



MARMARA UNIVERSITY
INSTITUTE FOR GRADUATE STUDIES
IN PURE AND APPLIED SCIENCES



**POLYPHENOL LOADED *HALOMONAS* LEVAN
BASED MICRONEEDLES FOR PREVENTION
OF JOINT INFLAMMATION IN RHEUMATOID
ARTHRITIS**

AYBÜKE TEKİN

MASTER THESIS

Department of Bioengineering

Thesis Supervisor

Prof. Ebru TOKSOY ÖNER

ISTANBUL, 2025



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ÖZET

ROMATOİD ARTRİT HASTALIĞINDA EKLEM İNFLAMASYONUNUN POLİFENOL YÜKLÜ *HALOMONAS* LEVAN BAZLI MİKROİĞNELER İLE ÖNLENMESİ

Sağlıklı bir bağışıklık sisteminin en önemli özelliği, kendi ve ben olmayanı ayırt etme yeteneği, yani bağışıklık toleransıdır. Otoimmün bozukluklar, bu toleransın bozulmasıyla bağışıklık sisteminin yanlışlıkla vücuttaki sağlıklı dokulara saldırmasıyla ortaya çıkan hastalıklardır. Romatoid artrit (RA), nüfusun %1'ini etkileyen, çoğunlukla eklemlerdeki inflamasyonla, ileri safhalarda ise cilt, göz, kalp, böbrek ve akciğer gibi vücudun diğer kısımlarında da komplikasyonlara neden olabilecek ciddi bir otoimmün hastalıktır. RA için mevcut tedavi yöntemleri, temel olarak bağışıklık sistemini baskılayarak semptomların giderilmesini amaçlamaktadır. Ancak çoğunlukla sürekli ve özellikle hastalığın alevlenme dönemlerinde yüksek doz kullanım gereksinimi, bu yöntemlerden kaynaklanabilecek yan etki riskini artırmaktadır. Levan, doğada çeşitli mikroorganizmalar ve bitkiler tarafından üretilen β -2,6 bağlı bir fruktoz ekzopolisakkaritidir. Biyoyumluluk, yüksek su tutma kapasitesi ve yapışkanlık özelliklerinin yanında antioksidan, antiinflamatuvar, antikanserojen vb. biyoaktiviteleri onu ilaç sektöründen gıda sektörüne ilgi odağı hale getirmektedir. Oleuropein (OLE), hidroksitirosol aktif maddesi ile kardiyovasküler ve nöro koruyucu, antikanser, antiinflamatuvar ve antioksidan özellikler sunan zeytin yağı ve yapraklarının ana fenolik bileşiklerinden biridir. Bu tez çalışmasında, levan bazlı foto çapraz bağlanmış mikroiğneleri DLP tekniği ile üretmek, bunların morfolojik, reolojik, şişme ve jel fraksiyonu özelliklerinin, oleuropein yüklenmiş mikroiğnelerin salınım davranışının ve mikroiğnelerin hücreler üzerindeki sitotoksitesinin incelenmesi hedeflenmiştir. Bu hususta, tamamen levan bazlı mikroiğneler (L1G0), farklı oranlarda LevMA-GelMA oranlarına sahip kompozitler (L3G1, L1G1, L1G3) ve GelMA mikroiğneleri (L0G1) biyoyumlulukları, mekanik ve morfolojik özellikleri bakımından karşılaştırılmıştır. Mikroiğnelerin tamamı PCS hücrelerinde %80'in üzerinde canlılık gösterirken, levan bazlı mikroiğneler GelMA mikroiğnelerine göre yüksek şişme kabiliyeti, jel fraksiyonu ve daha esnek bir yapı göstermiştir. Çalışma sonucunda, RA hastalığında eklem inflamasyonunu engelleyebilecek potansiyelde biyoyumlu, eklem bölgesinin dinamik yapısına uyum sağlayabilecek esneklikte ve antiinflamatuvar etki göstermesi beklenen oleuropeinin kontrollü ve yavaş salınımını sağlayabilecek levan bazlı mikroiğneler üretilmiştir.

ABSTRACT

POLYPHENOL LOADED *HALOMONAS* LEVAN BASED MICRONEEDLES FOR PREVENTION OF JOINT INFLAMMATION IN RHEUMATOID ARTHRITIS

The most important feature of a healthy immune system is the ability to distinguish between self and non-self, in other words, immune tolerance. Autoimmune disorders are diseases that occur when this tolerance is disrupted and the immune system mistakenly attacks healthy tissues in the body. Rheumatoid arthritis (RA) is a serious autoimmune disease that affects 1% of the population and can cause inflammation mostly in the joints and complications in other parts of the body, such as the skin, eyes, heart, kidneys, and lungs, in advanced stages. Current treatment methods for rheumatoid arthritis mainly aim to relieve symptoms by suppressing the immune system. However, the need for continuous and especially high-dose use during flare-ups of the disease increases the risk of side effects that may arise from these methods. Levan is a β -2,6-linked fructose exopolysaccharide produced by various microorganisms and plants in nature. In addition to its biocompatibility, high water retention capacity, and adhesive properties, its antioxidant, anti-inflammatory, anti-carcinogenic, etc., bioactivities make it a focus of interest from the pharmaceutical sector to the food sector. Oleuropein (OLE) is one of the main phenolic compounds of olive oil and leaves, which offers cardiovascular and neuroprotective, anticancer, anti-inflammatory, and antioxidant properties with its hydroxytyrosol active substance. In this thesis study, it was aimed to produce levan-based photo-crosslinked microneedles using the DLP technique, to investigate their morphological, rheological, swelling, and gel fraction properties, the release behavior of oleuropein-loaded microneedles, and the cytotoxicity of microneedles on cells. In this regard, completely levan-based microneedles (L1G0), composites with different LevMA-GelMA ratios (L3G1, L1G1, L1G3), and GelMA microneedles (L0G1) were compared in terms of their biocompatibility, mechanical and morphological properties. While all microneedles showed over 80% viability in PCS cells, levan-based microneedles showed higher swelling ability, gel fraction, and a more flexible structure compared to GelMA microneedles. As a result of the study, levan-based microneedles were produced that are potentially biocompatible, flexible enough to adapt to the dynamic structure of the joint area, and able to provide controlled and slow release of oleuropein, which is expected to have an anti-inflammatory effect and can prevent joint inflammation in RA disease.

SYMBOLS

%	: Percentage
μl	: Microliter
μm	: Micrometer
°C	: Celsius degree
CO₂	: Carbon dioxide
CV	: Column volume
kV	: Kilovolt
g/g	: Gram per gram
g	: Gram
M	: Molar
mg/ml	: Milligram per milliliter
ml	: Milliliter
nm	: Nanometer
rcf	: Relative centrifugal force
rpm	: Revolutions per minute
w/v	: Weight per volume
Pa	: Pascal

ABBREVIATIONS

3D	: Three Dimensional
API	: Active Pharmaceutical Ingredient
COX	: Cyclooxygenase
DDS	: Drug Delivery Systems
DLP	: Digital Light Processing
DM	: Degree of Methacrylation
DMARDs	: Disease-Modifying Antirheumatic Drugs
EDTA	: Ethylenediaminetetraacetic acid
FC	: Folin-Ciocalteu
FTIR	: Fourier Transform Infrared Spectroscopy
GCs	: Glucocorticoids
GelMA	: Gelatin Methacrylate
GR	: Glucocorticoid Receptor
GRE	: Glucocorticoid Responsive Genes
IMAC	: Immobilized Metal Affinity Chromatography
IPTG	: Isopropyl β -D-1-thiogalactopyranoside
LAP	: Lithium phenyl-2,4,6-trimethylbenzoylphosphinate
LB	: Luria Broth
LevMA	: Levan Methacrylate
MN	: Microneedle
NMR	: Nuclear Magnetic Resonance
NSAIDs	: Non Steroidal Antiinflammatory Drugs
OD	: Optical Density
OLE	: Oleuropein
PBS	: Phosphate Buffered Saline
PEG	: Polyethylene glycol
PLA	: Polylactic acid
PNIPAM	: Poly(N-isopropyl acrylamide)
PVA	: Poly (vinyl) alcohol
PVP	: Polyvinylpyrrolidone
RA	: Rheumatoid Arthritis

SEM : Scanning Electron Microscopy
TDDS : Transdermal Drug Delivery Systems
UV : Ultraviolet



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1. INTRODUCTION

1.1. Immune System

The immune system is a vital and complex defense mechanism that protects the body from diseases. The immune system consists of a large number of different cell types, all of which originate from hematopoietic stem cells in the bone marrow and have specific roles in protecting the body from microorganisms such as bacteria, viruses, parasites, abnormal or malignant cells, and foreign substances (Delves et al., 2000; Wang et al., 2024). The human body provides an ideal growth environment for many pathogens with its various macro- and micronutrients, high humidity, constant internal temperature, and pH (Qiu et al., 2022). These pathogens may reach the body through several routes, including open wounds and body openings. Organisms that can pass through barriers such as keratinized skin surfaces and mucus layers cause different immune responses in the body (Delves et al., 2000; Li, 2021; Soni et al., 2024).

Although the various immune cells, tissues, and organs that are involved in the formation of these responses produce different immune responses, they interact with each other and work in a highly coordinated manner (Weisberg et al., 2021). This interaction is provided by various receptors on the cell surfaces of macrophages, neutrophils, dendritic cells (cells of the innate immune system), and lymphocytes (cells of the adaptive immune system) that enable them to recognize each other and antigens, and by chemicals called cytokines that they secrete, which can regulate the immune response (Marshall et al., 2018; “Cell Biology of the Immune System,” 2020).

1.1.1. Autoimmunity

The immune system is a system that is constantly on alert against changes in and around the body due to the potential cancer risk that arises from every cell renewal and division that continues uninterruptedly in the body, as well as any situation that may lead to infection (Gonzalez et al., 2018; Hu et al., 2024). Different gene expressions that occur when cells that perceive signals from the environment and each other through their receptors activate some genes and turn off others constitute the ability of the immune system to constantly adapt to its environment and give different responses (Li & Wu, 2021; Nguyen et al., 2025). The most important of these abilities is immune tolerance, which is also the most important condition for a healthy and properly functioning immune system (Mazzone et al., 2019; Meng et al., 2023). Normally, bone marrow and the thymus are the two main training points that provide immune

tolerance in cells, and thus, cells that can distinguish between what belongs to them and what does not belong to them are produced (Shirafkan et al., 2024). Central tolerance, which occurs in the thymus and bone marrow, eliminates immune cells that react to themselves during their development, while peripheral tolerance mechanisms suppress self-reactive cells that escape into the circulation (Meng et al., 2023; Han et al., 2025). However, in some cases, this training and control mechanism may be disrupted. Autoimmune disorders are diseases characterized by the immune system wrongly producing autoantibodies that target and damage healthy tissues in the body (Chavda et al., 2023). The underlying causes of autoimmune disorders remain poorly known, yet they are believed to result from a combination of genetic and environmental factors that may impair immunological tolerance (Song et al., 2024). Autoimmune diseases are divided into two categories: specific ones, which mostly affect one organ, tissue, cell type, etc., and systemic ones, which affect multiple tissues and organs (Wang et al., 2015; Mazzone et al., 2019). It is known that autoimmune diseases, which often lead to chronic pain, poor quality of life, and even life-threatening conditions such as organ dysfunction, affect millions of people today (Nasir & Mak, 2024). However, despite advances in immunology, currently used treatment methods generally focus on immunosuppression, which only serves to eliminate existing symptoms.

1.2. Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a chronic, systemic, inflammatory autoimmune disorder marked by ongoing inflammation of synovial joints, impacting 0.5–1% of the population with a higher prevalence in women (Smolen et al., 2018b; Aletaha & Smolen, 2018; Wu et al., 2022; Zhang et al., 2025). The key difference between rheumatoid arthritis and diseases like osteoarthritis is that the abnormal immune response caused by autoimmune components leads to pannus formation, infiltration of inflammatory cells, and uncontrolled release of proinflammatory cytokines, which in turn causes inflammation of the synovial membrane (Zhao et al., 2021; Ding et al., 2023).

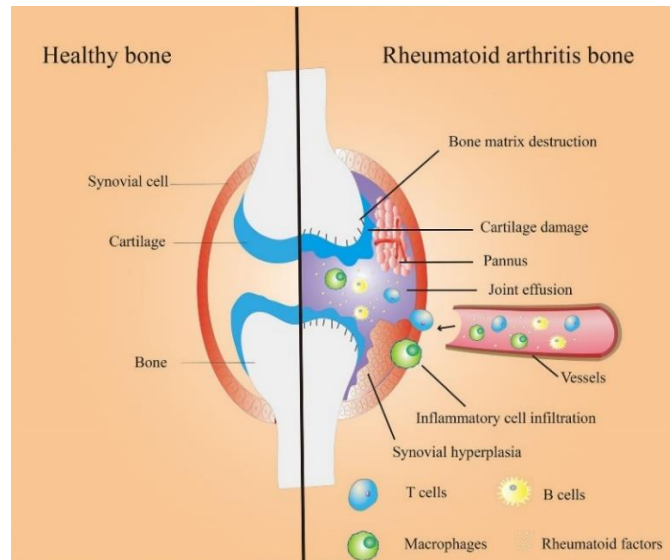


Figure 1.1 Comparison between the healthy bone and RA bone (Huang et al., 2022)

Prolonged morning stiffness, fever, weight loss, and swollen joints are some of the symptoms of RA. Although the joints most affected by RA are small joints such as the hands, wrists, and feet, many other joints can be affected in the advanced stages of the disease (Jahid et al., 2023). Rheumatoid arthritis results in breakdown, pain, stiffness, and ultimately a deficiency of joint function. In addition, the disease, which affects one or a few joints in the early stages of the disease, can progress to the spine in the advanced stages, causing advanced joint erosion (Evans et al., 1995) and disability (Mysler et al., 2021). In addition to the joints, RA can also affect the skin, eyes, heart, kidneys, and lungs, further shortening the quality and duration of life (Smollen et al., 2003; Bullock et al., 2018).

1.2.1. Current Treatment Methods

Current RA treatments do not offer a definitive cure for the disease, and almost all of them are associated with many possible side effects. A better understanding of the immunopathology of RA is needed, which can be used to develop new drugs and drug delivery systems and immunomodulatory strategies.

1.2.1.1. Glucocorticoids

Glucocorticoids (GCs) are steroid hormones secreted by the adrenal cortex. Their most basic function is to enable the body to cope with internal and external stressors and thus help maintain homeostasis (McGinn et al., 2021). They have functions such as creating constant blood

pressure in acute or chronic stress, regulating blood sugar levels, and regulating the immune response. The most famous of these hormones, also called stress hormones, is cortisol. Today's pharmacological GCs are drugs produced by the synthetic imitation of these natural hormones. Cortisone, produced as a cortisol imitation, is among the best-known GC (Fink, 2016; Kovačević et al., 2018). GCs, which was discovered in 1950 with its use for the treatment of RA, is still frequently used in the treatment of RA (Hua et al., 2020).

Synthetic GCs, like natural steroid hormones, reduce inflammation in a dose-dependent manner through genomic and non-genomic effects (Cutolo et al., 2021). GC binding to the glucocorticoid receptor (GR) changes the receptor structure and forms the GR-GC complex. The complex migrates into the cell and binds to the corticosteroid-responsive genes (GRE) of the DNA. The production of anti-inflammatory proteins is increased by this genomic pathway (Galon et al., 2002; Agarwal & Shanmugam, 2022). In genes without specific GRE sequences, the GC-GR complex works non-genomically by inhibiting the HAT enzyme responsible for the formation of pro-inflammatory proteins (Barnes, 2010). Glucocorticoid receptors (GR) are found in the tissues of almost all systems in the body (Androutsellis Theotokis et al., 2012).

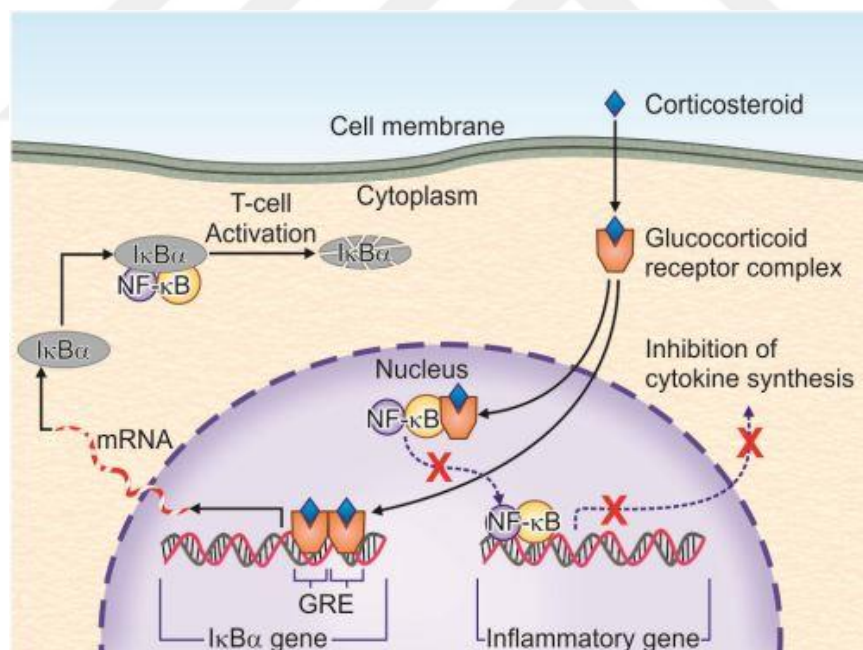


Figure 1.2 Glucocorticoids mechanism of action (Norris, 2005)

This is also the main reason for the side effects associated with GC treatment. Because this state of being recognized by almost every cell, whether related to inflammation or not, reduces the targeted functioning of the drug, and although it has immunomodulatory effects at low doses, it can cause suppression of the hypothalamic-pituitary-adrenal axis at high doses (Bullock et

al., 2018). However, there is also evidence that even low doses of GCs can have negative effects. Therefore, today they are used only in low doses and for short periods (Cutolo et al., 2021).

1.2.1.2. Non-Steroidal Antiinflammatory Drugs (NSAIDs)

Non-steroidal anti-inflammatory drugs (NSAIDs) are used in the treatment of RA because they are considered safer than GCs and are a fast-acting drug group (Quan et al., 2008). The main way NSAIDs work is by blocking an enzyme called cyclooxygenase (COX), which is responsible for the production of chemicals that cause inflammation, like prostaglandins, prostacyclin, and thromboxanes (Arfeen et al., 2024). This enzyme has two different isoforms called COX-1 and COX-2, and they have different biological functions. While COX-1 has functions that help maintain homeostasis, such as gastrointestinal mucosa lining, kidney function, and platelet aggregation, COX-2 is the isoform secreted in the event of inflammation (Faki & Er, 2020; Adamantidi et al., 2025).

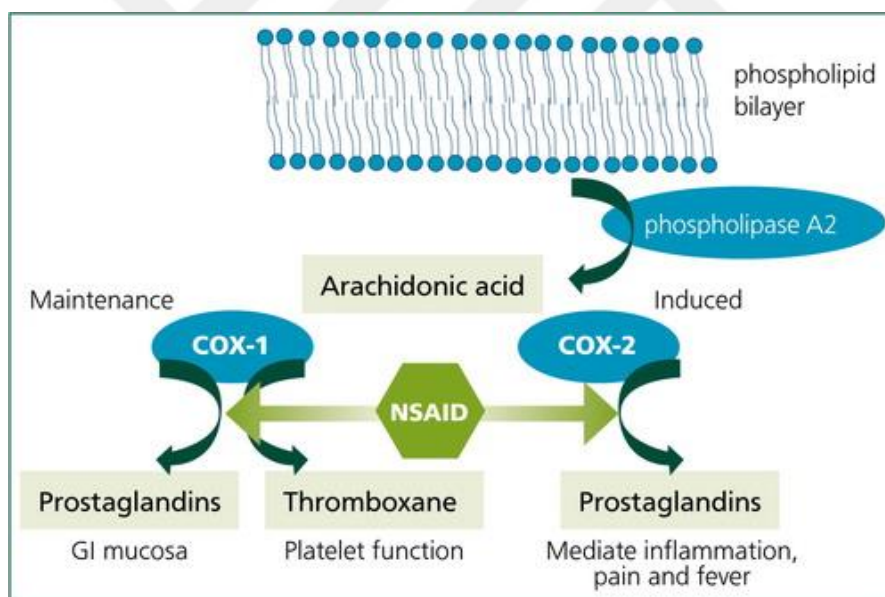


Figure 1.3 Targeted COX-2 inhibition expected from the NSAIDs group (Breen, 2017)

Although the main enzyme targeted by NSAIDs used for anti-inflammatory purposes is COX-2, most NSAIDs are not selective against COX enzymes and can inhibit both isoforms; this fact may be related to the side effects caused by NSAID drugs (Crofford et al., 2013; Gross et al., 2024; Khalil et al., 2024).

1.2.1.3. Disease-Modifying Antirheumatic Drugs (DMARDs)

Disease-modifying antirheumatic drugs (DMARDs) are now chosen as first-line pharmacological agents for people with RA owing to their capacity to diminish inflammation and prevent joint degradation (Bullock et al., 2018; Deshmukh, 2023). DMARDs can be classified differently according to the inflammation-related pathways they intervene in and their mechanisms of action.

Traditional DMARDs (csDMARDs) group, which is usually the first-choice group, methotrexate (MTX) reduces IL-6 and IL-8 levels and reduces joint destruction (Deshmukh, 2023; Feist & Nasonov, 2023). However, some clinical studies show that there is an increase in transaminases in patients treated with MTX, and this may lead to liver problems in the long term (Atallah et al., 2023).

Biologic DMARDs (bDMARDs) and targeted synthetic DMARDs (tsDMARDs), which are designed to have a more targeted effect, work by inhibiting proinflammatory cytokines such as tumor necrosis factor (TNF) and IL-6 and by restricting leukocyte activity (Riaz et al., 2025; Fazel et al., 2025).

Although currently considered a first-line treatment, these drugs are slow-acting and may take months to become effective, so they may need to be combined with other anti-inflammatory drugs initially (Smollen et al., 2003; Thakur et al., 2018). This may cause side effects that may vary from patient to patient, although their use together is considered safe.

1.3. Drug Delivery Systems

Active pharmaceutical ingredient, or API for short, is the main active ingredient that provides the therapeutic effect in a drug formulation. Depending on the therapeutic target of the drug, APIs may have different chemical compositions. The use of APIs in their free form is quite rare due to factors such as insufficient absorption, body temperature and pH, enzymes, etc. (Kumar et al., 2022). To provide the expected therapeutic effect from APIs, systems are needed that can protect them from the harsh conditions of the body and transport them to the targeted area.

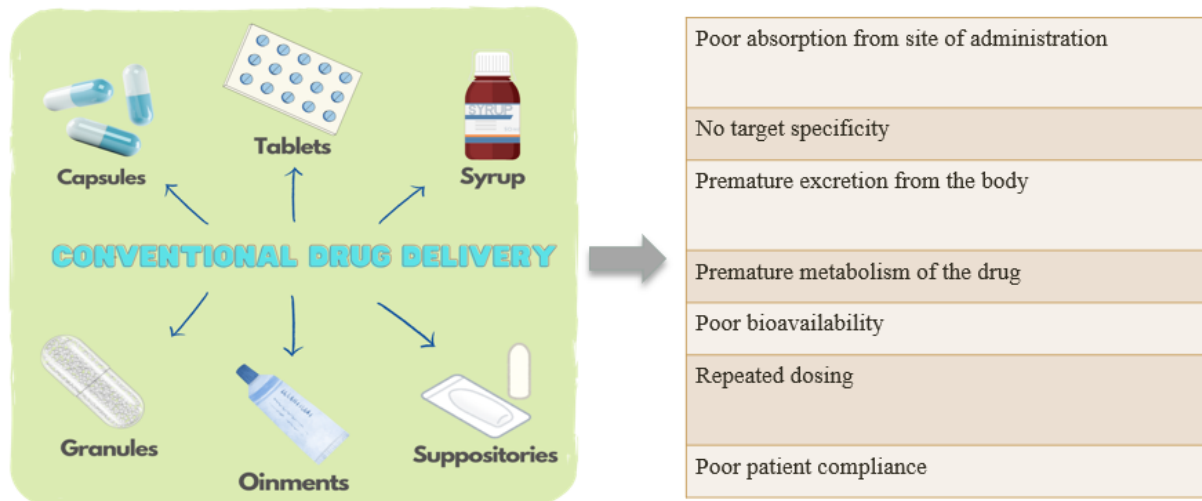


Figure 1.4 Conventional drug delivery systems and their disadvantages (Created by Canva)

Drug delivery systems (DDS) are devices or formulations designed to provide controlled and targeted transportation of APIs in the body. Thanks to DDSs, the drug is allowed to enter the body, be transported safely to the targeted area, and be released in a controlled manner (Ezike et al., 2023). The most well-known and used traditional DDS formulations, such as tablets, syrups, suppositories, etc., have disadvantages such as being non-target-specific, having low absorption and bioavailability, and being rapidly metabolized and excreted from the body. For this reason, they need to be used with repeated higher doses, which increases the possible side effects (Adepu & Ramakrishna, 2021).

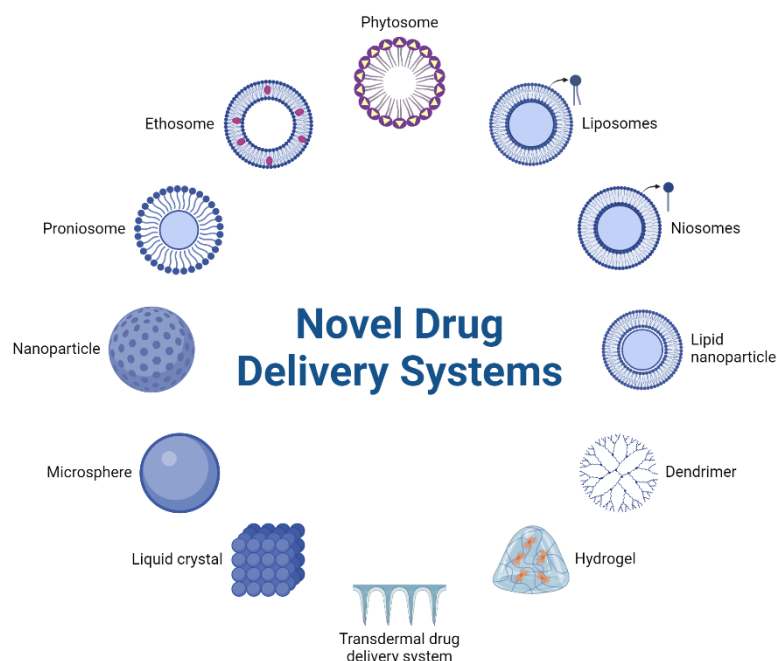


Figure 1.5 Novel Drug delivery systems (From Biorender templates)

On the other hand, novel DDS are systems designed to eliminate the problems associated with traditional methods and increase the therapeutic effect of drugs. These systems can be designed to be stimuli-responsive (e.g., pH- or temperature-sensitive) or to allow ligand-receptor-like interactions to provide controlled release to the target site (Ezike et al., 2023). This offers great flexibility in their design and route of administration, allowing them to be delivered via multiple routes such as oral, intravenous, nasal, ocular, transdermal, etc.

1.3.1. Transdermal Drug Delivery Systems

The skin is made up of three main layers. The first and outermost layer is the epidermis. In addition to preventing pathogens from entering the body, it protects the body from traumatic external factors such as impact, heat, UV, etc. (Rostkowska et al., 2023). The middle layer, the dermis, is 90% of the skin layer. Proteins such as collagen, elastin, etc., in its structure provide elasticity to the skin. It helps homeostasis by keeping body temperature constant with sweat production (Ye & De, 2016). In addition, nerve receptors and blood vessels start in this layer. In other words, this is the main drug release area that TDDS targets. The hypodermis layer, which is the lowest, protects muscle and bone tissue in the event of an impact with its fatty

structure, while connecting the skin to these tissues due to its connective tissue (Elalouf, 2021). Also, skin microcirculation is connected to the main circulation through the ongoing blood vessels by the hypodermis.

Transdermal drug delivery systems (TDDSs) are non-invasive methods that offer an alternative to other methods due to the ease of application and increased patient comfort, which are based on the principle that drugs are absorbed through the skin and passed into the bloodstream, reducing the risk of problems that may occur with traditional methods (Panda et al., 2025).

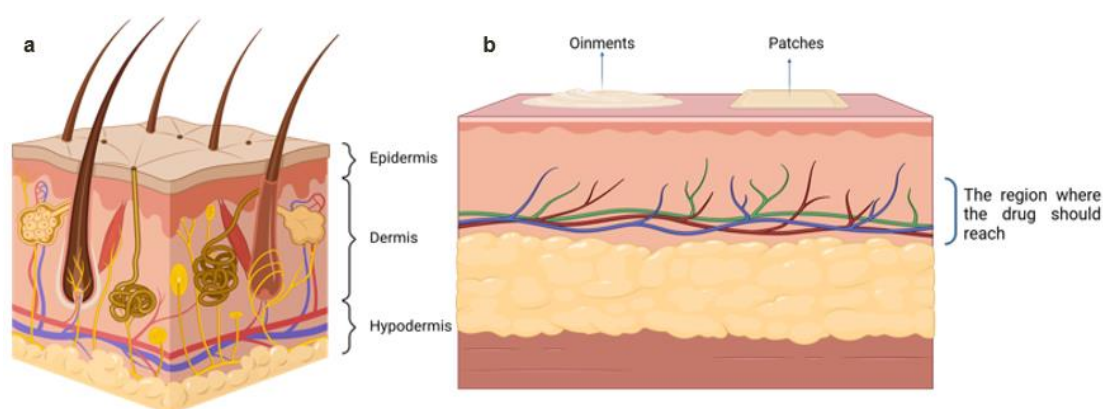


Figure 1.6 Skin anatomy **(a)** Skin Layers **(b)** Common traditional TDDS (Created by Biorender)

Nevertheless, conventional TDDSs possess significant drawbacks. The most important of these is the *stratum corneum* (SC), which is the outermost layer of the epidermis, composed of dead keratinocytes and an extracellular lipid matrix, and acts as a natural protective barrier (Yang et al., 2024; Hernandez-Serrano et al., 2024). SC is not permeable to topical formulations and prevents effective amounts of the drug from reaching the blood vessels. To address the limitations of conventional TDDSs, researchers have created innovative methods, including microneedles, which offer a minimally invasive yet highly efficient method for facilitating drug penetration through the SC.

1.4. Microneedles (MNs)

Microneedles are systems that work on the principle of penetrating the SC and introducing the drug into the bloodstream by creating microchannels between the external environment and the skin capillaries (Aldawood et al., 2021). The microchannels formed are larger than the size of drug molecules that would normally not pass through the SC, and thus the drug can be distributed evenly to the targeted area (Kumar et al., 2025). Unlike hypodermic needles, which

are frequently used today, microneedles do not contact the nerve endings, thus providing a more comfortable application. In addition, the microchannels formed in the skin are too small to cause microbial contamination (Oliveira et al., 2024; Kumar et al., 2025). MN types are divided into five main groups, namely solid, coated, dissolvable, hollow, and hydrogel-based, which can be designed according to the application site purpose (Nagarkar et al., 2020).

Although the first thing that comes to mind due to the design of MN is to overcome the SC barrier for transdermal drug delivery, their use has been tested in many different and new application areas. In addition to their use in bone (Shan et al., 2024), cardiac (He et al., 2025), ocular (Jiang et al., 2025), tissue regeneration, wound healing (Hou et al., 2025), and skin cancer (Wang et al., 2025), there are also quite recent studies on their use for depression (Patil et al., 2025). In addition, MNs are also used in vaccine applications (He et al., 2025), cosmetics (Don et al., 2025), diagnosis, and biosensing (Ben Halima et al., 2025; Dervisevic et al., 2025).

1.4.1. Microneedle Materials and Production

The manufacturing methods and materials used in the production of MNs are largely interdependent. While the material selection affects the applicable manufacturing methods, the selected manufacturing method also determines the types of materials that can be used effectively (Rad et al., 2021). Since the choice of material and fabrication method will affect the application area, drug release rate and mechanism, biocompatibility, and mechanical durability of hydrogel MNs, the selection of the appropriate method and material combination is of critical importance (Dave et al., 2024).

1.4.1.1. Microneedle Materials

Biocompatible, biodegradable, and flexible polymers are widely employed in producing functional microneedles (Kulkarni et al., 2023). These polymers may be either synthetic or natural. Polyvinylpyrrolidone (PVP), Polylactic acid (PLA), polyethylene glycol (PEG), and Poly Vinyl Alcohol (PVA) are synthetic polymers commonly used in the production process of microneedles (Koyani, 2020; Aroche et al., 2024).

PVP-based microneedles suitable for 3D printing that can adapt to complex tissues were produced, and pH-sensitive poly(lactic-co-glycolic acid) (PGLA) microspheres loaded onto microneedles made of PVP enabled controlled and stimulus-responsive release of different drugs (Ke et al., 2012). PVA, another frequently used polymer, was used for the transport of drugs such as MTX and DOX (Tekko et al., 2017; Nguyen et al., 2018). A recent study

examined the impact of PVA concentration on the physical characteristics and penetrating capability of the MN, revealing that increased concentrations resulted in enhanced mechanical stability and reduced dissolution rates (Saepang et al., 2025). Furthermore, PLA microneedles were observed to stimulate immune responses comparable to traditional vaccinations in non-human primates (DeMuth et al., 2013).

Although synthetic polymers provide advantages in terms of mechanical strength, the possibility of a high immune response in the body has increased the interest in biocompatible and biodegradable natural polymers in production (Pires et al., 2023; Satchanska et al., 2024). Natural polymers, including gelatin (Fan et al., 2025), chitosan (Liu et al., 2025), alginate (Ershaid et al., 2024), and hyaluronic acid (Lu et al., 2025), are essential for the fabrication of safe and effective microneedles for diverse biomedical applications.

Chitosan-based MN, which has antibacterial activity against *S. aureus* and *E. coli* for use in open wounds, increased collagen deposition and new blood vessel formation (Chi et al., 2020). In addition, glucose-responsive MN composed of chitosan and alginate could release high doses of insulin in a controlled manner (Wang et al., 2025). Additionally, MNs made of hyaluronic acid (HA) and sodium alginate (SA) for use in orthodontic treatments (Shan et al., 2024), dextran-based MNs for the treatment of vitiligo (Liang et al., 2023), and herbal extract-loaded hyaluronic acid-based MNs for hair regeneration (Hong et al., 2025) can be examples of natural polymer-based MNs used in different biomedical fields.

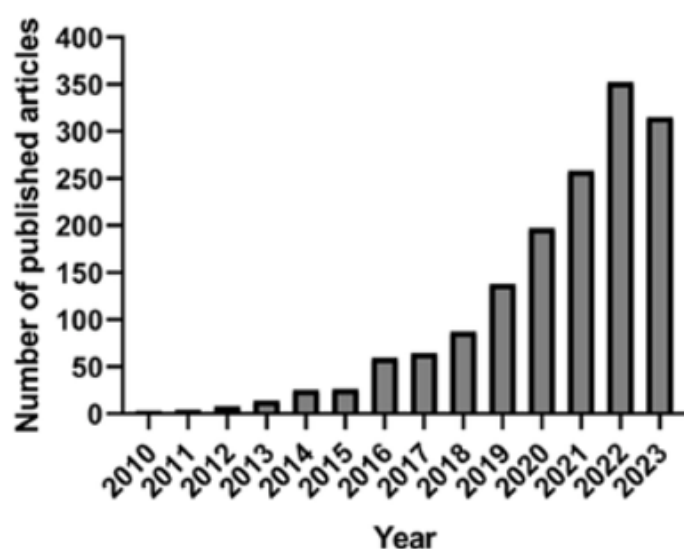


Figure 1.7 The number of published articles about GelMA (Zhang et al., 2023)

Gelatin Methacryloyl (GelMA) is a modified form of gelatin by adding methacryloyl groups. With this modification, GelMA is added properties such as adjustable mechanical strength and

stability, which are generally specific to synthetic polymers, in addition to the biocompatibility and biodegradability of gelatin (Kumar et al., 2020; Ghosh et al., 2023; Salisbury et al., 2024). Moreover, GelMA can create cross-linked hydrogels upon exposure to UV or visible light in the presence of a photoinitiator, attributable to its methacryloyl groups (Sharifi et al., 2021; Salisbury et al., 2024). Due to its characteristics of both natural and synthetic polymers, GelMA is regarded as the gold standard in hydrogel MN manufacturing (Boyd-Moss et al., 2021). The number of studies on GelMA has also increased, particularly notably (Zhan et al., 2023).

1.4.1.2. Production Methods

MNs have a wide range of production methods, including solvent casting, microelectromechanical systems, and photolithography (Tucak et al., 2020). However, despite the high cost and time required to apply traditional methods, the trend towards finding more effective methods has increased due to problems such as lack of penetration, insufficient drug loading and release capacity of MNs produced by traditional methods (Rad et al., 2021b; Erkus et al., 2023).

3D printing, also known as additive manufacturing (AM), is a widely used method to produce complex structures for specific applications. Thanks to its ability to be integrated with different artificial intelligence and software tools, 3D bioprinting has become increasingly popular as it can be used to produce complex objects directly from digital files such as CAD drawings (Zhao et al., 2025). It offers superior flexibility in the design of MN arrays, allowing for the creation of application-specific designs, rapid production, and increased mechanical durability through layer-by-layer deposition (Tucak et al., 2020; Biswas et al., 2024).

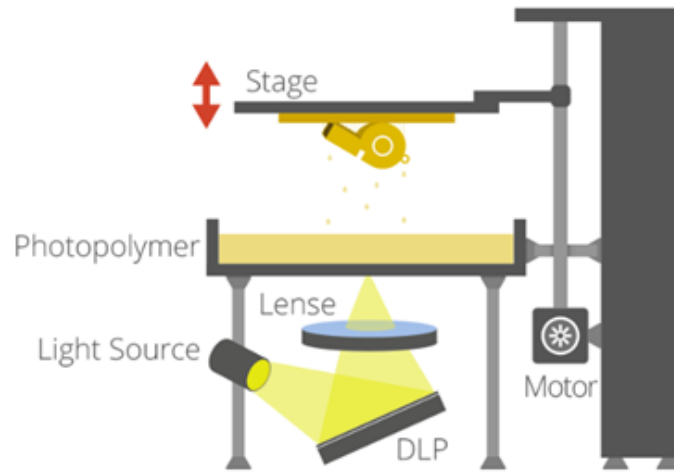


Figure 1.8 DLP technique working mechanism (*Digital Light Processing | 3DPrinting.Lighting*, 2019)

Among different 3D printing approaches, Digital Light Processing (DLP) stands out as a fast and effective method based on photopolymerization. DLP is based on the principle of layer-by-layer solidification of photocurable polymer as a result of exposure to UV or white light (Mu et al., 2017; Cheng et al., 2024).

1.4.2. Hydrogel Microneedles

Hydrogels are cross-linked hydrophilic polymer chains with 3D network structures. With their capacity to retain large amounts of water, swelling properties, and elastic structures, studies are being carried out on many different types of hydrogels, from drug delivery systems to the food sector (Thang et al., 2023). Compared to other types of MN, hydrogel MNs have various advantages according to their application areas (Mohite et al., 2024).

The first advantage is the elasticity provided by their hydrogel structure. Compared to materials such as polymers, metals, etc., used in traditional MN production, this feature reduces the possibility of allergy, irritation, etc., in the application area because it creates minimal damage to the skin (Aldawood et al., 2021). At the same time, thanks to this elasticity, they can also take the form of the tissue to which they will be applied and provide ease of application (Mohite et al., 2024). Also, the microchannels created by regular solid MNs, which usually close up quickly and make it hard for the drug to get through, can stay open longer with hydrogel MNs due to swelling, allowing enough drug to be released (Oliveira et al., 2024).

Another advantage of hydrogel MNs is the dynamism in drug release mechanisms. In traditional MNs, passive diffusion or dissolution of the polymer is required for drug release. In contrast, the swelling ability of hydrogel MNs, which is a result of their hydrogel composition, plays a fundamental role in the drug release mechanisms. Once you apply the dried drug-loaded hydrogel MN to the relevant skin area, it absorbs the skin's interstitial fluid, causing it to swell. The MN, which reaches the maximum swelling point, then begins to release the absorbed fluid, and the drug is released to the target area in a controlled manner (Chang et al., 2017; Shrinky et al., 2023).

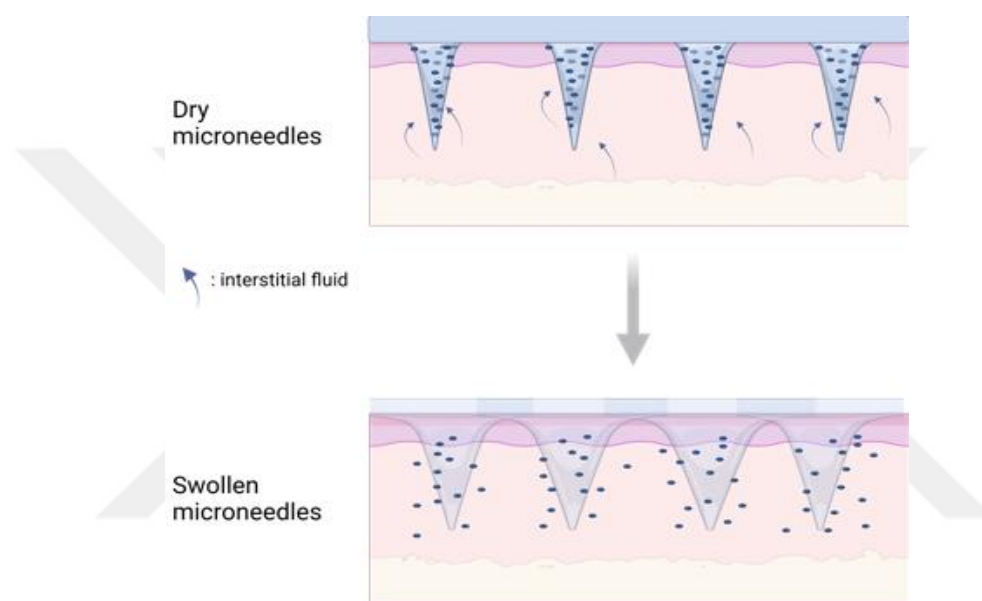


Figure 1.9 Drug release mechanism of hydrogel MN (Created by Biorender)

In addition to these advantages, when hydrogel MNs are made of polymers that are sensitive to pH, temperature, or external stimuli, more controlled release can be achieved (Omidian et al., 2025). As a result of the inclusion of microspheres consisting of NIR-responsive black phosphorus (BP) and poly(N-isopropylacrylamide) (PNIPAM) into the MN system, the system, whose temperature increased in the presence of NIR due to BP, swelled by absorbing liquid and provided controlled insulin release thanks to PNIPAM (Fan et al., 2021). In another study, Li et al. (2022) treated hypoglycemia in diabetic mice with insulin release by hydrogel MN composed of gelatin and thermosensitive PNIPAM.

Furthermore, HMNs have been used in areas such as early colorectal cancer diagnosis (Park et al., 2023) and breast cancer imaging (Huang et al., 2023) and also used for the treatment of melanoma (Zhu et al., 2025) and pancreatic cancer (Fu et al., 2021). Additionally, hydrogel MNs have been tested in diseases such as vitiligo (Liang et al., 2023) and alopecia areata

(Younis et al., 2024), as well as in inflammatory skin diseases such as psoriasis (Dawud et al., 2023).

1.5. Oleuropein (OLE)

Recently, phytochemicals have attracted the interest of biomedical researchers related to their potential benefits for human health and have been utilized in studies on tissue engineering and drug delivery systems in this field (Yadav & Puranik, 2024). Polyphenols, common phytochemicals in plants, are an essential subject in modern research and are found primarily in fruits, vegetables, tea, and wine (Prabhu et al., 2021; Bolat et al., 2024). Based on the 8000 structural varieties that differ according to the phenolic rings in their configurations (Yahfoufi et al., 2018; Aatif M., 2023), they may exhibit varying absorption rates in the body.

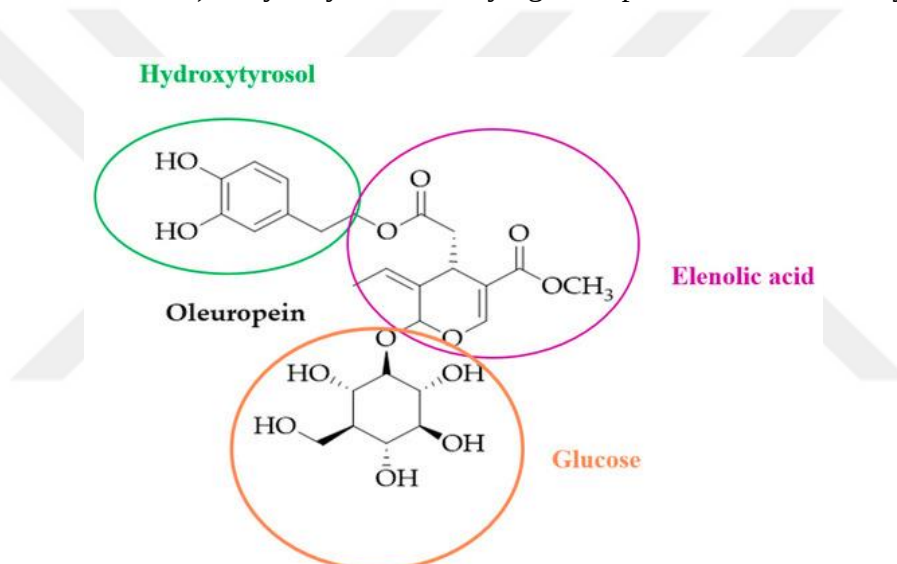


Figure 1.10 Chemical structure of oleuropein (Nediani et al., 2019)

Oleuropein (OLE), a glycoside with hydroxytyrosol as its active component, is the key phenolic chemical present in olive oil and leaves (Panou & Karabagias, 2025). They possess cardiovascular protective (Banaei et al., 2025), anticancer (Ramírez-Expósito et al., 2025), and neuroprotective effects (Riquelme & Pando, 2025). Moreover, they offer anti-inflammatory and antioxidant characteristics by neutralizing reactive oxygen species (ROS) (Hassen et al., 2014) and blocking transcription factors and pathways linked to proinflammatory genes (Li et al., 2025).

It has been reported that OLE may be an effective treatment for RA with these properties and may even show anti-inflammatory effects similar to drugs in the NSAID group (ibuprofen, etc.) (Beauchamp et al., 2005). Yousefi et al. (2022) revealed that OLE induces the production of

IL-10 and TGF- β in RA patients. An additional study by the same team revealed that OLE diminished the overexpression of miRNA-146aEk associated with RA (Mahi et al., 2023). Furthermore, OLE modulated inflammation in the human synovial fibroblast cell line SW982 (Castejón et al., 2017) and in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages (Ryu et al., 2015). Additionally, researchers reported polyphenols for their ability to control substances that disrupt homeostasis in the synovial joint and facilitate bone erosion (Christman & Gu, 2020; Rahaman et al., 2025). For example, OLE inhibits the progression of osteoarthritis lesions in cartilage *in vivo* (Horcajada et al., 2014). However, we need delivery systems that are biocompatible and biodegradable to improve OLEs' bioactivity and absorption compared to its free form.

1.6. Levan

Levan is a naturally formed fructose polysaccharide that can be produced by bacteria, archaea, fungi, and some plant species (González-Garcinuño et al., 2017; Kirtel et al., 2018). It comprises fructan chains linked by β -2,6 glycosidic linkages, exhibiting varying degrees of branching at the β -2,1 position (Toksoy Öner et al., 2016; Domżał-Kędzia et al., 2023). Levan, a neutral and amphiphilic polymer, is recognized for its high water retention capacity, self-assembly properties, strong adhesion, and biocompatibility (Gojgić-Cvijović et al., 2018; Toksoy Öner, 2023). Despite the limited research on levan relative to other fructans, interest in levan and the number of studies regarding it are progressively rising across many areas, including tissue engineering, drug delivery, cosmetics, and food, due to its structural and functional attributes (Toksoy Öner, 2023).

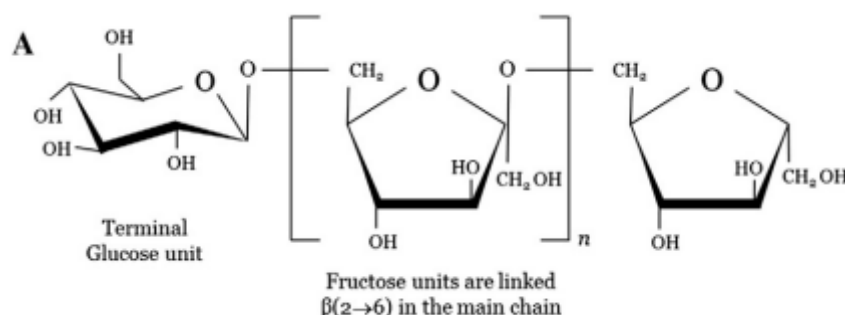


Figure 1.11 Chemical structure of linear levan (Toksoy Oner et al., 2016)

Halomonas levan (HL), produced by the halophilic bacteria *Halomonas smyrnensis*, has been reported as a bioactive polymer with properties such as anticancer, antioxidant, anti-inflammatory, cryoprotectant, and cell proliferating activity by our research group in many

studies (Toksoy Oner et al., 2016; Demirci et al., 2020; Cinan et al., 2021; Selvi et al., 2021; Peled et al., 2023; Tornacı et al., 2024; Akkulah et al., 2025).

Levan is a polymer suitable for chemical derivatization techniques, including sulfation, phosphonation, and oxidation (Kirtel and Öner, 2021). Reports indicate that its modified variants demonstrate distinct features, including anticoagulant action (Erginer et al., 2016) and anticarcinogenic activity (Sarilmiser and Toksoy Öner, 2014). It has also been reported that LevMA (Levan Methacrylate), synthesized by adding methacryloyl groups to the Levan polymer, may be a suitable product for 3D bioprinting applications (Berg et al., 2018). A fairly recent study has demonstrated the potential of 3D-printed LevMA scaffolds for tissue repair and regeneration applications by creating a suitable microenvironment for bone regeneration (Waidi et al., 2025b).

1.7. Aim

Oleuropein-loaded *Halomonas* levan MNs were investigated in this thesis for their potential to prevent joint inflammation in RA. The study hypothesized that oleuropein-loaded, levan-based MNs would penetrate the skin effectively and enable a controlled release of oleuropein at non-toxic concentrations without an initial burst release. The MNs we designed for application to inflamed tissues or more sensitive tissues prone to inflammation were designed to have needles small enough for penetration but not to reach the nerves.

In the scope of this thesis, the first LevMA and LevMA-GelMA composite needles were printed using the DLP 3D printing technique. For characterization, swelling, gel fraction, and rheology tests of full LevMA, LevMA-GelMA composites, and full GelMA MNs were examined. In addition, MNs were analyzed morphologically. Cell viability assays with PCS cells were conducted using empty MNs to evaluate biomaterials' cytocompatibility. In characterization and cytotoxicity studies, using full GelMA MNs, which are currently considered the gold standard in the literature, an objective analysis of full LevMA and composite LevMAs was performed to reveal their potential advantages and disadvantages. Release test was performed to assess the oleuropein release profile.

Compared to the MNs and polyphenol-loaded MNs used for RA, which have appeared very recently in the literature, as discussed in Section 1.1, the incorporation of oleuropein into HL MNs introduces a novel approach to addressing this clinical challenge. This study revealed that

these MNs demonstrated appropriate swelling behavior, controlled oleuropein release, and biocompatibility, offering new insights into potential therapeutic strategies for RA.

2. MATERIALS AND METHODS

2.1. Materials

All chemicals and materials used during the experiments, along with their suppliers, are given in Table 2.1.

Table 2.1 Chemicals and Materials

Chemical or Material	Supplier
Chemicals that were used in the medium for levan production	MERCK (Germany) and Sigma (USA)
İzmir Çamaltı Salt	Estuz company (Turkey)
Methacrylic anhydride	Sigma Aldrich (USA)
Oleuropein	Sankara Brain and Biotechnology Research Center
PBS Tablets	MP Biomedicals
Trypsin/EDTA	PAN Biotech (Germany) Cat No: P10-019100
Peniciline-Streptomycin	PAN Biotech (Germany)
Trypan Blue	Sigma-Aldrich (USA)
DMEM	PAN Biotech (Germany) Cat No: P04-03590
FBS	PAN Biotech (Germany)
Folin Ciocalteu Phenol Reagent	Sigma Aldrich (USA)
DPPH	Apollo Scientific (UK)
LAP	MERCK (Germany)
CCK-8 Kit	Abbkine (China)

2.2. Instruments

All equipment used during the experiments, along with their supplier company, are given in Table 2.2.

Table 2.2 List of Equipments

Equipment	Brand
Autoclaves	• Nüve OT32V S (Turkey) • Biolab Systec 3870 (Systec, Germany)
Magnetic stirrers	• Heidolph Mr3003s (Germany) • Heidolph MR3001 K (Germany)
Refrigerators -	• +4 °C Arçelik (Turkey) • +4 °C Beko (Turkey)
Freezer	• 20 °C Arçelik (Turkey) • -20 °C Beko (Turkey) • -80 °C Thermo Fischer Scientific (USA)
Shaker incubators	• Certomat BS1(Germany) • New Brunswick Scientific Excella E24 (Germany)
Drying Oven	• Binder Ed115 (Germany)
Centrifuges	• Hermle/Z36HK (Germany) • Nüve/NF800 (Turkey) • Nüve/NF 4000R (Turkey)
Biosafety cabinets	• Nüve MN090 (Turkey) • Thermo Scientific MSC Advantage (USA)
Precise vacuum	• Visd ThermoStable OV-30 (South Korea)
Lyophilizer	• Martin Christ Alpha 1-2 LDplus (Germany)
Spectrophotometer	• 5100 UV-VIS Spectrophotometer (Perkin Elmer, USA)
Sonicator	• Bandelin Sonopuls HD 4200 (Germany)

NMR spectrometer	Ascend 500 MHz, Avance NEO (Germany)
Fourier Transform Infrared (FTIR) Spectrometer	FT/IR-4700, Jasco (Japan)
Rheometer	Anton Paar Rheometer (Germany)
DLP 3D printer	ASIGA MAX X, ASIGA (Australia)
Multimode Microplate Reader and Imager Cytation 3	BioTek (USA)
Microscope	SOIF XDS-1B
Grinder	Kiwi KSPG-4820 (Turkey)
pH meter	Mettler Toledo MP 220 (Germany)
Automatic micropipette sets	4500080 Finnpipette (Finland) 4500110 Finnpipette (Finland)

2.3. Methods

2.3.1. Production and Purification of Levan

The production of levan polymer was carried out using the enzymatic production procedure from Kirtel et al. (2018). First, LB media prepared with sodium chloride (NaCl), casein peptone, and yeast extract was prepared and sterilized. Then, *E. coli* BL21DE3 containing the levansucrase gene taken from *H. smyrnensis* was pre-cultured in 10 ml LB medium supplemented with kanamycin at 37°C and 180 rpm. At the end of approximately 3 hours, the OD600 nm value of the preculture was measured, and when it reached the range of 0.6-0.8, 1% inoculation from the preculture was made into 200 ml of LB media. When the culture reached the target turbidity and the optimum range (0.6-0.8) after the OD600 measurement, IPTG was added to the medium for gene expression and incubated overnight at 26°C. On the following day, the culture was centrifuged at 4°C at 8000 rcf for 20 minutes, and the pellet was collected. The cell pellets were solubilized in 6.6 pH phosphate (cell lysis) buffer containing KH₂PO₄, K₂HPO₄, and NaCl. The mixture was placed on ice to prevent enzyme degradation and sonicated at 30% power for 15 min. After sonication, the mixture was centrifuged at 8000 rcf for 20 min, and the pellet was discarded. Meanwhile, 10 CV equilibration buffer (7.4 pH) was used to prepare immobilized metal affinity chromatography (IMAC). The supernatant obtained

after centrifugation was mixed with equilibration buffer at a ratio of 1:1 and passed through the column. Then, 10 CV wash buffer (7.4 pH) was passed through the column, and non-targeted proteins were eluted. Afterwards, 10 CV elution buffer (7.4 pH) was passed through the column to collect the main targeted levan sucrase enzyme. The necessary substrate solution was prepared simultaneously in the IMAC step. The substrate solution was prepared with KH_2PO_4 , K_2HPO_4 , sucrose, and salt taken from the Çamaltı salt pan. The prepared 2 liters of substrate was filtered, and 0.5 M EDTA was added to the solution, and the solution pH was adjusted to 5.9. The pure enzyme obtained was mixed with the substrate and incubated at 15°C overnight. The next day, 2:3 ethanol was added to the mixture for precipitation and taken to 20°C to wait overnight. The following day, the ethanol in the top phase was extracted using a peristaltic pump, and the precipitate was obtained via centrifugation at 4°C at 8000 rcf for 20 minutes. The collected precipitate was dissolved in distilled water and transferred to dialysis tubes to remove salt impurities by changing the water twice a day for 4-5 days. After dialysis, a DEAE Sepharose weak ion exchange column was used to remove protein impurities that may exist in the levan solution. Finally, levan was dried at 50°C, crushed, and stored at -20°C for later use.

2.3.2. Synthesis of Hydrolyzed Levan

The goal was to break down the levan polymer into smaller pieces to be ready for the methacrylation process in the next steps. For the hydrolyzation of levan, the method found in the literature was applied with minor modifications (Avsar et al., 2018). First, 10 g of levan was dissolved in 171.5 ml of distilled water. Then, 3.5 ml of acetic acid was added to the solution and left for hydrolysis for 10 minutes. Then, the solution was placed in a microwave at 60% power, and every 30 seconds, 100 μL of the sample was taken and diluted with 900 ml of distilled water. The OD of the collected samples was measured with a UV spectrophotometer until the reading of $A_{600} = 0.1-0.06$. After the 30-second microwave treatment, the solution was cooled in an ice bath until the sampling, measurement, and microwave re-application process. Ethanol was added to the hydrolyzed levan in an amount three times the volume of the solution and left to settle overnight at -20°C. The next day, the precipitated hydrolyzed levan was collected by centrifugation at 8000 rcf for 30 minutes. It was dried in a 45°C vacuum oven for 24 hours. The meringue-like hydrolyzed levan obtained after drying was then crushed and stored at 20°C to be used.

2.3.3. Synthesis of LevMA

LevMA was synthesized by making some modifications to a previously described protocol (Berg et al., 2018). To prepare the borate buffer, 1.24 g of boric acid was dissolved in 100 ml of distilled water. Then, the pH was adjusted to 9 with NaOH. 2 g of hydrolyzed levan was mixed into 100 ml of borate buffer, and the pH of the mixture was changed to 8.5 using HCl, which activates the methacrylate groups. Since Methacrylic anhydride (MAA) is a light-sensitive chemical, the shot bottle containing the solution was foiled, and its contact with light was prevented. Then, the shot bottle was placed in ice, and the solution was cooled to approximately 13 °C. 30 mg of hydroquinone was added to the cooled solution in the fume hood as a polymerization inhibitor and stirred with a magnetic stirrer for approximately 30 min. 9 ml of MAA was added dropwise for 3 hours, 2 ml at 30-min intervals. The solution was then stirred at 13 °C for 24 h and then at room temperature for another 2 h. The reaction mixture was left to settle with 400 ml of acetone for 3–4 days. The acetone in the upper phase was discarded, and 50 ml of distilled water was added and mixed. After the precipitate was dissolved, LevMA was transferred to dialysis tubes and dialyzed against distilled water by changing the water twice a day for 3-4 days. After dialysis, LevMA was added to the petri dishes, not exceeding half. The petri dishes were kept at -20 °C and then -80 °C for at least one night. Finally, LevMA was taken to the lyophilizer and dried.

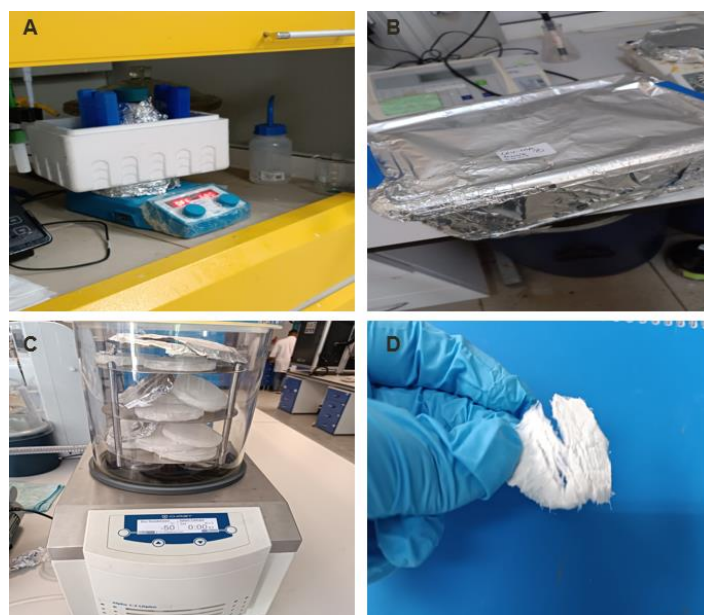


Figure 2.1 LevMA production process (A) Methacrylation of hydrolyzed Levan (B) Dialysis (C) Drying with a lyophilizer (D) LevMA as a final product

2.3.4. Synthesis of GelMA

10 g of type A porcine skin gelatin was dissolved in 100 ml of Dulbecco's phosphate-buffered saline (DPBS) to make 10% (w/v). The solution was stirred at 60°C for approximately 1 h until the gelatin dissolved. The pH of the solution was adjusted to 9 with NaOH, where the MA groups are more active. 2 ml of MAA was then added dropwise at a rate of 0.5 mL/min, making up to 8 ml at half-hour intervals. After each half-hour, the pH was adjusted to 9 before further additions. The mixture was allowed to react for 2 h at 50°C under controlled stirring conditions. To stop the reaction, 100 ml of pre-warmed (50°C) DPBS was added, and stirring was continued for another 10 min. The resulting mixture was transferred to molecular dialysis membranes and dialyzed against distilled water with water changes twice a day for 7 days. After dialysis, GelMA was added to the petri dishes, not exceeding half. The petri dishes were kept at -20°C and then at -80°C for at least one night. Finally, GelMA was taken to the lyophilizer and dried. The dried GelMA was stored at -20 °C for later use (Hutson et al., 2011).

2.3.5. 3D Printing of MNs

MN solutions were prepared in the amounts specified in the **Table 2.3** in 1 ml PBS, totalling 100 mg of sample. The solution was stirred at 37°C until LevMA/GelMA was completely dissolved. After dissolution, the beaker was covered with foil to prevent light exposure and 5 mg of Lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) was added in the dark and stirred for approximately 20 min until completely dissolved.

Table 2.3 Different LevMA/GelMA concentrations of MN solutions.

Name	LevMA (mg)	GelMA (mg)	LAP (mg)
L1G0	100	0	5
L3G1	75	25	5
L1G1	50	50	5
L1G3	25	75	5
L0G1	0	100	5

Microneedle designs were created using SolidWorks software, featuring a 7×7 array of 49 needles. Each microneedle was designed with a height of 1500 μm and a base diameter of 800 μm . The completed design was saved in ".stl" format and subsequently converted into a G-Code

file compatible with a 3D DLP printer. This G-Code file was imported into the Asiga Composer software and transmitted to the printer. The prepared precursor solutions were then loaded into the printer's reservoir, and the microneedles were fabricated using an Asiga Max X printer (Figure 2.2).

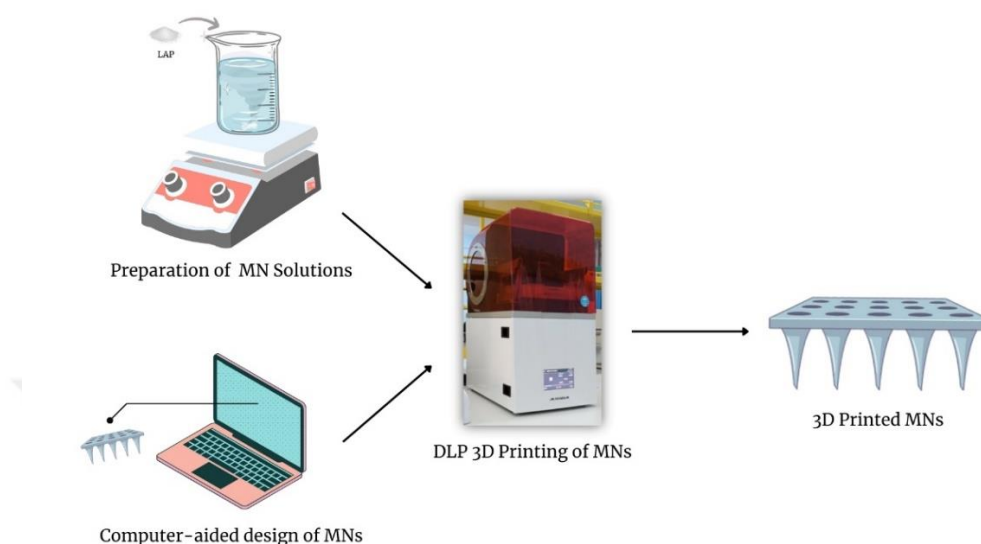


Figure 2.2 3D Printing of MNs with Digital Light Processing (Created by Canva)

2.3.6. Characterization of MNs

2.3.6.1. Fourier Transform Infrared (FTIR) spectroscopy

FTIR spectroscopy (Jasco FT/IR-4700, Tokyo, Japan) was employed to examine the chemical characteristics of LevMA and the synthesized MNs (L1G0, L3G1, L1G1, L1G3, and L0G1). FTIR spectra were acquired at 25°C in the mid-infrared band (4000 to 400 cm^{-1}) with a resolution of 4.

2.3.6.2. Nuclear Magnetic Resonance (NMR)

The structure of the produced LevMA was further verified using ^1H NMR and ^{13}C NMR spectroscopy (Ascend 500 MHz, Avance NEO). The sample of LevMA was dissolved in D_2O .

2.3.6.3. Rheology of MNs

Rheological analysis of microneedles (L1G0, L3G1, L1G1, L1G3, and L0G1) was carried out at ambient temperature to evaluate the viscoelastic properties of the chemically cross-linked polymer network. Measurements were carried out using an Anton Paar RheoCompass rheometer equipped with a parallel plate arrangement having a 15 mm diameter disk. The evaluation was carried out in three stages as time, frequency, and strain amplitude sweeps. In the first stage, the temporal variation of storage modulus (G') and loss modulus (G'') were evaluated by keeping frequency and strain amplitude constant at 1% and 0.01%, respectively. In the second stage, a frequency sweep was performed in the range of 0.1-100 Hz at a fixed strain amplitude of 0.1%. In the third stage, a strain amplitude sweep was performed, including gamma ranging from 0.01% to 100% at a fixed frequency of 1 Hz.

2.3.6.4. Scanning Electron Microscopy (SEM)

Morphological characterization of the produced MNs was performed by scanning electron microscopy (SEM, EVO MA 10, ZEISS). First, SEM samples were stored at 25 °C for 48 h and then coated with gold using the Quorum SC7620 Mini Sputter Coater. Voltages were determined at 10 kV while scanning at magnifications of 60 to 125X with working distances ranging from 13.5 to 35 mm..

2.3.7. Swelling Capacity of Microneedles

The swelling properties of MNs were tested according to a previously described protocol (Saglam et al., 2023). To evaluate the swelling capacity of the LevMA MNs and GelMA MNs, dried MN samples were weighed and immersed in 1× PBS (pH 7.4) at 37 °C for 72 h. At various times, the swelled samples were withdrawn from the buffer and rapidly wiped with filter paper; the weights were recorded and reintroduced to the buffer. To ensure statistical reliability, this procedure was conducted with three samples tested per group. This procedure was repeated until the swelling reached a state of equilibrium. The swelling degree (g/g) of each MN was estimated according to **Eq. (1)**:

$$\text{Swelling degree} \left(\frac{g}{g} \right) = \frac{W_s}{W_d} \quad (1)$$

W_s: weight of swollen MN at specified time intervals

W_d: weight of dry MN

2.3.8. Gel Fraction

The efficiency of network formation in MNs was tested with the previously applied gel fractionation protocol, with slight differences that allowed us to measure the crosslinking efficiency (Sabri et al., 2022). For this, dried MNs were weighed to obtain the initial dry weight (m_0). Then, MNs immersed in 5 ml of distilled water were left at room temperature until they reached equilibrium swelling. The swollen MNs were placed in an oven at 37 °C for 24 h. The weight of the MNs removed from the oven (m_d) was measured. The percentage of gel fraction of the samples was then calculated using **Eq. (2)**:

$$\text{Gel Fraction}\% = \frac{m_d}{m_0} \times 100 \quad (2)$$

m_0 : Initial dry weight of MNs

m_d : Dried weight of MNs

2.3.9. *In vitro* Oleuropein Release

The L1G0 MN was selected to test the drug release dynamics due to its existing swelling, flexible rheological properties, and suitable morphology. To prepare OLE-loaded MNs, 100 mg LevMA and 1 mg OLE were dissolved in 1 ml PBS at 37°C. Then, LAP was added to the solution and mixed until a homogeneous mixture was obtained. Then, the solution was added to the vat of the DLP device for 3D printing. Finally, OLE-loaded MNs were dried at room temperature for one day (Erkuş et al., 2023).

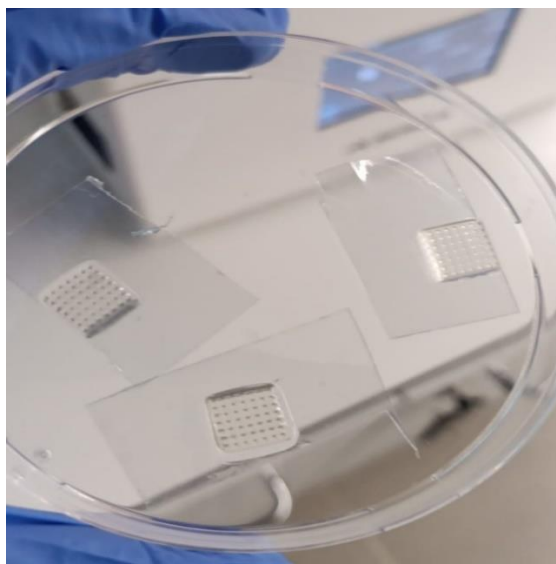


Figure 2.3 OLE-loaded L1G0 MNs

OLE-loaded MNs were taken into 2 ml of PBS solution and shaken at 37°C at 50 rpm (Dawud & Ammar, 2023; Erkuş et al., 2023). At certain time intervals (5 min, 10 min, 15 min, 30 min, 45 min, 1 h, 1.5 h, 2 h, 2.5 h, 3 h, 4 h, 5 h, 6 h, 24 h), 500 µL samples were taken, and same amount of PBS was added. The samples were kept at 4°C until the next stage.

The Folin-Ciocalteu (FC) test was applied to analyze the release profile of oleuropein from L1G0 MN and to learn the total phenolic content. Samples collected at certain time intervals were collected in 400 µl and 3 ml of distilled water, and 250 µl FC were added. Samples were protected from darkness for 8 min, and then 750 µl of 7.5% sodium carbonate solution was added. Distilled water was included in order to reach a total volume of 5 ml. Solutions were manually mixed and kept in a dark place at the ambient temperature for 2 hours. Oleuropein solutions ranging from 1 mg/ml (as much as the amount of oleuropein in the drug-loaded needle) to 0.005 were prepared by serial dilution in PBS to create the oleuropein standard curve. The FC test steps mentioned above were applied to these samples. At the end of 2 hours, the absorbance of the standards and release samples at 765 nm was measured with a UV spectrophotometer (5100 UV-VIS Spectrophotometer) (Topuz & Bayram, 2021; Luaces et al., 2021).

2.3.10. Cell Viability

Biocompatibility of the microneedles was assessed on skin fibroblast cell line (PCS-201-012) and were performed according to ISO 10993-5:2009 cytotoxicity test. Briefly, microneedles were sterilized under UV light for 30 minutes. Then each one of the microneedles (height of

1500 µm and a base diameter of 800 µm) were putted into the 10 ml of Dulbecco's Modified Eagle Medium (DMEM) and sample extracts were obtained. On the other hand, PCS 201 012 cells were grown under optimal condition (37 °C, 5% CO₂ and stabile humidity incubator) within 10% Fetal bovine serum (FBS) and 1% penicillin/ streptomycin supplemented DMEM. After 80% confluence was reached cells were washed with PBS and were detached from the plate surface with trypsin/EDTA. Cells were collected by centrifugation and then the cell pellet was dissolved in complete DMEM. Finally, a sample from cell suspension was mixed with 4% trypan blue stain to determine the viable cell concentration with counting on hemocytometer. Counted cells were seeded on 96 well plate as 10⁴ per well and attachment to the surface was waited for 1 day. After attachment sample extracts were applied according to elution day, and after incubation for 24 h, Cell Counting Kit (CCK-8) was used to determine the cell viability. CCK-8 was applied according to the manufacturer's recommendation and incubated for 2 hours before measurement at 450 nm. The measurement was taken using BioTek Cytation3 Multimode Microplate Reader.

2.3.11. Statistical Analysis

One-way ANOVA and Tukey tests of the results were performed by using software (GraphPad Prism 8.0) to interpret the data.

3. RESULTS AND DISCUSSION

3.1. Synthesis of LevMA

In a study conducted by Berg et al. (2018), three different levan derivatives were obtained by methacrylating *Bacillus subtilis* levan with glycidyl methacrylate (GMA), methacrylic anhydride (MAA) and 2-isocyanatoethyl methacrylate (IEM). As a result of the study, it was reported that the obtained levan derivatives were photo-crosslinkable. This makes them a potential 3D printing material. Although the procedure was the same, successful MN 3D printing could not be achieved in the first attempts. The first reason for this is thought to be the source of levan. It is known that the chemical structure of levan produced from different sources differs in terms of properties such as molecular weight and degree of branching (Erginer et al., 2023). The unmodified enzymatic *Halomonas* levan used in the first methacrylation experiments in this study has a more branched structure than the almost completely linear microbial *Halomonas* levan with a branching degree of 10% and is similar to *Bacillus* levan in

this respect (Kirtel et al., 2019). However, the environmental conditions of enzymatic production, which can be controlled more easily than microbial production, may cause the formation of higher MW levan, i.e., longer chain levan (Tanaka et al., 1980; Hou et al., 2020). In this case, the closure of the long chains of enzymatic levan in alkaline conditions (pH = 9) during methacrylation may have prevented the MAA groups from reaching the existing -OH groups. To solve this problem, the enzymatic HL chains were hydrolyzed before methacrylation (Selvi et al., 2021).

Another problem we encountered in LevMA production that affects 3D printability is adjusting the optimum pH. MAA groups are very sensitive to pH changes and work at pH=9 (Zhu et al., 2019). When this pH value could not be achieved, 3D printing could not be obtained, and the MNs came out in a more fluid and amorphous form. After pH optimization, 3D printing of solid, regular and transparent MN was possible from LevMA synthesized with the same hydrolyzed levan.

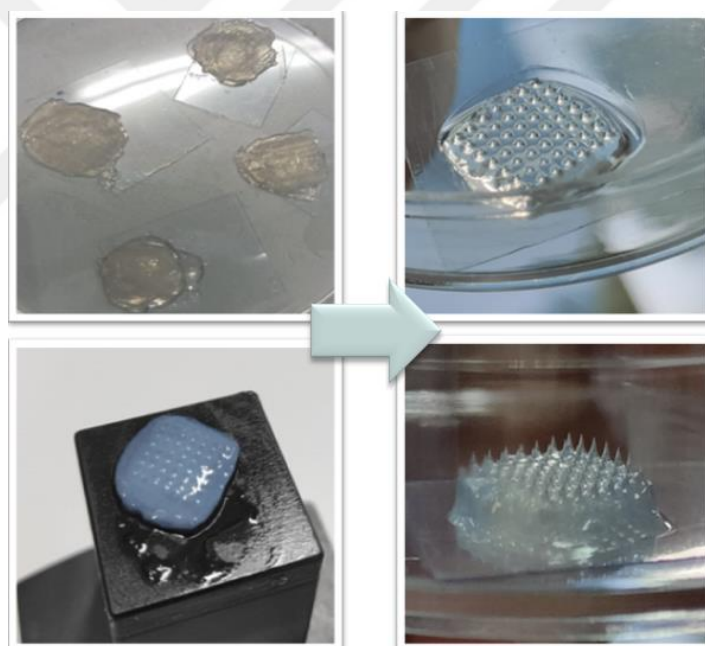


Figure 3.1 Effect of pH on 3D printability

In addition to 3D printability, some pre-printing data showing the success of methacrylation were also obtained. The first of these is that the color of the solution turned orange after the addition of the polymerization inhibitor hydroquinone during synthesis. There was no such change in the color of the solution after the addition of hydroquinone after pH optimization .



Figure 3.2 Acetone collected after the precipitation stage of a successful LevMA production (clear and transparent) and acetone collected after an unsuccessful LevMA production (orange)

In addition, in the reaction where the precise pH range cannot be provided, the color of acetone collected from the upper phase after precipitation is between orange and brown, while it is colorless at the optimum pH value (Figure 3.2). Moreover, it was understood that another indicator was the smell emitted during dialysis. In an unsuccessful methacrylation process, an intense MAA smell spreads during the dialysis water change, while the smell is almost absent in a successful MA process. This can be attributed to the absence or very little free MAA in the environment.

3.2. Characterization of MNs

3.2.1. FTIR

The structural characterization of the synthesized LevMA was performed by FTIR analysis. Peaks related to levan and new peaks resulting from methacrylation were observed in LevMA (Figure 3.2).

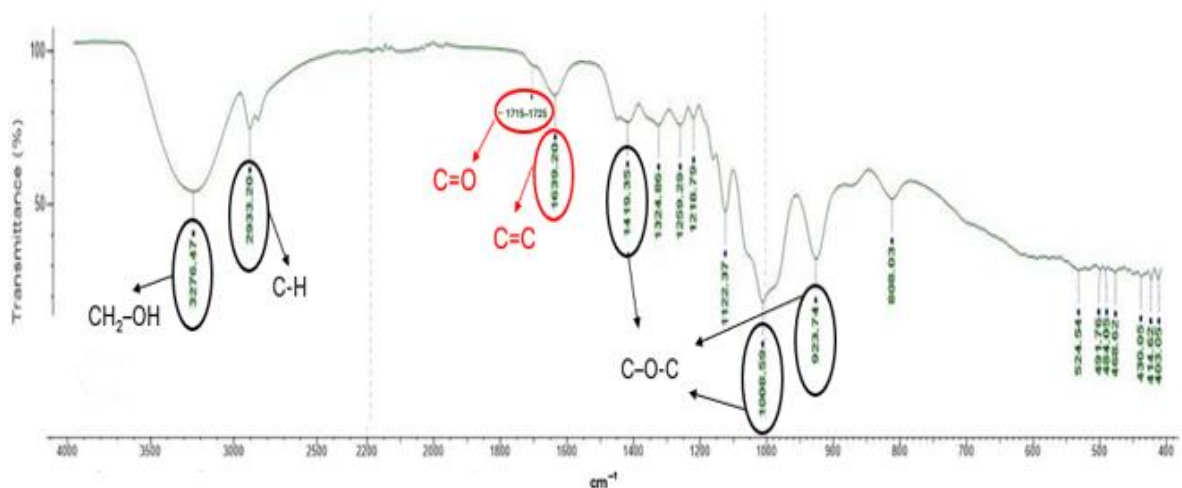


Figure 3.3 FTIR spectra of LevMA

The band at 3276.47 cm in LevMA shows the OH stretching of CH₂-OH groups and is characterized by the fructofuranose rings of levan (Erginer et al., 2016). In addition, the band at 2933.20 cm shows the C–H vibration of fructose residues, and the bands at 1419.35, 1008.59 and 923.75 cm show the C-O-C stretching of the glycosidic bond (Sarilmiser et al., 2014). These peaks are an indication that the polymer structure of levan is protected from degradation after methacrylation. In addition, the band observed at approximately 1715-1725 cm indicates C=O stretching (occurring at 160-170 ppm in ¹³C NMR) and the band at 1639.20 cm indicates C=C stretching, and these two bands are associated with methacrylation (Berg et al., 20218).

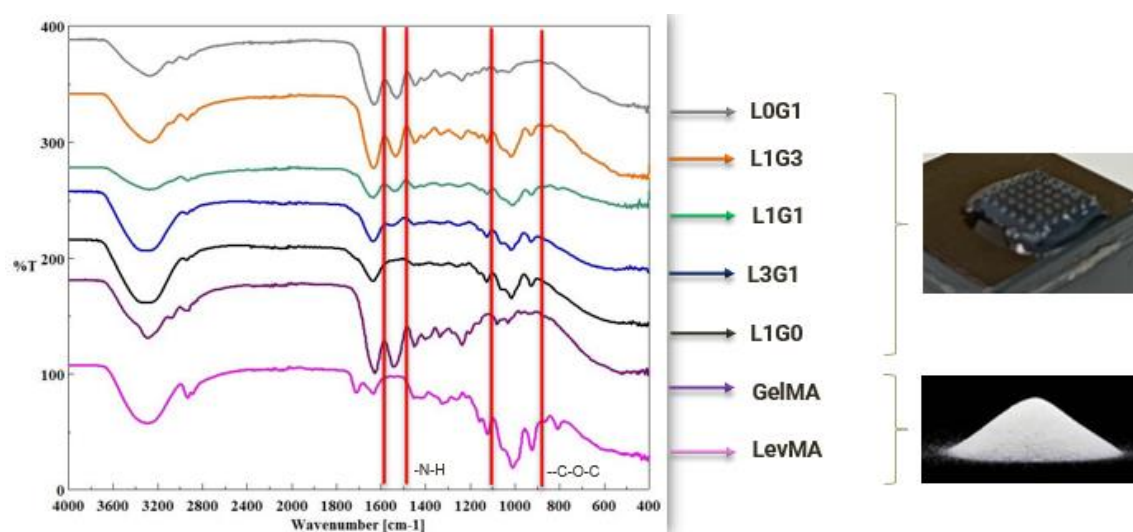


Figure 3.4 FTIR spectra of Microneedles

When microneedle formulations are evaluated in terms of Amide II (N–H), which is seen in the approximately 1550–1600 cm^{-1} band characterized by gelatin and GelMA (Jamshidifar et al., 2022), it is observed that the peak intensity gradually decreases as the GelMA concentration decreases and LevMA increases. It is also observed that increasing GelMA concentration decreases the C–O–C peak intensity, indicating the glycosidic bond of levan in microneedles (Figure 3.3).

3.2.2. NMR

Methacrylation was confirmed by ^1H NMR and ^{13}C NMR analyses by comparing the unmodified enzymatic levan (EL) and LevMA spectra. Signals characterized by fructofuranosyl groups in the levan backbone, observed primarily in the 3.2–4.5 ppm region, were seen in the LevMA spectrum. In addition, a peak indicating the anomeric proton of levan was seen at approximately 5.2 ppm (Tornacı et al., 2023). The preservation of these peaks, characterized by levan, indicates that the polysaccharide structure was preserved after methacrylation.

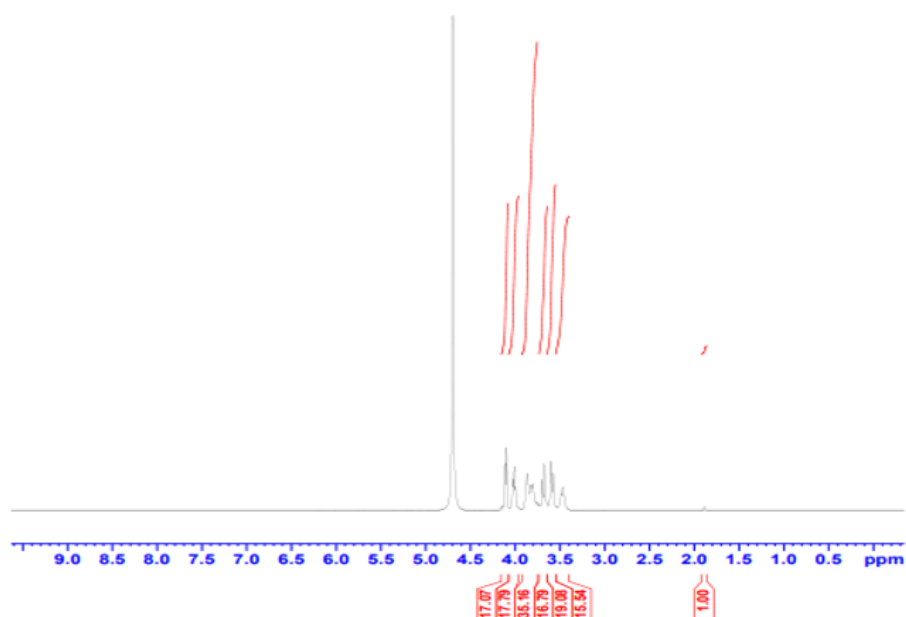


Figure 3.5 ^1H NMR Spectrum of LevMA

Also, two different new signals were detected only in the LevMA spectrum. These are the vinyl proton peaks at ~ 5.6 and ~ 6.1 ppm (they are not visible in the graph because they are low signals. These are signals between 0.11 and 0.20) and a methyl proton peak at ~ 1.88 ppm. These new peaks indicate the selective addition of methacrylate groups at hydroxyl groups in the levan backbone (Berg et al., 2018).

The degree of substitution of methacrylate groups (DS_{MA}), which indicates the average number of methacrylate groups per C3 proton of the levan anhydrofructose repeating unit, was calculated as 0.27% in this study, which is lower than the 0.95% DS_{MA} reported for LevMA produced by Berg et al. (2018). We can attribute this decrease in DS_{MA} to the differences in polymer structure as mentioned in section 3.1. Although the DS_{MA} value in this study is low, Berg et al. stated that 0.95% DS_{MA} is sufficient to form stable, photo-crosslinkable hydrogels. At this point, LevMA with a lower DS_{MA} degree was evaluated in terms of photo-crosslinkability, and the produced MNs may have different properties in terms of swelling, drug release, mechanical strength, etc.

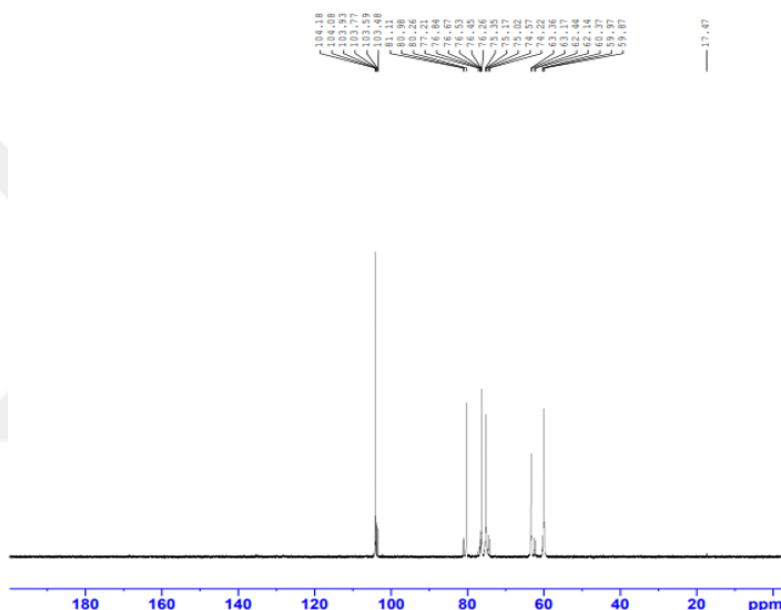


Figure 3.6 ^{13}C -NMR spectrum of LevMA

In common with the ^{13}C -NMR spectrum of unmodified EL, carbon signals were observed predominantly between 60 and 105 ppm. These peaks correspond to various carbon atoms of fructofuranosyl rings forming the β -(2 $\rightarrow$ 6)-linked polysaccharide backbone and confirm that the main fructan structure is intact (Tornaci et al., 2023). Carbon signals at 165–170 ppm ($\text{C}=\text{O}$) and 125–136 ppm ($\text{CH}_2=\text{C}$ and $\text{C}=\text{CH}_2$), characterized by the presence of methacrylate groups, were not displayed in the ^{13}C NMR spectrum of our sample, probably due to the low methacrylate degree, which may cause weak or undetectable ^{13}C signals. The low signal ~ 1715 – 1725 cm^{-1} in FTIR indicates the presence of the $\text{C}=\text{O}$ group. In addition, when the H peaks corresponding to these carbons are examined in the ^1H NMR spectrum, it is confirmed that they exist in the 5.6–6.2 ppm ($=\text{CH}_2$ and $\text{CH}=\text{}$) region, despite the low signal. Additionally, the signal at 17.47 ppm belongs to the methyl group (CH_3) added after methacrylation.

3.2.3. Rheological Properties of MNs

Frequency sweep tests were measured between 0-100 Hz, but after 10 Hz, these values were disabled in the evaluation due to the error in the measurement (operator or device). This test shows how the materials respond to oscillatory stress at different frequencies.

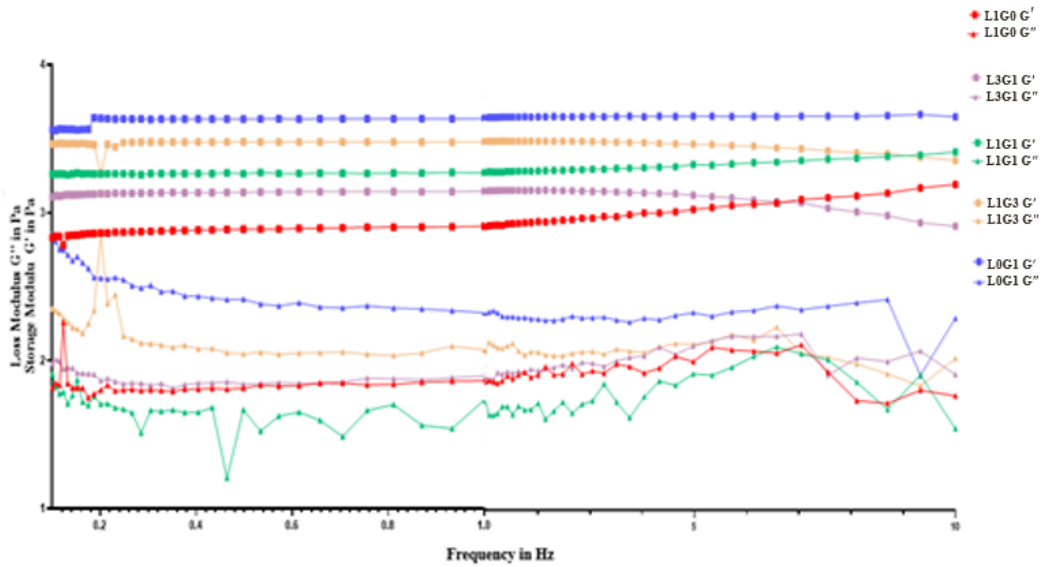


Figure 3.7 Frequency sweep analysis of MNs (Red: L1G0, Purple: L3G1, Green: L1G1, Yellow: L1G3, Blue: L0G1)

Frequency sweep analysis shows that the storage modulus (G') is consistently greater than the loss modulus (G'') in the frequency range (0-10 Hz) outside the faulty region for all microneedle formulations, regardless of LevMA or GelMA concentrations. This is an important result obtained for the newly designed LevMA MNs because the $G'=G''$ point and the subsequent $G''>G'$ interval are not observed, indicating that there is no deformation in the MNs (Nguyen et al., 2024). In other words, the MN formulations maintained their elasticity under dynamic stress and did not switch to viscous behavior. This analysis explains one of the important properties sought in MNs, as it will provide information about the resistance to the force to be applied during MN penetration (Medico et al., 2024). In addition, increasing the GelMA concentration increased the G' value in MNs. MNs with increasing G' values have a more rigid structure, and this increase can be attributed to the high crosslinking rate of GelMA (Young et al., 2020). Conversely, when the evaluation is made, the decrease in G' experienced in needles with increasing LevMA ratio caused less rigid but flexible needle formation due to lower crosslinking (Zhou et al., 2019). Despite this difference, the $G' > G''$ relationship in all

formulations, as explained above, shows that LevMA can form structurally stable hydrogel MNs suitable for MN applications.

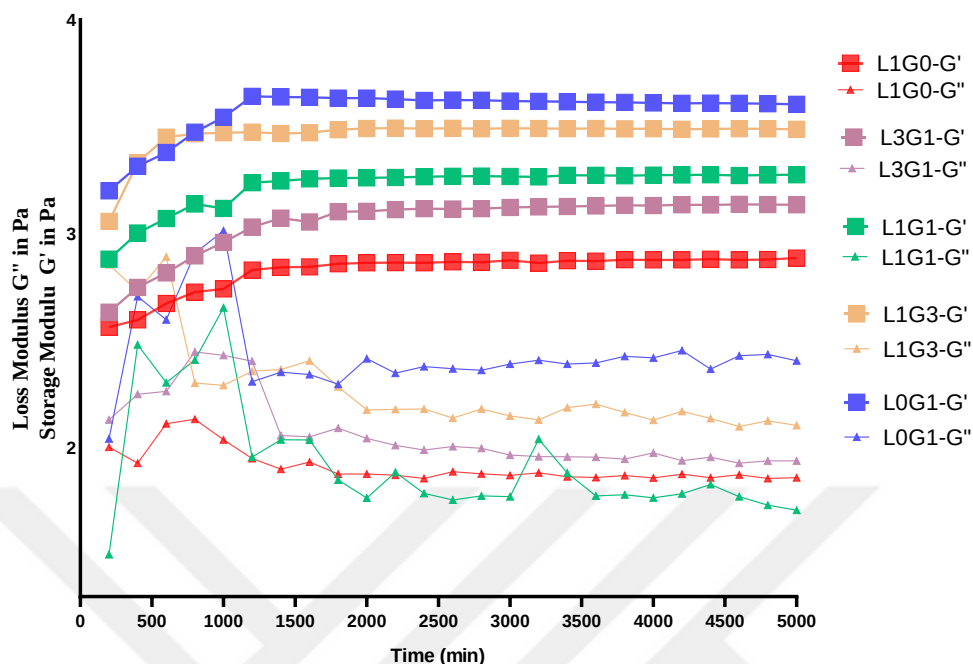


Figure 3.8 Time sweep analysis of MNs (Red: L1G0, Purple: L3G1, Green: L1G1, Yellow: L1G3, Blue : L0G1)

Time sweep rheological analysis was performed at constant strain and frequency for different time intervals. The results confirmed the viscoelastic stability of the microneedle formulations throughout the tested period. Here again, $G' > G''$ was observed in all MN formulations throughout the test period, indicating that the MNs maintained their solid-like behavior, i.e., their structural integrity over time under constant oscillatory stress, throughout the test period (Zhang et al., 2023). Consistent with the frequency sweep results, MNs with higher GelMA concentration exhibited a more rigid structure with higher G' values, while increased LevMA resulted in a more flexible structure with lower G' values in the MNs.

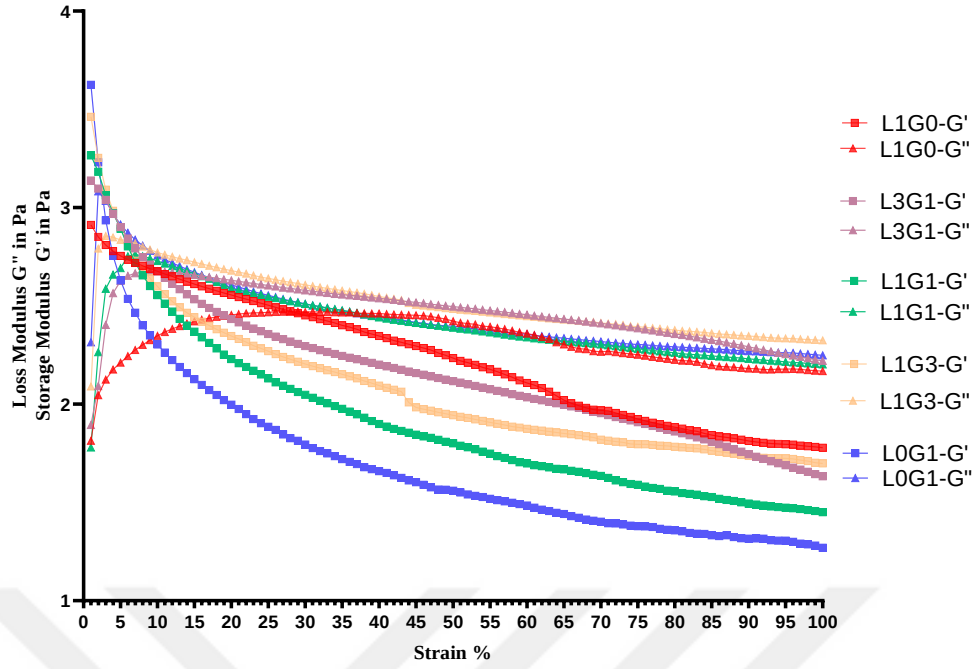


Figure 3.9 Strain sweep analysis of MNs (Red: L1G0, Purple: L3G1, Green: L1G1, Yellow: L1G3, Blue: L0G1)

When designing Mn, it is important to look not only at frequency and time stress (initial stress) but also at shear stress to analyze how resistant the material is to stress. Therefore, stress sweep analysis was used to determine the G' and G'' moduli of an MN at constant frequency and increasing deformation (strain) levels and to determine the linear viscoelastic region (LVER), which is the region before the $G' = G''$ point where materials protect their elastic behavior. The final G'' value of all needle samples was higher than the final G' value, indicating that they were all closer to a viscous state than an elastic state (Zhang et al., 2023). At low stress levels, MNs with high GelMA concentration exhibited higher initial G' compared to those with high LevMA. These initial G' values coincided with the frequency and time tests, indicating that MNs with high GelMA had a greater initial stiffness. However, GelMA high MNs reached $G' = G''$ (the point at which degradation begins) earlier at lower stress values, indicating that they lose their elastic structure and become viscous more rapidly under stress. In contrast, LevMA-based microneedles maintained their elastic dominance ($G' > G''$) over a wider stress range, despite starting with a lower G' . When the MNs were compared, the $G' = G''$ point was 25-30% stress in L1G0, while this value was in the range of 0-5% in L0G1. This conclusion means that the L1G0 needle started to deteriorate later than L0G1, i.e., it became viscous later and maintained its elasticity for a longer time.

Furthermore, when higher stress levels and final G' values were compared, L1G0 had the highest G' value, while L0G1 had the lowest G' value. The later reached the $G'=G''$ point, and the high final G' value may be due to the fact that the L1G0 contains entirely levan polymer. Due to the branching and hydrogen bonding in the polysaccharide chains, which are more abundant than proteins, during shear sweep, the hydrogen bonds in the polysaccharide chains of L1G0 started to break during the deformation, but then, under increasing stress levels, the broken bonds between the complex chains were reengage or reform, and the MN may have exhibited self-healing properties. This property is important for the MN to maintain its stability for the longest time during and after deformation. Zhang et al. (2024) reported that the ratio of hydrogen bond donors to acceptors directly affects the self-healing property of the material. So, although the L1G0 MN initially had a low G' value, the more porous 3D network of levan polysaccharide via hydrogen bonds compared to protein gelatin may have allowed it to become stiffer over time, thus preserving its elastic structure for a longer duration.

3.2.4. Morphology of Produced MNs

SEM was employed to examine the morphological structures of five groups of MNs. Figure 3.10 presents the SEM images of these MNs generated at a magnification of 60-125X, with a working distance (WD) ranging from 13.5 to 35 mm, at an electron high voltage of 10kV.

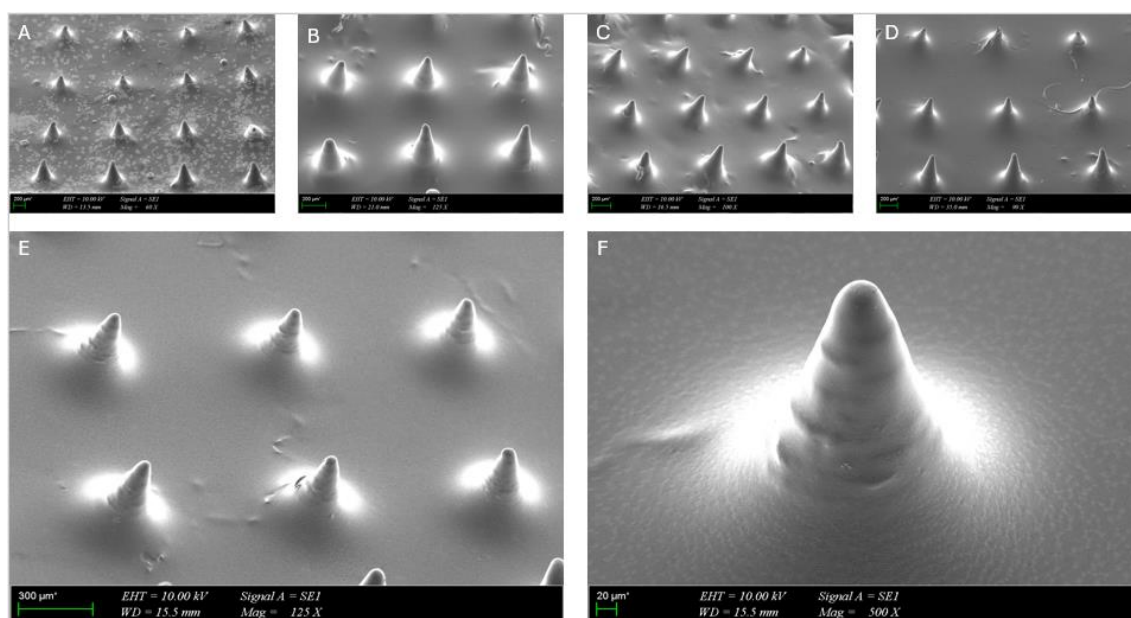


Figure 3.10 SEM images of produced MNs. (A) L0G1 (B) L1G3 (C) L1G1 (D) L3G1 Scale Bar: 200 μ m. (E) L1G0 Scale Bar: 300 μ m. (F) L1G0 MN at 500X. Scale Bar: 20 μ m.

The produced MNs had conical shapes and smooth surfaces except for L0G1. The conical shape of the MN is characterized by sufficient sharpness, which means that these needles can be penetrated without applying too much force and thus causing skin irritation. The length range of the needles was measured in the range of 240–608. Among these needles, the length of L1G0 ranges from approximately 240 to 250 μm , which falls within a favorable range for transdermal drug delivery that spans from 150 to 1500 μm (Jung & Jin, 2021). This data indicates that the needles are sized to penetrate the *stratum corneum* (SC), which is approximately 10–20 μm thick, but they cannot reach the nerves, which are typically located beyond $\sim 700\mu\text{m}$ (Larrañeta et al., 2016). Verbaan et al. (2006) found that microneedles with a length above 550 μm penetrate the skin better, especially for the delivery of large molecular agents (Verbaan et al., 2006). Considering that L1G0 needle lengths are between 240 and 250 μm , these results may suggest problems with penetration (Oliveira et al., 2024). However, in terms of autoimmune diseases such as RA, the targeted immune-active microenvironment is localized in the upper dermis and epidermis. This means that the range of 240 to 250 μm would be sufficient (Guo et al., 2017; Guo et al., 2023; Zhao et al., 2023).

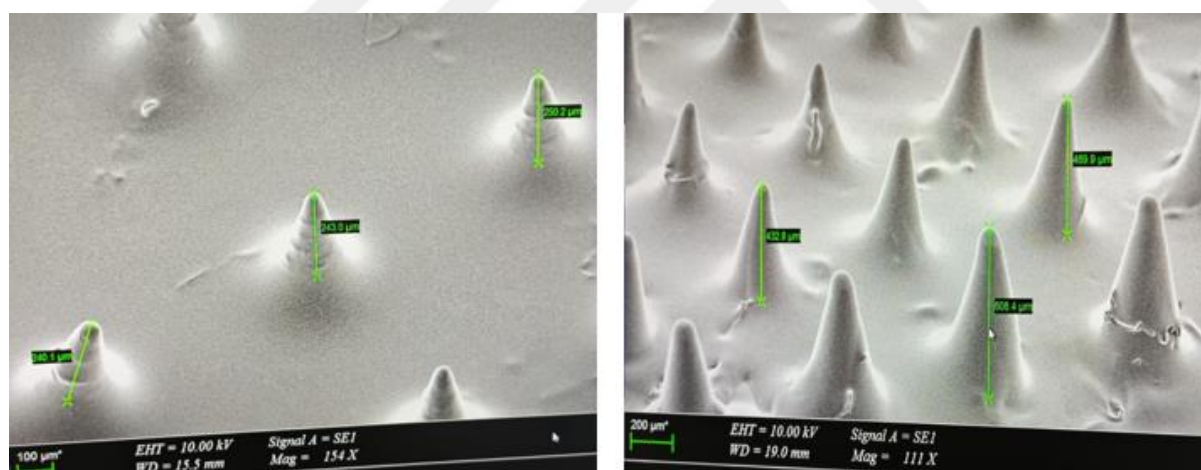


Figure 3.11 Length of L1G0 and L1G1 MNs. **(A)** L1G0 MN lengths with the range of 240 to 250. Scale Bar: 100 μm . **(B)** L1G1 MN lengths with the range of 432.8 to 608.8. Scale Bar: 200 μm .

In addition, when the uniformity of needle sizes is examined, since the 240-250 range is fairly close, this may prevent penetration-related problems due to varying needle lengths. When the needle lengths of L1G1 are examined, it is seen that the maximum value is 608 μm . Although this length suggests a better penetration idea, when the other needle lengths in the patch are examined, these are 432.8 and 469.9 μm ; the resulting non-uniformity problem may reduce the penetration ability of MN (Donnelly et al., 2010). As a result, the produced L1G0 needle has a needle length that can reach the targeted immune region in RA and does not cause pain, and it

has a morphology that can provide ease of application when the shape and sharpness of its needles are taken into account.

3.3. Swelling Capacity of MNs

Hydrogels are three-dimensional hydrophilic polymer networks with water absorption capacity. These polymeric networks are capable of holding the loaded drug for the required time and then providing controlled release (Ho et al., 2022). The swelling capacity gives us an idea about how much interstitial fluid the hydrogel MN can absorb in the area where it is applied. In other words, the drug release profile is directly dependent on the swelling capacity (Carbinatto et al., 2013). Therefore, L1G0/L3G1/L1G1/L1G3 and L0G1 MNs were tested for their swelling capacity. For this purpose, dried MNs were immersed in PBS solution (pH = 7.4) at 37 °C for three days and taken out of the solution at certain time intervals to measure their weights. According to the results obtained, all MNs reached the equilibrium swelling point within approximately 2-3 hours.

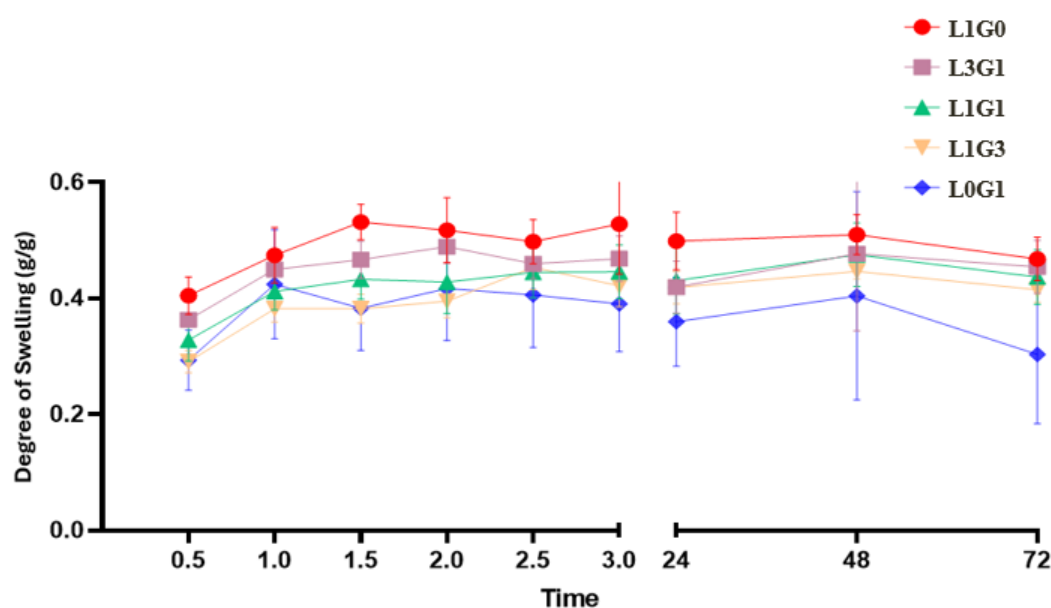


Figure 3.12 Swelling degrees of MNs (Red: L1G0, Purple: L3G1, Green: L1G1, Yellow: L1G3, Blue: L0G1)

It is observed that increasing levan concentration (L1G0>L3G1>L1G1>L1G3>L0G1) causes an increase in MN swelling capacity. Levan has a high water retention capacity due to its structural and physicochemical properties. The β -(2,6) bonds in the structure of levan allow the polymer to form an amorphous structure that will facilitate easier interaction with water molecules, and being a polymer rich in hydroxyl (-OH) groups is another feature that increases

water absorption (Angel et al., 2022; Domżał-Kędzia et al., 2023). However, an intriguing situation that we encountered here is that the swelling degree of the hydrogels in Berg et al. (2018), which we took as an example for LevMA production, is higher than the MNs in this study. The reason why this situation is interesting is that the methacrylation degree (DM) of the LevMA produced in this study due to the levan source is lower than that in Berg et al. (2018) (as mentioned in section 3.1); therefore, a lower methacrylation degree means that less cross-linking will be obtained, and theoretically, low cross-linking is expected to result in higher swelling capacity. This brings to light another factor that influences the swelling capacity of MNs: the production method. Berg et al. (2018) also used the LAP photoinitiator in the hydrogels they created to test the swelling capacity of LevMA; however, they produced bulk hydrogels by using a shorter UV curing process. In this study, the DLP technique was used for MN production, and this is a technique that provides a longer photocuring layer by layer. The layer-by-layer curing strategy of DLP printing may lead to more curing of each layer individually (including the surrounding layers where diffusion will start) compared to bulk curing (Shanjani et al., 2015; Soullard et al., 2024). This means that the LevMA in this study may have more cross-linking, despite its lower DM. In addition, Xin et al. (2025) used DLP to produce organohydrogels with anti-swelling properties for soft robotics in a relatively recent study. The conclusion we can draw from this is that the swelling capacity of LevMA MNs depends on the cross-linking ratio, but this is also closely related to the production method.

The significant decrease in swelling behavior when GelMA concentration in MNs increases (L0G1>L1G3>L1G1>L3G1>L1G0) is also parallel to the study of Shen et al. (2023), where the swelling capacity of MNs decreases when high GelMA concentration is used. The decrease can be explained by the high cross-linking ratio that forms the tight structure of GelMA. When the cross-linking ratio of the polymer is increased, more compact and mechanically strong hydrogels are obtained. However, this increase in density will cause the formation of smaller pores in the hydrogel structure, which limits the swelling capacity (Hyun et al., 2018; Shen et al., 2023). It can be seen that the existing swelling capacity of GelMA is increased with the addition of Levan. As mentioned above, less cross-linking should be done to increase the swelling capacity, which will reduce the mechanical strength. According to the results, the existing swelling capacity of GelMA is increased with the addition of Levan without changing the crosslinker ratio.

Since MNs containing LevMA-GelMA at varying concentrations have different swelling properties, their physical properties will also differ in the same way. This means that they can

be applied in different areas where more swelling, more mechanical strength or both are desired (Shen et al., 2023). Accordingly, it can be said that L1G0 and L3G1 can be ideal for areas such as wound healing, tissue regeneration, and cosmetics, where increased skin hydration is desired due to their high swelling capacity (Olteanu et al., 2024). In addition, since they can have a sponge effect against biofluids due to their higher swelling capacity, they can also be used as a continuous and comfortable biosensor in the analysis of health indicators such as glucose (Saifullah et al., 2025). On the other hand, L0G1 (completely GelMA MN) can be ideal for bone and cartilage tissue engineering, where mechanical strength is sought rather than swelling (Piao et al., 2021). When it comes to RA, the joint surrounding tissues where the MNs are planned to be applied are already inflamed and edematous or prone to inflammation and edema (Deshmukh, 2023). This scenario may be an ideal situation for the use of MNs with high levam concentration because MNs, which are expected to absorb more interstitial fluid from edematous tissue.

3.4. Gel Fraction

In total, as shown in Table 3.1, the gel fraction of MN formulations varied between 98.84% and 99.29% (Table 3.1), and there was no significant difference between them ($P > 0.05$).

Table 3.1 Gel fraction ratios of MNs in distilled water

MN Samples	Concetrations	Gel Fraction (%)	SD
L1G0	100% LevMA/0%GelMA	99.29%	±2.70
L3G1	75% LevMA/25%GelMA	98.97%	±0.36
L1G1	50% LevMA/50%GelMA	98.96%	±0.83
L1G3	25% LevMA/75%GelMA	98.79%	±0.20
L0G1	0% LevMA/100%GelMA	98.84%	±0.50

Gel fraction is directly related to crosslinking. Increasing gel fraction means a higher degree of crosslinking within the hydrogel 3D network (Xing et al., 2023). It should be noted here that, although different microneedle formulations have similar gel fractions, the degree of crosslinking is significantly affected by the structural properties of the polymers and their interactions with each other (Liu et al., 2012; Larrañeta et al., 2016). As shown in tests that measure rheology and swelling, even if MNs have similar gel fractions (Figure 3.13), their mechanical and physical properties can differ based on the type of polymer used or how the polymers interact with each other (Shie et al., 2020).

3.5. Oleuropein Release

To test the release properties of the L1G0 needle, an oleuropein-loaded microneedle was placed in PBS, and the total phenolic compound amount in released samples was calculated by FC analysis of samples taken at certain time intervals. Accordingly, L1G0 showed a linear release phase in the first 6 hours, and this release is probably related to the diffusion of oleuropein near the surface of the microneedle matrix (Erkuş et al., 2023). Then, the release reached a plateau, remained relatively constant for a week, and minimal release behavior was observed, with only approximately 12% of oleuropein released at the end of 7 days. One of the factors that may cause this release rate is the oleuropein loading method. The inclusion of oleuropein in the polymer solution before 3D printing and the subsequent photocuring step may have caused the oleuropein to be more trapped in the cross-linked polymer network. This limitation in diffusion may be advantageous for autoimmune diseases that require continuous and controlled release. Minimal but continuous and long-term release after release may ensure that the drug is retained in the tissue microenvironment for a long time and reduce the risk of systemic toxicity. It may also increase patient comfort by reducing the need for frequent applications.

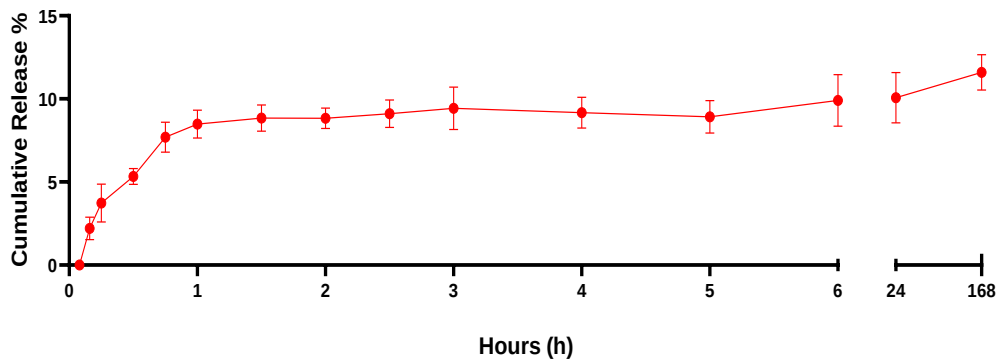


Figure 3.13 Release of Oleuropein from L1G0 Microneedles within a week

3.6. Cell viability

Cell viability of the hydrogels were tested with CCK-8 kit for 24, 48, and 72 hours. Figure 3.13. shows the cell viability percentages of PCS cells for each day.

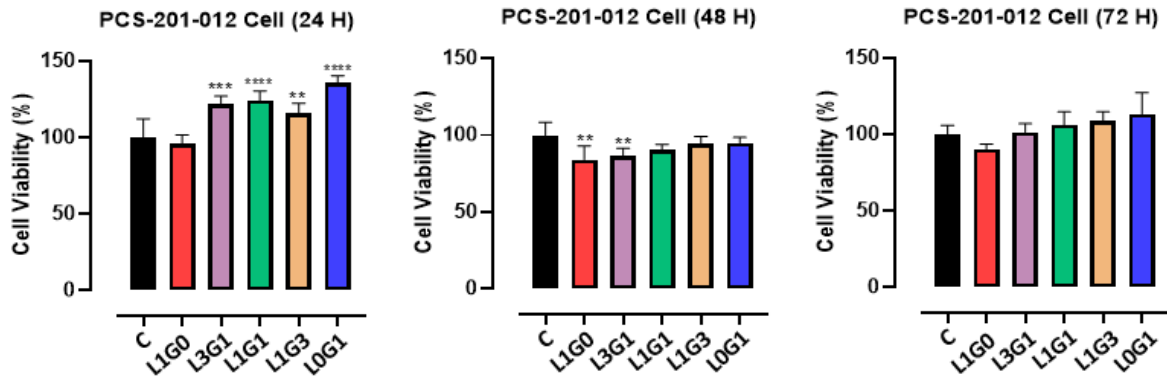


Figure 3.14 Cell viability of PCS cells treated with MN extracts

Control group was calculated as 100%. Cell viability at 24 hours extract was calculated as 95.78% for L1G0, 121.97% for L3G1, 124.41% for L1G1, 115.80% for L1G3 and 136.05% for L0G1. For the 48 hours extract, it was calculated as 84.09% for L1G0, 86.50% for L3G1, 90.30% for L1G1, 94.28% for L1G3 and 94.90% for L0G1. Cell viability at 72 hours extract was calculated as 90.30% for L1G0, 101.45% for L3G1, 106.38% for L1G1, 108.91% for L1G3 and 113.39% for L0G1. A significant difference in cell viability of PCS cells was observed at 24 and 48 hours extracts.

No significant difference was observed in the cell viability of PCS cells in L1G0 extracts except for the 48th hour. Therefore, L1G0 extract was found to be biocompatible with PCS cells (Selvi et al., 2021). However, considering the previous studies conducted by our team, this viability percentage was lower than expected. It is thought that this may be due to the UV-cured hydrogel disks used in the preparation of the extracts, not the MN itself. There are some differences in the photocuring reaction between bulk hydrogel obtained by UV curing and layer-by-layer curing (performed by the DLP method). Normally, the LAP photoinitiator used in MN production activates MAA groups by forming free radicals when exposed to UV light and initiates polymerization (Fairbanks et al., 2009). Crosby et al. (2020) observed that LAP alone did not cause cytotoxicity in CD34+ iPSC-EPs, but viability decreased to 20% as a result of UV exposure. In this case, it can be said that free radicals formed as a result of activation of the photoinitiator with UV light negatively affect cell viability. The duration of photoinitiator exposure to UV light (bulk hydrogel: 10 min; MN: 60 layers, 30 seconds each layer) may not cause sufficient crosslinking and may cause some of these radicals or partially reacted photoinitiators to be trapped in the hydrogel network (the area in the bulk hydrogel that is less exposed to UV than the environment) (Kamoun et al., 2018). These may have caused oxidative

damage in cells transferred to DMEM during the extraction process, resulting in lower cell viability than expected in L1G0. The higher DM of GelMA compared to LevMA caused more cross-linking and therefore decreased swelling capacity in MNs with high GelMA concentration. Theoretically, the same can be said for bulk hydrogels used in cell tests. Hydrogels with higher swellability may have absorbed more solution and caused the radicals and LAP residues trapped in the polymer network to pass to the extract at a higher rate. When the 24th, 48th, and 72nd hour viability percentages were examined, the viability increased with the increase in GelMA concentration, and this was inversely proportional to the swelling capacity.



4. CONCLUSIONS AND RECOMMENDATIONS

RA is an autoimmune disease characterized by inflammation and deterioration in the joints. Today, the primary goal of RA treatments is to suppress symptoms, which can lead to common side effects even at low dosages. It is an undeniable fact that new drugs and drug delivery systems are needed to relieve the symptoms of RA with the least possible side effects for the patient (Deshmukh, 2023). Levan is a polysaccharide known for its biocompatible, anti-inflammatory, and antioxidant properties, and its use in many different areas has been investigated in the literature (Tekin et al., 2025). On the other hand, considering the need for more natural and bioactive substances, oleuropein (OLE) may be a suitable compound for use in RA and also in different autoimmune diseases with its immunomodulatory effect (Mahi et al., 2023). The ability to apply OLE directly to the inflammation area is an approach that increases its effect and eliminates problems such as low bioabsorption encountered in oral administration, and delivery systems that can provide localized release are needed. Microneedles (MNs) are delivery systems that create microchannels in the stratum corneum, the outermost layer of the skin, and allow drugs to enter the circulation through these channels. Among many different types, hydrogel-based MNs stand out with their biocompatibility and mechanical properties that can be adapted to the area where they are applied (Hamdan, 2024).

In this study, OLE-loaded *Halomonas* levan-based MNs were developed to prevent joint inflammation in RA. The morphological and mechanical properties and biocompatibility of the produced MNs were analyzed. It was observed that L1G0 MN were 240-250 nm in size and uniform to deliver the drug to the synovial tissue in RA. These dimensions can be enlarged to provide delivery to deeper tissues as needed. Rheological tests showed that Lev100 started with lower G' in frequency and time tests, but the fact that the $G'=G''$ point was not reached indicated that there was no degradation. In the strain sweep test, where the beginning of degradation was observed, it degraded later than the GelMA MN and maintained its elastic structure for a longer time during high strains. According to the swelling test results performed in PBS (7.4), increasing the levan concentration increased the swelling ability of the MN. This test, which was performed based on the neutral pH of the synovial fluid to see the potential of microneedles to prevent inflammation, should be repeated with an acidic pH (6.8–7) to evaluate the swelling behavior during inflammation.

When the low-dose (1 mg/ml) OLE release of L1G0 in PBS was examined, a controlled but slow release profile was observed. Accordingly, it can be said that it has potential as a system

that can prevent possible inflammation by providing long-term and continuous release in RA. It should be tested how higher OLE doses, loading methods, and acidic pH, which will simulate the moment of inflammation, will change this release profile. Considering the high gel fraction, low swelling capacity compared to levan hydrogels, and slow OLE release, it can be said that crosslinking in levan-based MNs is high. Although this seems to contradict the NMR (low DM) results, it is possible to say that DM alone does not determine crosslink efficiency but increases its probability, and the MN production method is also effective in this. Different production methods can be tried to obtain more swelling or more rigid MNs, or different variants such as oxidized levan can be used in the levMA production process to change DM.

In addition, to better understand the anti-inflammatory effect of OLE-loaded levan-based MNs on RA, they should be subjected to ELISA tests to determine pro-inflammatory and anti-inflammatory cytokines.

As a result, biocompatible, flexible, morphologically uniform, and controlled-release levan-based MNs were produced in this thesis. It is thought that this thesis will inspire more *in vitro* and *in vivo* tests and introduce the first examples of levan-based MNs to the literature that can be used not only in RA but also in different disease types or application areas.

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Publications

- Tekin, A., Tornacı, S., Boyacı, D., Li, S., Calligaris, S., Maalej, H., & Öner, E. T. (2025). Hydrogels of levan polysaccharide: A systematic review. *International Journal of Biological Macromolecules*, 144430. <https://doi.org/10.1016/j.ijbiomac.2025.144430>