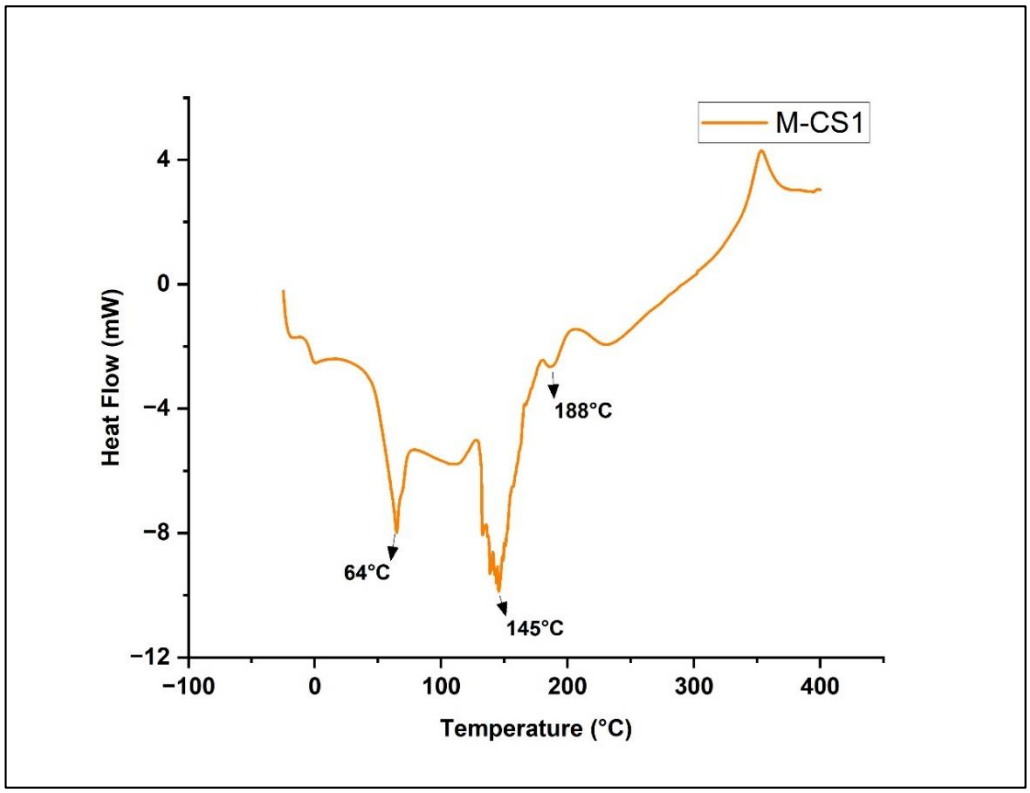


Figure 4.11: DSC analysis of microfluidically produced hydrogels(M-CS1).

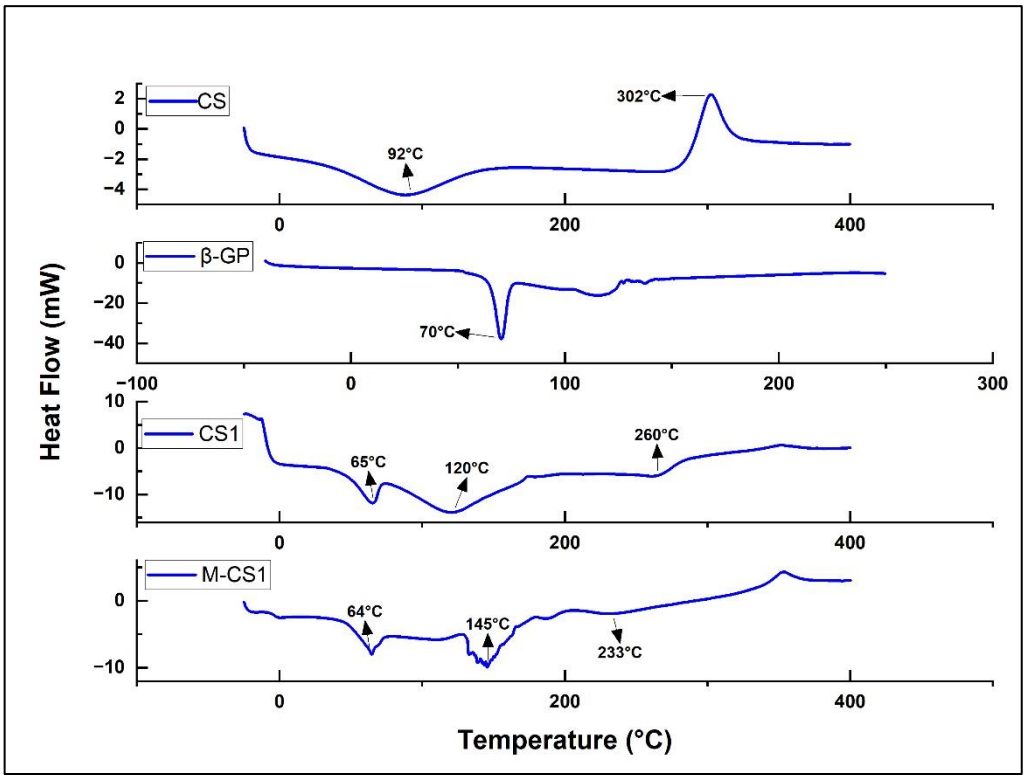


Figure 4.12: DSC analysis of chitosan powder (CS), pure beta glycerophosphate (β-GP), manually prepared CS/β-GP hydrogel (CS 1), microfluidically produced hydrogels(M-CS1).

4.5. Antibacterial Activity Results of Produced Hydrogels Using Antibiotic Disk Diffusion Assay

Inhibition zone values of the test materials used against microorganisms as a result of disk diffusion analysis were measured in mm and are given in Figure 4.16. Amoxycillin was used as the positive control group and phosphate buffered saline solution was used as the negative control group (Figure 4.13).

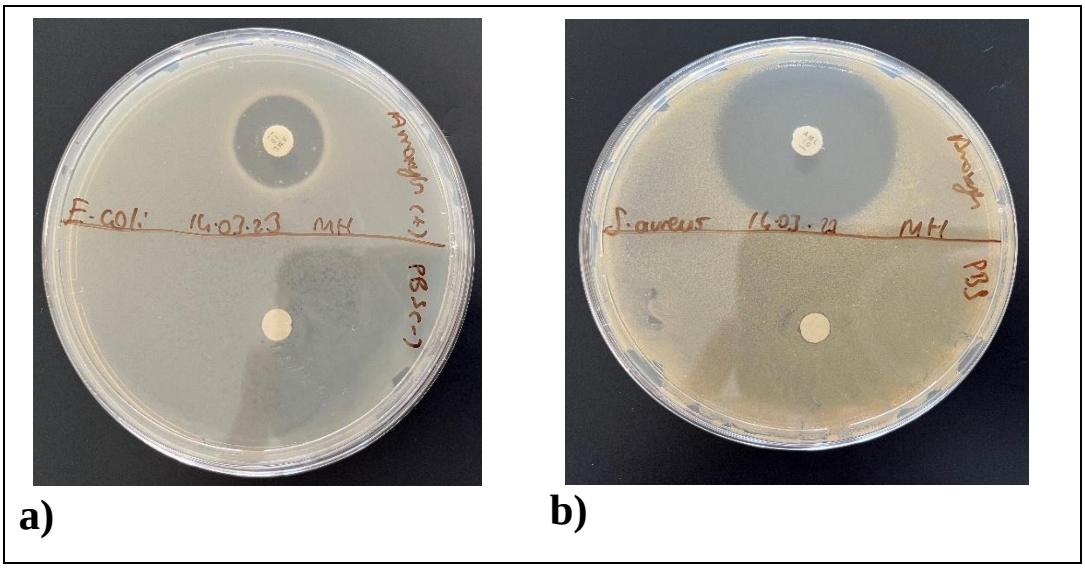


Figure 4.13: The gram-negative(a)/positive(b) antibacterial activity result against to positive control (amoxycillin) and negative control (PBS).

Antibacterial susceptibility of hydrogels produced by conventional method and microfluidic method was investigated by performing two repetitive experiments with 6.2 mm discs as shown in Figure 4.14 and Figure 4.15.

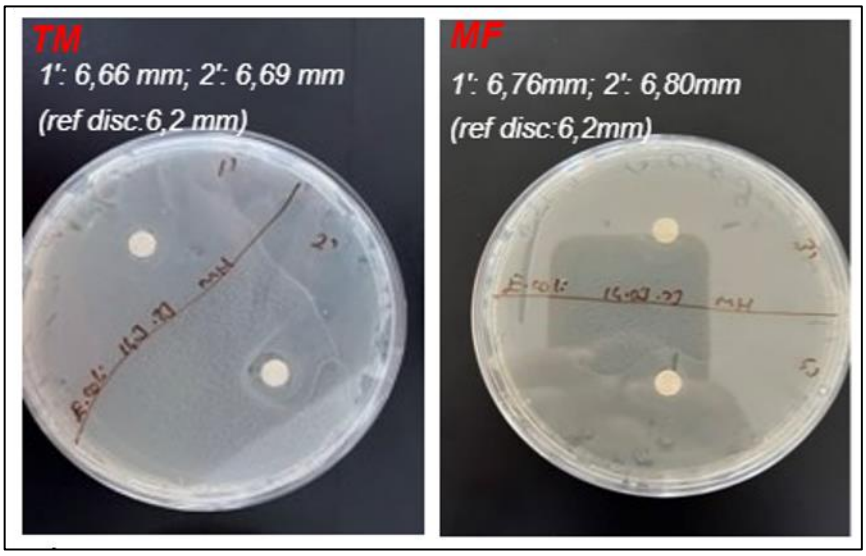


Figure 4.14: Antibacterial activity of hydrogels against *E.coli.*

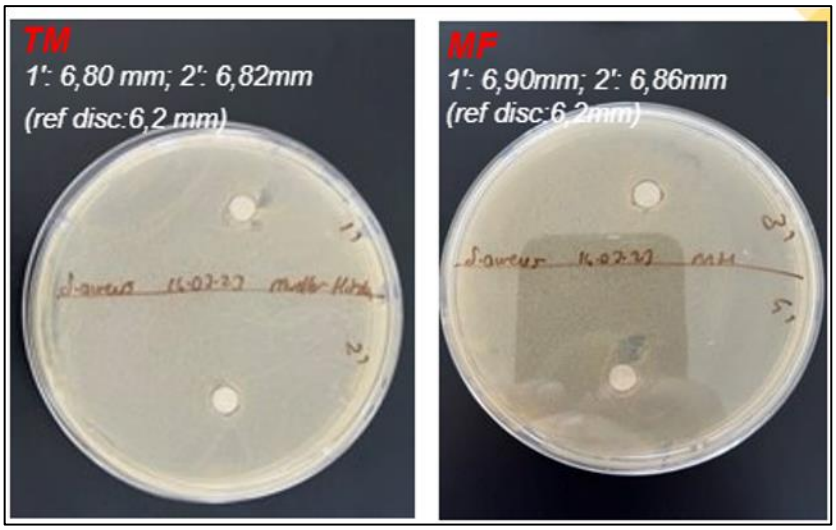


Figure 4.15: Antibacterial activity of hydrogels against *S.aureus.*

The maximum zone expansion created statistically more inhibition areas in the hydrogels produced with the microfluidic system. Hydrogels prepared by conventional method and microfluidic method formed a very small inhibition area against gram-positive and gram-negative microorganisms. This area of inhibition is significantly lower compared to amoxycillin. The inhibitory effect of hydrogels produced by two different methods do not have a significant effect as shown in Figure 4.16.

Disk diffusion test result of the hydrogels produced using the T-junction microfluidic device system (MF) and traditional manually produced (TM) showed poor antibacterial effect against to the gram-negative(a)/positive(b) bacteria.

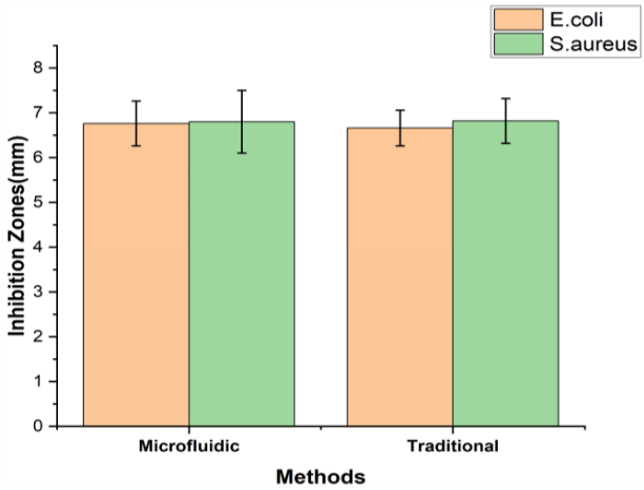


Figure 4.16: The gram-negative(a)/positive(b) antibacterial performance using the T-junction microfluidic device system (MF) and traditional manually produced (TM).

4.6. Cytotoxicity Analysis Results of Produced Hydrogels Using Resazurin Cell Viability Assay

The cytotoxic effects of hydrogels prepared with microfluidic and manual production methods at different concentrations were investigated using the resazurin cell viability assay [86]. In order to determine the presence of any potential toxic leakage from the hydrogels intended for wound care applications, they were kept in the cell culture medium for 24 hours at 37 ºC [87]. Afterwards, the cell culture medium was transferred to another medium containing the extraction solution for cell viability assessment, and the cells were incubated for an additional 24 hours. The live cells were incubated with Alamarblue reagent. Resazurin is an active ingredient in Alamarblue solution and by viable cells it is converted to its reduced state (resorufin) which indirectly reveals the viability and proliferative state of the cells. The control group consisted of cells treated with cell culture medium without any treatment, while acetic acit was used as a positive control. The results obtained are shown in Figure 4.17.

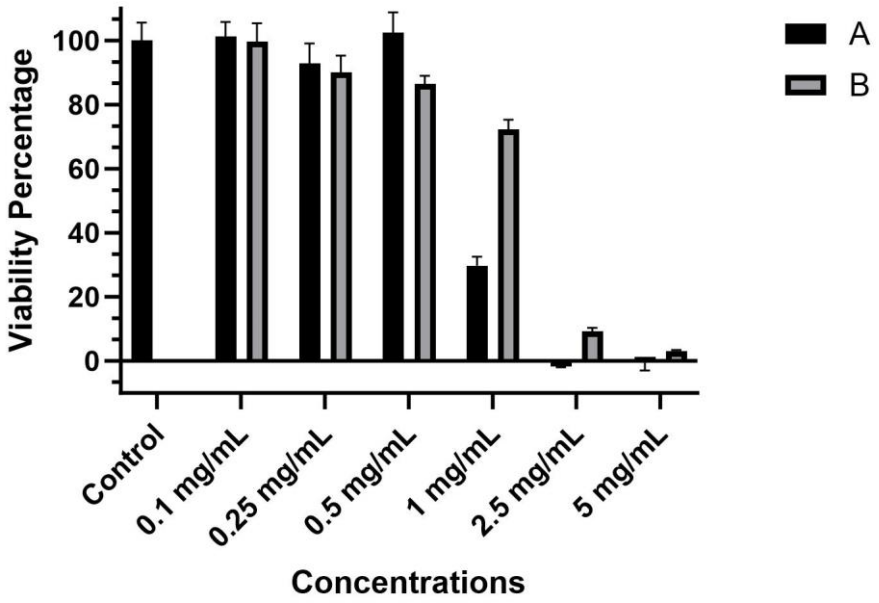


Figure 4.17: Cell viability percentage of sample A (manual manufacturing) and sample B (microfluidic device system manufacturing) against L929 cell line.

The results indicate that the hydrogels did not exhibit high cytotoxic effects up to a concentration of 1 mg/mL [88],[89]. However, hydrogels produced by the manual method showed toxic effects at concentrations of 2.5 mg/mL and 5 mg/mL. It can be observed that hydrogels produced by the microfluidic system were less toxic compared to those produced by the manual method. When the results were evaluated, the highest cell viability rate was observed in hydrogels with concentrations of 0.1% to 0.5%. Increasing the concentration of hydrogels had a negative effect on cell viability. In the hydrogel containing 0.1% CS/β-GP produced by the microfluidic system, cell viability was 9.6% higher compared to the hydrogel containing 0.25% CS/β-GP. In the hydrogel containing 0.1% CS-BGP produced by the manual system, cell viability was 8.5% higher compared to the hydrogel containing 0.25% CS/β-GP.

Overall, when all the data were evaluated, IC50 was calculated as a 1.38 for microfluidically produced hydrogel and 0.94 for manually prepared hydrogel. It can be seen that hydrogels produced by the microfluidic method increased cell viability and proliferation compared to those produced by the manual method. These results are consistent with the cytotoxicity results of other chitosan-based hydrogels produced using different methods in the literature [90], [91] but a comparison could not be made

since no study using the microfluidic method has been conducted [92]. Considering the positive effects of stable uniform microbubbles in wound healing applications, especially in the microfluidic system, the biocompatibility of these structures produced in the microfluidic system and the positive results obtained in cytotoxic experiments should be carefully considered. A preliminary investigation on in vitro cytotoxicity showed that the hydrogels produced were non-cytotoxic to L929 cells when the correct concentrations were used [93]. Although many studies have shown that chitosan is non-cytotoxic, biologically soluble, and biocompatible, the cytotoxic effects of formulations created with different surfactants need to be controlled.

The antibacterial activity and cytotoxicity of chitosan have been widely studied. While some of these studies have shown that chitosan has potent antibacterial activity against a wide range of bacteria, some studies have reported little or no antibacterial activity[94]. Chitosan has been found to cause cell death by disrupting the cell membrane of bacteria. In addition, it has been found as a result of studies that chitosan has a positive charge that can attract and trap negatively charged bacteria.

In terms of cytotoxicity, some studies have reported that chitosan has low cytotoxicity against a number of cell lines, including human skin fibroblasts and blood cells, making it a promising material for use in biomedical applications. However, other studies have reported that chitosan may be toxic to certain cell lines, especially when used at high concentrations [39].

In summary, literature results regarding the antibacterial activity and cytotoxicity of chitosan are not yet clear. More studies on chitosan will shed light on the scientific world.

5. FUTURE PROSPECTS

Future studies should focus on water intake capacity of injectable %1 (v/v) CS/β-GP hydrogels which are produced by manually and microfluidically. The acid used to dissolve chitosan also has a toxic effect. Cytotoxicity experiments should be carried out by developing alternative solvents. The effects of the samples obtained from cytotoxicity results should be observed by in-vivo animal studies. 1% w/v CS and 7.5% w/v β-GP was used in this study; it is important to evaluate the antibacterial activity and cytotoxicity results using different concentrations [95]. Although it is possible to obtain stable results with structures produced with microfluidic, technological developments are needed in order to produce fast and efficient enough to be used in industry and clinic. Because of the diversity of wound, there is also significant need to improve pH sensitive multifunctional hydrogels for further studies.

The hydrogels have antibacterial properties because they contain elements like medicines, nanoparticles, and other things. But concurrently, some cytotoxic problems are generated. Further research is required to determine how to increase the solubility of chitosan materials so they may be easily performed or shaped, reduce their toxicity, and maximize all of their benefits while preserving their intrinsic properties. We anticipate the creation of multifunctional chitosan hydrogels with bright possibilities for treating wounds. Due to the wide variety of wounds, the hydrogels to be used in the treatment should be multifunctional. pH-sensitive hydrogels, injectable hydrogels, self-healing hydrogels, conductive hydrogels, etc., for use under various environmental conditions. Multifunctional hydrogels that respond to stimuli such as to minimize the distress of doctors and patients, multifunctional hydrogels would be a more attractive wound healing material. More importantly, more attention should be paid to simple, economical, practical, useful and degradable materials in future studies [20].

6. CONCLUSION

In this thesis, an injectable chitosan-based hydrogel that can be used in wound healing treatments was produced by both manual and microfluidic methods, then characterized in terms of morphological, structural, thermal behaviour properties using scanning electron microscopy (SEM), Differential Scanning Calorimetry (DSC), Fourier transform infrared spectroscopy (FTIR) and Optical microscope. We also evaluated the effect of produced hydrogels on cell viability status in vitro condition using immortalized mouse fibroblast L929 cell line along with their antimicrobial properties. The aim of this thesis study is to develop a rapidly produced, biocompatible, non-toxic, easy to use, antimicrobial and inexpensive method for wound healing, which is one of the biggest health problems in the world in recent years. Injectable chitosan-based hydrogels provide better tissue penetration, high water content, biocompatibility and antimicrobial properties, thereby reducing the risk of infection, scarring and pain. Our hypothesis is microfluidically prepared injectable chitosan-based hydrogels might show significantly important antibacterial effect without use extra additional composite and promote cell growth because of microbubbles which are uniform, sponges' structure similar with tissue.

In line with this hypothesis, the chitosan-based injectable hydrogel produced by microfluidic and manual method, it was characterized by DSC, SEM, FTIR, Optical microscope. The antibacterial activity of the hydrogels, whose density, viscosity and surface tension were examined, were examined and their biocompatibility was evaluated by cytotoxicity test. The important results obtained from the microfluidic system method production of chitosan-based injectable hydrogel structures used for wound healing applications are summarized below.

- There is no unexpected chemical reaction was observed in the structure of the hydrogels examined by FTIR, DSC and SEM. The results of characterization studies showed that both hydrogels have very similar outputs. In SEM images, more homogeneous and distinct pore of the hydrogels produced with the microfluidic system was observed.

- The hydrogel structure produced with T-junction microfluidic passing through channels with a diameter of 200 μm, which was examined under an optical

microscope, was observed to have a homogenous bubble distribution with an average size of 135 μm and the PDI value was 17%, as intended.

- Disk diffusion test result of the hydrogels produced using the T-junction microfluidic device system (MF) and traditional manually produced (TM) showed poor antibacterial effect against to the gram-negative and gram-positive bacteria. Compared to manually prepared hydrogel, microfluidically prepared hydrogels showed more inhibition zone. For manually produced hydrogel, this result very similar with the literature, however there is no any study which examine the antibacterial activity of microfuildically prepared CS/β-GP hydrogel.

- The mouse fibroblast L929 cell line and resazurin cell viability assay experiments performed to evaluate the biocompatibility showed that the hydrogel produced with microfluidic was less toxic. The reason of less toxic effect of microfluidically prepared hydrogel might be associated with the better penetration of microbubbles in the cell.

Overall, the research points to the possibility that chitosan-based hydrogels may be effective in the treatment of wounds due to their strong antibacterial capabilities. To fully grasp their potential and establish the ideal usage circumstances, more research is necessary. This is, to the best of our knowledge, the first national and international thesis in terms of the originality and scope of the subject about bacterial behaviour analysis and cytotoxicity analysis of microfluidically produced hydrogel. Thanks to this study, it was showed that the microfluidic system is an effective method that can be used for the production of chitosan-based injectable hydrogel.

REFERENCES

[1] Pankongadisak P., Suwantong O., (2018), "The Potential Use of Thermosensitive Chitosan/Silk Sericin Hydrogels Loaded with Longan Seed Extract For Bone Tissue Engineering", RSC Advances, 8(70), 40219–40231.

[2] Torkaman S., Rahmani H., Ashori A., Najafi S. H. M., (2021), "Modification of Chitosan Using Amino Acids for Wound Healing Purposes: a Review", Carbohydrate Polymers, 258, 117675.

[3] Suarato G., Bertorelli R., Athanassiou A., (2018), "Borrowing From Nature : Biopolymers and Biocomposites as Smart Wound Care Materials", 6, 1–11.

[4] Chen H., Cheng J., Ran L., Yu K., Lu B., Lan G., Dai F., Lu F., (2018), "An injectable self-healing hydrogel with adhesive and antibacterial properties effectively promotes wound healing", Carbohydrate Polymers, 201, 522–531.

[5] Moeini A., Pedram P., Makvandi P., Malinconico M., Gomez d'Ayala G., (2020), "Wound healing and antimicrobial effect of active secondary metabolites in chitosan-based wound dressings: A review", Carbohydrate Polymers, 233, 115839.

[6] Zhang R., Yu B., Tian Y., Pang L., Xu T., Cong H., Shen Y., (2022), "Diversified antibacterial modification and latest applications of polysaccharide-based hydrogels for wound healthcare", Applied Materials Today, 26, 101396.

[7] Broughton G., Janis J. E., Attinger C. E., (2006), "The basic science of wound healing", Plastic and Reconstructive Surgery, 117(7).

[8] Li M., Liang Y., He J., Zhang H., Guo B., (2020), "Two-Pronged Strategy of Biomechanically Active and Biochemically Multifunctional Hydrogel Wound Dressing to Accelerate Wound Closure and Wound Healing", Chemistry of Materials, 32(23), 9937–9953.

[9] Su J., Li J., Liang J., Zhang K., Li J., Hydrogel J., Walles H., (2021), "Hydrogel Preparation Methods and Biomaterials for Wound Dressing", Life (Basel), 11(10): 1016.

[10] Suarato G., Bertorelli R., Athanassiou A., (2018), "Borrowing From Nature: Biopolymers and Biocomposites As Smart Wound Care Materials", Frontiers in Bioengineering and Biotechnology, 6, 137.

[11] Bhattarai N., Gunn J., Zhang M., (2010), "Chitosan-based hydrogels for controlled, localized drug delivery", Advanced Drug Delivery Reviews, 62(1), 83–99.

[12] Qu J., Zhao X., Liang Y., Zhang T., Ma P. X., Guo B., (2018), "Antibacterial adhesive injectable hydrogels with rapid self-healing, extensibility and compressibility as wound dressing for joints skin wound healing", Biomaterials, 183, 185–199.

[13] Yang Z., Huang R., Zheng B., Guo W., Li C., He W., Wei Y., Du Y., Wang H., Wu D., Wang H., Yang Z., Huang R., Guo W., Li C., Wei Y., Du Y., Wang H., Zheng B., He W., Wu D., (2021), "Highly Stretchable, Adhesive, Biocompatible, and Antibacterial Hydrogel Dressings for Wound Healing", Advanced Science, 8(8), 2003627.

[14] Deng P., Jin W., Liu Z., Gao M., Zhou J., (2021), "Novel multifunctional adenine-modified chitosan dressings for promoting wound healing", Carbohydrate Polymers, 260, 117767.

[15] Liu H., Wang C., Li C., Qin Y., Wang Z., Yang F., Li Z., Wang J., (2018), "A functional chitosan-based hydrogel as a wound dressing and drug delivery system in the treatment of wound healing", RSC Advances, 8(14), 7533.

[16] Feng P., Luo Y., Ke C., Qiu H., Wang W., Zhu Y., Hou R., Xu L., Wu S., (2021), "Chitosan-Based Functional Materials for Skin Wound Repair: Mechanisms and Applications", Frontiers in Bioengineering and Biotechnology, 9, 111.

[17] Deng P., Yao L., Chen J., Tang Z., Zhou J., (2022), "Chitosan-based hydrogels with injectable, self-healing and antibacterial properties for wound healing", Carbohydrate Polymers, 276, 118718.

[18] Zhang X., Li L., Luo C., (2016), "Gel integration for microfluidic applications", Lab on a Chip, 16(10), 1757–1776.

[19] Zhou H. Y., Jiang L. J., Cao P. P., Li J. B., Chen X. G., (2015), "Glycerophosphate-based chitosan thermosensitive hydrogels and their biomedical applications", Carbohydrate Polymers, 117, 524–536.

[20] Zhang X., Qin M., Xu M., Miao F., Merzougui C., Zhang X., Wei Y., Chen W., Huang D., (2021), "The fabrication of antibacterial hydrogels for wound healing", European Polymer Journal, 146, 110268.

[21] (1993), "First Joint Meeting of The Wound Healing Society and the European

Tissue Repair Society.", Wound repair and regeneration : official publication of the Wound Healing Society [and] the European Tissue Repair Society, 1(2), 84–84, Amsterdam, The Netherlands, August 22-25, 1993.

[22] Chen X., Zhang M., Chen S., Wang X., Tian Z., Chen Y., Xu P., Zhang L., Zhang L., Zhang L., (2017), "Peptide-Modified Chitosan Hydrogels Accelerate Skin Wound Healing by Promoting Fibroblast Proliferation, Migration, and Secretion", Cell Transplantation, 26(8), 1331.

[23] Heimbuck A. M., Priddy-arrington T. R., Padgett M. L., Llamas C. B., Barnett H. H., Bunnell B. A., Caldorera-moore M. E., (2019), "Development of Responsive Chitosan–Genipin Hydrogels for the Treatment of Wounds", ACS Applied Bio Materials, 2, 2879–2888.

[24] Niranjan R., Koushik C., Saravanan S., Moorthi A., Vairamani M., Selvamurugan N., (2013), "A novel injectable temperature-sensitive zinc doped chitosan/β-glycerophosphate hydrogel for bone tissue engineering", International journal of biological macromolecules, 54(1), 24–29.

[25] Stefanov I., Pérez-Rafael S., Hoyo J., Cailloux J., Santana Pérez O. O., Hinojosa-Caballero D., Tzanov T., (2017), "Multifunctional Enzymatically Generated Hydrogels for Chronic Wound Application", Biomacromolecules, 18(5), 1544–1555.

[26] Hemmingsen L. M., Julin K., Ahsan L., Basnet P., Johannessen M., Škalko-basnet N., (2021), "Chitosomes-In-Chitosan Hydrogel for Acute Skin Injuries : Prevention and Infection Control", 1–21.

[27] Chouhan D., Dey N., Bhardwaj N., Mandal B. B., (2019), "Emerging and innovative approaches for wound healing and skin regeneration: Current status and advances", Biomaterials, 216.

[28] Domenech T., Doyle P. S., (2020), "High Loading Capacity Nanoencapsulation and Release of Hydrophobic Drug Nanocrystals from Microgel Particles", Chemistry of Materials, 32(1), 498–509.

[29] Li J., Yao F., (2016), "Environment-stimuli response of chitosan-based hydrogels", Chitosan-Based Hydrogels: Functions and Applications, 235–261.

[30] Zhong Y., Xiao H., Seidi F., Jin Y., (2020), "Natural Polymer-Based Antimicrobial Hydrogels without Synthetic Antibiotics as Wound Dressings", Biomacromolecules, 21(8), 2983–3006.

[31] Sowmya S., Bumgardener J. D., Chennazhi K. P., Nair S. V., Jayakumar R.,

(2013), "Role of nanostructured biopolymers and bioceramics in enamel, dentin and periodontal tissue regeneration", Progress in Polymer Science, 38(10–11), 1748–1772.

[32] Lü S., Gao C., Xu X., Bai X., Duan H., Gao N., Feng C., Xiong Y., Liu M., (2015), "Injectable and Self-Healing Carbohydrate-Based Hydrogel for Cell Encapsulation", ACS Applied Materials and Interfaces, 7(23), 13029–13037.

[33] Kharkar P. M., Kiick K. L., Kloxin A. M., (2013), "Designing degradable hydrogels for orthogonal control of cell microenvironments", Chemical Society Reviews, 42(17), 7335–7372.

[34] Szekalska M., Sosnowska K., Zakrzeska A., Kasacka I., Lewandowska A., Winnicka K., (2017), "The Influence of Chitosan Cross-linking on the Properties of Alginate Microparticles with Metformin Hydrochloride-In Vitro and In Vivo Evaluation", Molecules, 22(1).

[35] Sivashanmugam A., Arun Kumar R., Vishnu Priya M., Nair S. V., Jayakumar R., (2015), "An overview of injectable polymeric hydrogels for tissue engineering", European Polymer Journal, 72, 543–565.

[36] Do N. H. N., Pham T. H., Le P. K., Ha A. C., (2023), "Thermo-responsive Chitosan/β-glycerophosphate hydrogels directly post-loading anti-inflammatory diclofenac sodium", Journal of Sol-Gel Science and Technology, 105(2), 451–460.

[37] Kazemi-Aghdam F., Jahed V., Dehghan-Niri M., Ganji F., Vasheghani-Farahani E., (2021), "Injectable chitosan hydrogel embedding modified halloysite nanotubes for bone tissue engineering", Carbohydrate Polymers, 269.

[38] Wu J., Zheng K., Huang X., Liu J., Liu H., Boccaccini A. R., Wan Y., Guo X., Shao Z., (2019), "Thermally triggered injectable chitosan/silk fibroin/bioactive glass nanoparticle hydrogels for in-situ bone formation in rat calvarial bone defects", Acta Biomaterialia, 91, 60–71.

[39] Martinotti S., Gheorghit D., Moldovan iu, Robu A., Bit A.-I., Grosu E., Antoniac A., Corneschi I., Antoniac I., Dănut A., Ionut C., (2023), "Chitosan-Based Biomaterials for Hemostatic Applications: A Review of Recent Advances", International Journal of Molecular Sciences, 24(13), 10540.

[40] Sahariah P., Másson M., (2017), "Antimicrobial Chitosan and Chitosan Derivatives : Antimicrobial Chitosan and Chitosan Derivatives : A Review of the Structure-Activity Relationship".

[41] Areevijit K., Dhanesuan N., Luckanagul J. A., Rungsiyanont S., (2021), "Biocompatibility study of modified injectable hyaluronic acid hydrogel with mannitol/BSA to alveolar bone cells", Journal of Biomaterials Applications, 35(10), 1294–1303.

[42] Zou W., Chen Y., Zhang X., Li J., Sun L., Gui Z., Du B., Chen S., (2018), "Cytocompatible chitosan based multi-network hydrogels with antimicrobial, cell anti-adhesive and mechanical properties", Carbohydrate Polymers, 202(March), 246–257.

[43] Yin H., Yan Z., Bauer R. J., Peng J., Schieker M., Nerlich M., Docheva D., (2018), "Functionalized thermosensitive hydrogel combined with tendon stem/progenitor cells as injectable cell delivery carrier for tendon tissue engineering", Biomedical Materials (Bristol), 13(3).

[44] Li S., Dong S., Xu W., Tu S., Yan L., Zhao C., Ding J., Chen X., (2018), "Antibacterial Hydrogels", Advanced Science, 5(5).

[45] Rizzotto M., (2012), "Metal Complexes as Antimicrobial Agents", InTech.

[46] Varçin M., Şener B. B., Bayraç C., (2021), "Adsorption of resazurin by poly(acrylic acid) hydrogels and evaluation of its use in reduction assay for quantification of cell viability", Dyes and Pigments, 186, 109038.

[47] Raina N., Pahwa R., Thakur V. K., Gupta M., (2022), "Polysaccharide-based hydrogels: New insights and futuristic prospects in wound healing", International Journal of Biological Macromolecules, 223, 1586–1603.

[48] Deng P., Yao L., Chen J., Tang Z., Zhou J., (2022), "Chitosan-based hydrogels with injectable, self-healing and antibacterial properties for wound healing", Carbohydrate Polymers, 276.

[49] Almawash S., Osman S. K., Mustafa G., El Hamd M. A., (2022), "Current and Future Prospective of Injectable Hydrogels-Design Challenges and Limitations", Pharmaceuticals (Basel, Switzerland), 15(3).

[50] Li S., Dong S., Xu W., Tu S., Yan L., Zhao C., Ding J., Chen X., (2018), "Antibacterial Hydrogels", 1700527.

[51] Wang Q., Chen D., (2016), "Synthesis and characterization of a chitosan based nanocomposite injectable hydrogel", Carbohydrate Polymers, 136, 1228–1237.

[52] Cheng Y. H., Yang S. H., Lin F. H., (2011), "Thermosensitive chitosan-gelatin-glycerol phosphate hydrogel as a controlled release system of ferulic acid for nucleus pulposus regeneration", Biomaterials, 32(29), 6953–6961.

[53] Wei Z., Wang S., Hirvonen J., Santos H. A., Li W., (2022), "Microfluidics Fabrication of Micrometer-Sized Hydrogels with Precisely Controlled Geometries for Biomedical Applications", 2200846, 1–23.

[54] Zhang X., Li L., Luo C., (2016), "Gel integration for microfluidic applications", Lab on a Chip, 16(10), 1757–1776.

[55] Laurenti M., Cauda V., "Biodegradable polymer nanocomposites for tissue engineering: Synthetic strategies and related applications", Materials for Biomedical Engineering: Absorbable Polymers, 2019, 157–198.

[56] Shang L., Cheng Y., Zhao Y., (2017), "Emerging Droplet Microfluidics", Chemical Reviews, 117(12), 7964–8040.

[57] Mutlu B., Farhan M., Kucuk I., (2019), "T-Shaped Microfluidic Junction Processing of Porous Alginate-Based Films and Their Characteristics", Polymers 2019, Vol. 11, Page 1386, 11(9), 1386.

[58] Griffin D. R., Archang M. M., Kuan C. H., Weaver W. M., Weinstein J. S., Feng A. C., Ruccia A., Sideris E., Ragkousis V., Koh J., Plikus M. V., Di Carlo D., Segura T., Scumpia P. O., (2021), "Activating an adaptive immune response from a hydrogel scaffold imparts regenerative wound healing", Nature materials, 20(4), 560–569.

[59] Griffin D. R., Weaver W. M., Scumpia P. O., Di Carlo D., Segura T., (2015), "Accelerated wound healing by injectable microporous gel scaffolds assembled from annealed building blocks", Nature materials, 14(7), 737.

[60] Elmas M., (2022), "Microfluidic Preparation of Metformin-Encapsulated Thermo-Sensitive Nanocomposite Hydrogel Systems for Topical Vaginal Applications", Master Thesis, Gebze Technical University.

[61] DOĞAN M., (2022), "Preparation of Chitosan-Polyvinyl Prolidone (PVP) Hydrogels with Fibroblast Growth Factor (FGF) and Investigation of in Vitro Characteristics", Clinical and Experimental Health Sciences, 12(2), 418–422.

[62] Wang X., Song R., Johnson M., He Z., Milne C., Wang X., Lara-Sáez I., Xu Q., Wang W., (2021), "An Injectable Chitosan-Based Self-Healable Hydrogel System as an Antibacterial Wound Dressing", Materials.

[63] Li Y., Zhang Y., Wei Y., Tao L., (2017), "Preparation of chitosan-based injectable hydrogels and its application in 3D cell culture", Journal of Visualized Experiments, 2017(127), 1–7.

[64] Kanimozhi K., Khaleel Basha S., Sugantha Kumari V., Kaviyarasu K., Maaza

M., (2018), "In vitro cytocompatibility of chitosan/PVA/methylcellulose – Nanocellulose nanocomposites scaffolds using L929 fibroblast cells", Applied Surface Science, 449, 574–583.

[65] Sinha M. K., Gao J., Stowell C. E. T., Wang Y., (2015), "Synthesis and biocompatibility of a biodegradable and functionalizable thermo-sensitive hydrogel", Regenerative Biomaterials, 2(3), 177–185.

[66] Behera S. S., Das U., Kumar A., Bissoyi A., Singh A. K., (2017), "Chitosan/TiO2 composite membrane improves proliferation and survival of L929 fibroblast cells: Application in wound dressing and skin regeneration", International Journal of Biological Macromolecules, 98, 329–340.

[67] De Souza Costa E., Pereira M. M., Mansur H. S., (2009), "Properties and biocompatibility of chitosan films modified by blending with PVA and chemically crosslinked", Journal of materials science. Materials in medicine, 20(2), 553–561.

[68] Mansur H. S., de S. Costa Jr. E., Mansur A. A. P., Barbosa-Stancioli E. F., (2009), "Cytocompatibility evaluation in cell-culture systems of chemically crosslinked chitosan/PVA hydrogels", Materials Science and Engineering C, 29(5), 1574–1583.

[69] Nafee N., Zewail M., Boraie N., (2017), "Alendronate-loaded, biodegradable smart hydrogel: a promising injectable depot formulation for osteoporosis", 26(7), 563–575.

[70] Queiroz M. F., Melo K. R. T., Sabry D. A., Sassaki G. L., Rocha H. A. O., (2015), "Does the use of chitosan contribute to oxalate kidney stone formation?", Marine Drugs, 13(1), 141–158.

[71] Pankongadisak P., Suwantong O., (2018), "The potential use of thermosensitive chitosan/silk sericin hydrogels loaded with longan seed extract for bone tissue engineering", RSC Advances, 8(70), 40219–40231.

[72] Zhou F., Xu T., Zhao Y., Song H., Zhang L., Wu X., Lu B., (2018), "Chitosan-coated liposomes as delivery systems for improving the stability and oral bioavailability of acteoside", Food Hydrocolloids, 83, 17–24.

[73] Skwarczynska A., Kaminska M., Owczarz P., Bartoszek N., Walkowiak B., Modrzejewska Z., (2018), "The structural (FTIR, XRD, and XPS) and biological studies of thermosensitive chitosan chloride gels with β-glycerophosphate disodium", Journal of Applied Polymer Science, 135(27),

46459.

[74] Xiao Y., Gu Y., Qin L., Chen L., Chen X., Cui W., Li F., Xiang N., He X., (2021), "Injectable thermosensitive hydrogel-based drug delivery system for local cancer therapy", Colloids and Surfaces B: Biointerfaces, 200.

[75] Qin H., Wang J., Wang T., Gao X., Wan Q., Pei X., (2018), "Preparation and characterization of chitosan/β-glycerophosphate thermal-sensitive hydrogel reinforced by graphene oxide", Frontiers in Chemistry, 6, 421666.

[76] Saeednia L., Usta A., Asmatulu F. R., (2013), "Injectable Thermosensitive Hydrogels as Drug Delivery Systems", Techniical Report No: 9, 69–70, Department of Mechanical Engineering, College of Engineering, Wichita State University,USA.

[77] Do N. H. N., Pham T. H., Le P. K., Ha A. C., (2023), "Thermo-responsive Chitosan/β-glycerophosphate hydrogels directly post-loading anti-inflammatory diclofenac sodium", Journal of Sol-Gel Science and Technology, 105(2), 451–460.

[78] Kim M. A., Shin S. R., Kim H. J., Lee J. S., Lee C. M., (2022), "Chemo-photothermal therapeutic effect of chitosan-gelatin hydrogels containing methotrexate and melanin on a collagen-induced arthritis mouse model", International journal of biological macromolecules, 218, 1013–1020.

[79] Nair L. S., Starnes T., Ko J. W. K., Laurencin C. T., (2007), "Development of injectable thermogelling chitosan-inorganic phosphate solutions for biomedical applications", Biomacromolecules, 8(12), 3779–3785.

[80] Zhou H. Y., Jiang L. J., Cao P. P., Li J. B., Chen X. G., (2015), "Glycerophosphate-based chitosan thermosensitive hydrogels and their biomedical applications", Carbohydrate polymers, 117, 524–536.

[81] Boido M., Ghibaudi M., Gentile P., Favaro E., Fusaro R., Tonda-Turo C., (2019), "Chitosan-based hydrogel to support the paracrine activity of mesenchymal stem cells in spinal cord injury treatment", Technical Report No: 9, 6402, Neuroscience Institute, University of Torino, Italy.

[82] Supper S., Anton N., Seidel N., Riemenschnitter M., Curdy C., Vandamme T., (2014), "Thermosensitive chitosan/glycerophosphate-based hydrogel and its derivatives in pharmaceutical and biomedical applications", Expert Opinion on Drug Delivery, 11(2), 249–267.

[83] Pereira R. M., Andrade G. S. S., De Castro H. F., Campos M. G. N., (2017),

"Performance of chitosan/glycerol phosphate hydrogel as a support for lipase immobilization", Materials Research, 20, 190–201.

[84] Maiz-Fernández S., Guaresti O., Pérez-Álvarez L., Ruiz-Rubio L., Gabilondo N., Vilas-Vilela J. L., Lanceros-Mendez S., (2020), "β-Glycerol phosphate/genipin chitosan hydrogels: A comparative study of their properties and diclofenac delivery", Carbohydrate polymers, 248.

[85] Kucuk I., (2016), "Microfluidic Engineered Polymer Nanovehicles for Drug Delivery", Doctoral thesis, UCL (University College London).

[86] Woehrling E. K., Hill E. J., Torr E. E., Coleman M. D., (2011), "Single-cell ELISA and flow cytometry as methods for highlighting potential neuronal and astrocytic toxicant specificity", Neurotoxicity research, 19(3), 472–483.

[87] Fiorentini F., Suarato G., Grisoli P., Zych A., Bertorelli R., Athanassiou A., (2021), "Plant-based biocomposite films as potential antibacterial patches for skin wound healing", European Polymer Journal, 150, 110414.

[88] Sahariah P., Benediktssdóttir B. E., Hjálmarsdóttir M. A., Sigurjonsson O. E., Sørensen K. K., Thygesen M. B., Jensen K. J., Másson M., (2015), "Impact of chain length on antibacterial activity and hemocompatibility of quaternary N-alkyl and N, N-dialkyl chitosan derivatives", Biomacromolecules, 16(5), 1449–1460.

[89] Zhong H., Liang H., Yan Y., Chen L., Zhao T., Liu L., Chen Y., (2022), "Nucleic Acid-Scavenging Hydrogels Accelerate Diabetic Wound Healing", Biomacromolecules, 23(8), 3396–3406.

[90] Ma G., Fang D., Liu Y., Zhu X., Nie J., (2012), "Electrospun sodium alginate/poly(ethylene oxide) core–shell nanofibers scaffolds potential for tissue engineering applications", Carbohydrate Polymers, 87(1), 737–743.

[91] Lee C., Shin J., Lee J. S., Byun E., Ryu J. H., Um S. H., Kim D. I., Lee H., Cho S. W., (2013), "Bioinspired, calcium-free alginate hydrogels with tunable physical and mechanical properties and improved biocompatibility", Biomacromolecules, 14(6), 2004–2013.

[92] Ramírez Barragán C. A., Macías Balleza E. R., García-Uriostegui L., Andrade Ortega J. A., Toríz G., Delgado E., (2018), "Rheological characterization of new thermosensitive hydrogels formed by chitosan, glycerophosphate, and phosphorylated β-cyclodextrin", Carbohydrate Polymers, 201, 471–481.

[93] Muir V. G., Burdick J. A., (2021), "Chemically Modified Biopolymers for the

Formation of Biomedical Hydrogels", Chemical Reviews, 121(18), 10908–10949.

[94] Reakasame S., Boccaccini A. R., (2017), "Oxidized Alginate-Based Hydrogels for Tissue Engineering Applications: A Review", Biomacromolecules, 19(1), 3–21.

[95] Wang Y., Pang X., Luo J., Wen Q., Wu Z., Ding Q., Zhao L., Yang L., Wang B., Fu S., (2019), "Naproxen Nanoparticle-Loaded Thermosensitive Chitosan Hydrogel for Prevention of Postoperative Adhesions", ACS Biomaterials Science and Engineering, 5(3), 1580–1588.

BIOGRAPHY

Sümeyye Danış graduated from Department of Biomedical Engineering, in the Faculty of Engineering, at Karabuk University with a B.Sc. degree in 2019. During her undergraduate education, she was entitled to participate in the exchange program for one semester in the Faculty of Engineering, at Lublin University of Technology in Poland and successfully completed the 2016-2017 fall semester. She was entitled to participate in the Erasmus internship program in 2018 and successfully completed his internship for 3 months in the Faculty of Engineering at Brunel University of London in UK. She started working with leading companies in the medical field right after her graduation. As of fall 2020, she started her master's degree in Department of Nanoscience and Nanoengineering at Gebze Technical University, Institute of Nanotechnology.