

**DEVELOPMENT OF GELATIN-CARBOXYMETHYL
CELLULOSE BASED 3D-PRINTED SCAFFOLDS FOR
CARDIAC AND BONE TISSUE ENGINEERING**

by

Şule Arıcı

B.S. in Biomedical Engineering, Erciyes University, 2014

M.S., in Biomedical Engineering, Boğaziçi University, 2019

Submitted to the Institute of Biomedical Engineering

in partial fulfillment of the requirements

for the degree of

Doctor

of

Philosophy

Boğaziçi University

2025

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APPROVED BY:

Assoc. Prof. Dr. Duygu Ege
(Thesis Advisor)

Assoc. Prof. Dr. Banu İyisan

Assoc. Prof. Dr. Batur Ercan

Assoc. Prof. Dr. Özgül Gök Özatay

Assoc. Prof. Dr. Ceyda Öksel Karakuş

DATE OF APPROVAL: 22 Jul 2025

ACKNOWLEDGMENTS

First of all, I would like to express my sincere gratitude to my supervisor, Assoc. Prof. Dr. Duygu Ege, for her continuous support, motivation, and valuable guidance throughout this journey. It has been a great privilege to conduct my doctoral research under her supervision. I consider it a true opportunity to have worked with her, and I am deeply grateful for the academic insight and encouragement she has provided.

I would also like to thank my thesis committee members, Assoc. Prof. Dr. Özgül Gök Özatay, Assoc. Prof. Banu İyisan, Assoc. Prof. Dr. Batur Ercan, and Assoc. Prof. Dr. Ceyda Öksel Karakuş for their valuable time, constructive feedback, and contributions to the improvement of my thesis. I am grateful to my colleagues and friends at the Institute of Biomedical Engineering for their support and collaboration during my studies. Their presence made this challenging journey more manageable and enjoyable. Secondly, I extend my heartfelt thanks to my parents, Bekir and Sevil, for their endless support and encouragement. I am especially thankful to my husband, Mehmet Ali, for always standing by my side with patience and understanding throughout this demanding process. Lastly, I would like to express my deepest gratitude to my daughter, Rüya, who has accompanied me during the final and most intense stage of this journey. Even before entering this world, she filled my heart with strength, hope, and endless joy. Her presence gave me the motivation I needed to keep going, and I feel incredibly lucky to be completing this path with her. I would also like to express my sincere gratitude for the support provided by the Council of Higher Education (YÖK) 100/2000 PhD Scholarship program. This doctoral study was supported by the Scientific and Technological Research Council of Turkey (TÜBİTAK) under Grant No. 117M231 and by the Boğaziçi University Scientific Research Projects (BAP) unit under Grant No. 18001.

ACADEMIC ETHICS AND INTEGRITY STATEMENT

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ABSTRACT

DEVELOPMENT OF GELATIN-CARBOXYMETHYL CELLULOSE BASED 3D-PRINTED SCAFFOLDS FOR CARDIAC AND BONE TISSUE ENGINEERING

Tissue defects affecting both soft and hard tissues remain a significant challenge in regenerative medicine. In this study, specifically, cardiac tissue was studied as a representative soft tissue model, while bone tissue served as the hard tissue model. Gelatin (Gel) and carboxymethyl cellulose (CMC) formed the biocompatible, printable backbone, while graphene oxide (GO) and nano-hydroxyapatite (HA) were added to tailor scaffold properties for cardiac and bone tissue applications, respectively. For cardiac applications, extrusion-based 3D printing was used to produce CMC/Gel and CMC/Gel/GO scaffolds crosslinked with different concentrations (100:20 mM, 50:10 mM, 25:5 mM) of N-(3-dimethylaminopropyl)-n'-ethylcarbodiimide hydrochloride (EDC)/N hydroxysuccinimide (NHS) to optimize mechanical integrity and structural stability. While C15/G7.5/GO1 demonstrated a substantial increase in tensile strain (61.03%), C10/G7.5/GO1 exhibited conductivity values within the range of native myocardium. Furthermore, H9C2 cell viability significantly improved in GO-containing groups, confirming their suitability for cardiac tissue applications. For hard tissue engineering, the effects of nano-HA and Gel concentrations on 3D-printed bone scaffolds were investigated in terms of mechanical performance, bioactivity and biocompatibility. HA10/G15/C7.5 showed the highest compressive strength (2.6 MPa), controlled degradation, favorable water uptake, and notable apatite formation after 28 days in SBF. MC3T3-E1 cell studies showed excellent adhesion, proliferation, and mineralization, especially in nano-HA enriched scaffolds. Overall, this thesis shows that Gel/CMC-based scaffolds are promising for soft and hard tissue repair due to their tunable biochemical, mechanical, and structural properties.

Keywords: Gelatin, Carboxymethyl Cellulose, Tissue Scaffold, 3D-printing, Bone Tissue Engineering, Cardiac Tissue Engineering.

ÖZET

KARDİYAK VE KEMİK DOKU MUHENDİSLİĞİ İÇİN JELATİN VE KARBOKSİMETİL SELÜLOZ BAZLI 3B-BASILMIŞ DOKU İSKELELERİNİN GELİŞTİRİLMESİ

Yumuşak ve sert dokuları etkileyen doku defektleri, rejeneratif tıpta hala önemli bir zorluk oluşturmaktadır. Bu çalışmada, yumuşak doku modeli olarak kalp dokusu, sert doku modeli olarak ise kemik dokusu ele alınmıştır. Her iki sistemde de, biy uyumlu ve baskıya uygun yapıları nedeniyle jelatin (Gel) ve karboksimetil selüloz (CMC) temel polimerler olarak kullanılmış; dokuya özgü özellikler için kalp iskelelerine grafen oksit (GO), kemik dokusu iskelelerine ise nano-hidroksiapatit (HA) eklenmiştir. Kalp dokusu uygulamaları için, farklı konsantrasyonlardaki (100:20 mM, 50:10 mM, 25:5 mM) N-(3-dimetilaminopropil)-nn'-etilkarbodiimid hidroklorür (EDC) ve N-hidroksisüksinimid (NHS) kullanılarak çapraz bağlanmış CMC/Gel ve CMC/Gel/GO iskeleleri, ekstrüzyon tabanlı 3D baskı yöntemiyle üretilmiştir. C15/G7.5/GO1 formülasyonu % 61.03 oranında önemli bir gerinme artışı sağlarken, C10/G7.5/GO1 formülasyonu elektrik iletkenliği ile doğal miyokard dokusuna benzer iletkenlik göstermiştir. GO içeren gruplarda H9C2 hücre canlılığı anlamlı şekilde artarak iskelelerin kalp doku mühendisliğine uygunluğunu göstermiştir. Sert doku mühendisliği kapsamında, nano-HA ve jelatin konsantrasyonlarının 3D baskılı kemik iskelelerinin mekanik performansı, biy uyumluluğu ve biyolojik etkinliği üzerindeki etkileri incelenmiştir. HA10/G15/C7.5 formülasyonu, yük taşımayan uygulamalar için uygun 2.6 MPa basma dayanımı, kontrollü degradasyon ve 28 gün sonunda SBF (simüle vücut sıvısı) ortamında belirgin apatit oluşumu göstermiştir. MC3T3-E1 pre-osteoblast hücreleri ile yapılan hücre kültürü çalışmaları, özellikle nano-HA ile zenginleştirilmiş gruplarda mükemmel hücre tutunması, proliferasyonu ve mineralizasyon potansiyelini ortaya koymuştur. Gel/CMC bazlı iskeleler hem yumuşak ve sert doku onarımı için umut vadeden platformlardır.

Anahtar Sözcükler: Jelatin, Karboksimetil Selüloz, Grafen Oksit, Hidroksiapatit, Kardiyak Doku Mühendisliği, Kemik Doku Mühendisliği, 3B Baskı .

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LIST OF SYMBOLS

COO^-	Carboxyl ion
Ca^{2+}	Calcium ion
PO_4^{3-}	Phosphate ion
OH^-	Hydroxide ion
CO_2	Carbon dioxide
g	Gram
$^{\circ}C$	Degree Celsius
ρ	Resistivity
σ	Electrical conductivity
V	Volt
μg	Mikrogram
μm	Mikrometer
μl	Mikroliter
mm	Milimeter
mL	Mililiter
mM	Milimolar
nm	Nanometer
O	Oxygen
P	Phosphate
rad	Radian
S	Siemens

LIST OF ABBREVIATIONS

3D	Three dimensional
ALP	Alkaline phosphate collagen
AM	Additive Manufacturing
ANOVA	Analysis of variance
AO	Acridine orange
BSA	Bovine serum albumin
CAD	Computer-aided design
CMC	Carboxymethyl cellulose
CMs	Cardiomyocytes
CT	Computed Tomography
CVD	Cardiovascular disease
DI	Deionized
EDC	N-(3-dimethylaminopropyl)-n'-ethylcarbodiimide hydrochloride
FDM	Fused Deposition Modeling
FTIR	Fourier Transform Infrared Spectroscopy
G-code	Geometric code
Gel	Gelatin
G'	Storage modulus
G''	Loss modulus
GO	Graphene Oxide
GPa	Gigapascal
hMSCs	Human mesenchymal stem cells
LVR	Linear viscoelastic region
LV	Left ventricular
MEM- α	α -Minimal Essential Medium
min	Minute
MPa	Megapascal
MRI	Magnetic Resonance Imaging

MWCNT	Multi-walled Carbon Nanotube
NHS	N-hydroxysuccinimide
OCN	Osteocalcin
PBS	Phosphate buffered saline
PCL	Polycaprolactone
PFA	Paraformaldehyde
PI	Propidium iodide
PLGA	Poly (Lactide-co-glycolide) acid
Runx2	Runt-related transcription factor 2
SBF	Simulated body fluid
SEM	Scanning Electron Microscopy
STL	Stereolithography
TCP	Tissue culture plate
UV	Ultraviolet
XRD	X-ray Diffraction

1. INTRODUCTION

1.1 Motivation

Tissue defects resulting from trauma, cancer, or degenerative diseases remain a major significant medical challenge due to the natural healing limitation of native tissues. Traditional methods like autografts and allografts have several drawbacks, such as complications at the donor site, potential immune system rejection, and limitation of donor tissue [1]. To overcome these issues, the development of biomaterials has been gained as a promising alternative to support and guide tissue regeneration. Among various strategies, tissue engineering scaffolds which are three-dimensional, biocompatible structures designed to imitate the structure of native extracellular matrix provide the support of tissue regeneration and mechanical integrity [2]. Recent advances in biomaterial science for scaffold fabrication techniques, particularly 3D printing, have made it possible to design the functional scaffolds. These advancements have strengthened the interest in exploring novel biomaterial combinations to improve scaffold systems that meet specific requirements of various tissue types [3].

Cardiovascular disease (CVD) is estimated to cause approximately 18.6 million deaths annually, which is one of the leading causes of death worldwide [4]. Among these diseases, myocardial infarction (MI) known as heart attack, the main cause of heart failure, usually initiates with coronary artery blockage and results in loss of cardiomyocytes. After the MI, scar formation occurs and the heart cannot regenerate cardiomyocytes to heal injured cardiac tissue [5]. Cell therapy is a common treatment method to obtain functional improvement in cardiac tissue regeneration. However, this method has many limitations such as cell death and inability to mimic the composition of native myocardium [6],[7],[8]. Cardiac scaffolds, named as cardiac patches, utilizing natural materials produced by 3D printing method have been a popular study field that develop a tissue scaffold to enhance the ability of myocardial regeneration and to prevent left ventricular (LV) remodeling after MI that causes the changes in size,

shape, and function of the heart [9, 10]. The major tissue defects for hard tissue are bone defects due to injuries, trauma and osteoporosis, which reduces the quality of patient's life [11, 12]. Autografts and allografts are implanted as bone substitutes which lead to mechanical support and new tissue regeneration [13]. However, they have high costs with long recovery periods after surgery [14]. Moreover, immune and inflammatory responses, limited donor site, morbidity and infectious disease are their major drawbacks [15]. To overcome these limitations, suitable biomaterials are produced by 3D printing, focusing on promoting cell regeneration and enhancing effective bone healing [16]. Although 3D printing has been successfully applied for fabrication of tissue scaffold for bone regeneration; selection of materials and mechanical properties are still challenges for many applications [17]. Hydrogels have started to have an important role in 3D printing tissue applications due to their capability to mimic the natural tissue environment [18]. Scaffolds based on gelatin (Gel) produced by 3D printing method have been investigated for their potential in regenerating a range of tissues, including skin, cartilage, bone, and cardiac tissue [19],[20],[21]. Gel, extracted from collagen derived from bone and skin of animals through denaturation, is known for its strong support of cell growth and proliferation [22]. Gel is highly preferred for extrusion-based 3D printing in biomedical applications due to its favorable rheological properties, biocompatibility, cell affinity, and biodegradation [23],[24],[25]. However, a critical limitation of gelatin hydrogels is their poor mechanical integrity. Additionally, introducing a macroporous structure within these bulk hydrogels can further compromise their structural stability and mechanical strength. Hence, the challenge lies in creating mechanically stable 3D-printed gelatin hydrogels with a predefined internal pore structure [26],[27]. Considering that only gelatin usage is inappropriate to increase mechanical properties and enhance printability, there is a need of co-polymer to provide stability of hydrogels [28],[29],[30].

Carboxymethyl cellulose (CMC) is extensively studied due to its biocompatibility, mechanical rigidity, and viscosity property in 3D printing applications [31],[32]. Janarthanan et al. [33] studied on the printability of hyaluronic acid-CMC with different compositions for the tissue regeneration. Their results showed that composition of hydrogel and crosslinking capacity have great impacts on final products. Habib et

al. [34] investigated the printability of hydrogel including CMC and alginate. They showed that the increase of CMC in hydrogel to some extent provide well shape fidelity of 3D-printed scaffolds without collapse between lines. In this study, we were first motivated to produce tissue scaffolds with proper mechanical properties and biocompatible using the viscosity promoter effect of carboxymethyl cellulose for cardiac and bone tissue defects [31].

Yang et al. [35] has shown that scaffolds including gelatin is required to be crosslinked to obtain the high hydrolysis resistance which maintain their stability during implantation. N-(3-dimethylaminopropyl)- α -ethylcarbodiimide hydrochloride (EDC)/ N-hydroxysuccinimide (NHS) which is a zero-length crosslinker is commonly utilized to improve the mechanical properties and stability of Gel [36]. EDC/NHS conjugates amino groups with activated carboxylic groups within Gel chains, resulting in the formation of covalent bonds. Kuijpers et al. [37] showed that 5:1 molar ratio of EDC/NHS was the most effective ratio for activation of carboxylic acid. Above 5:1 molar ratio, NHS reacts with EDC and reduces concentration of active carboxylic acid groups for formation of amide bonds which decreases stability of the cardiac patches. Another motivation was to ensure the molar ratio of EDC:NHS to obtain more stable and durable tissue scaffolds in body simulated fluids.

Hydroxyapatite (HA) is commonly used in bone tissue regeneration due to its similarity to natural bone tissue constituents [29],[38]. HA scaffolds have the ability to effectively connect with their surrounding bone tissue [39],[40]. However, HA is hard and brittle, and its rate of biodegradation is slow under in vivo conditions [41]. Therefore, effective polymeric hydrogel solutions have provided the homogenous distribution of HA particles within the final product in the 3D printing applications, which improve their mechanical properties and compatibility with natural bone tissue. Qian et al. [42] demonstrated that CMC was an effective polymeric additive to inhibit HA particle aggregation in the. Moreover, only one study in the literature, CMC and HA were printed with low percentage of polymeric additives. Although they investigated morphological and mechanical properties of 3D-printed scaffold, the biocompatibility of scaffolds was not studied which is required for cell proliferation [19].

This thesis broadly addresses both soft and hard tissue applications; specifically, cardiac tissue was targeted as a representative soft tissue model, while bone tissue served as the model for hard tissue engineering. The study aims to demonstrate the versatility of Gel/CMC-based hydrogel systems in supporting regeneration across distinct tissue types with differing biological and mechanical demands. The fundamental motivation of this thesis is investigating the biocompatibility, mechanical and electrical properties of Gel/CMC cardiac scaffolds to heal scarred myocardial tissue. Then, incorporating with HA, HA/Gel/CMC bone scaffolds were examined in terms of printability, scaffold morphology, mechanical characteristics and cytocompatibility.

1.2 Objectives and Outline of the Thesis

The growing demand for regenerative strategies in cardiac and bone tissue engineering has accelerated the development of bioinspired materials with tunable mechanical and biological properties. In this thesis, two distinct yet complementary 3D printing approaches were explored to address critical limitations in current tissue scaffold designs: one targeting myocardial repair, the other aimed at enhancing bone regeneration.

The cardiac segment of this study focused on the fabrication of flexible, conductive, and biocompatible patches based on CMC, Gel, and graphene oxide (GO). These inks were optimized for extrusion-based 3D printing, and their rheological behavior was systematically evaluated to establish optimal printing conditions. The effects of GO incorporation and EDC/NHS crosslinking ratios on mechanical stability, electrical conductivity, and H9C2 cardiomyoblast viability were comprehensively studied. Among various formulations, a balanced composition of CMC/Gel/GO with moderate crosslinking yielded cardiac patches that demonstrated enhanced flexibility, cytocompatibility, and physiologically relevant conductivity.

In parallel, bone scaffolds were developed using nanohydroxyapatite (nano-HA), gelatin, and CMC, aiming to replicate the hierarchical architecture and mineral compo-

sition of native bone tissue. The scaffolds were printed using the same extrusion-based platform and were evaluated for their printability, structural integrity, compressive strength, and bioactivity. MC3T3-E1 pre-osteoblasts were employed to evaluate the ability of the scaffolds to support bone regeneration, while mineralization and apatite formation were examined through Alizarin Red staining and Stimulated body fluid (SBF) immersion. The results demonstrated that 10% nano-HA in combination with 15% gelatin and 7.5% CMC produced scaffolds with optimal mechanical performance and excellent bioactivity.

Throughout the thesis, rheological and physicochemical characterizations served as a foundation for controlling the microstructure and performance of the printed constructs. By systematically varying the composition and processing parameters, it was possible to tailor scaffold properties to meet the specific demands of either cardiac or bone tissue environments.

In conclusion, this work presents two novel hydrogel systems—CMC/Gel/GO and nano-HA/Gel/CMC—each designed for a different tissue application but sharing a common strategy of material tunability and process optimization. Together, these findings contribute to the field of 3D printing by demonstrating how the integration of nanomaterials, crosslinking chemistry, and advanced printing techniques can lead to the generation of tissue-specific scaffolds with translational potential.

The outline of the thesis with the main objectives is presented as in the following: The main objectives and outlines of this thesis were presented in Chapter 1. To understand the approach of our study, background information on cardiac and bone tissue engineering were explained in Chapter 2. This thesis focusses on both soft tissue and hard tissue. So, this study was divided into 2 main target tissues.

In Chapter 3, the main objectives are:

- Investigation of critical impact of EDC/NHS concentration and the presence

of GO and CMC on the mechanical properties of cardiac scaffolds, including stability, flexibility, tensile strength, and stiffness.

- Evaluation of conductivity of cardiac scaffolds with varying CMC concentration
- Confirmation of the scaffolds' biocompatibility, indicating their potential to support cardiac tissue regeneration.

In Chapter 4, we focus on investigation of nano-HA/Gel/CMC hydrogel-based 3D-printed bone scaffolds. This chapter included these main objectives:

- To fabricate nano-hydroxyapatite nano-HA/Gel/CMC composite bone scaffolds using extrusion-based 3D printing technology.
- To optimize the nano-HA concentration in scaffold formulations, aiming to achieve enhanced shape fidelity and mechanical strength.
- To analyze the bioactivity of the scaffolds through alizarin red staining and SBF incubation, supported by SEM, FTIR, and XRD characterization techniques.
- To evaluate the scaffold materials for their suitability in a biological environment by investigating MC3T3-E1 pre-osteoblast cell adhesion and proliferation on days 1, 3, 5, and 7.

2. BACKGROUND

2.1 Cardiac Tissue Engineering Applications

2.1.1 Heart Structure

The heart is a crucial organ, which maintains homeostasis of the whole organism. The heart wall consists of epicardium, myocardium, and endocardium, as shown in Figure 2.1. The heart muscles, called myocardium, consist of cardiomyocytes in the range of 2-3 billion, accounted for 30% total cells of the human heart, which also provides the function of cardiac contraction driven by the propagation of bioelectrical activity [43],[44],[45].

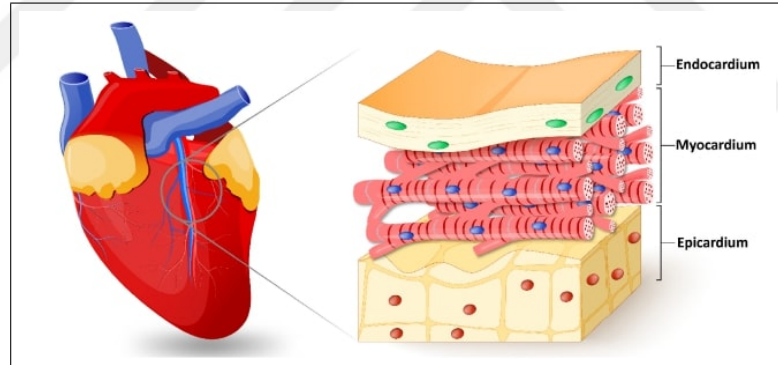


Figure 2.1 Human heart wall [45].

2.1.2 Myocardial Infarction

In the case of hearth attack (myocardial infarction), blood flow is interrupted, and oxygen cannot be delivered to the cells. This interruption causes permanent damage on the myocardium due to the poor self-renewal ability of cardiomyocytes. In a healthy heart, approximately only 1% of cardiomyocytes are regenerated annually at 25 years of age and 0.45% at 75 years of age [46]. Thus, structural and functional changes are formed, known as left ventricular (LV) remodeling. Patients with LV remodeling

is under risk of cardiovascular fatalities since the heart cannot maintain its ability of mechanical strength due to dilation and distortion of cavity shape, as shown in Figure 2.2 [47, 48].

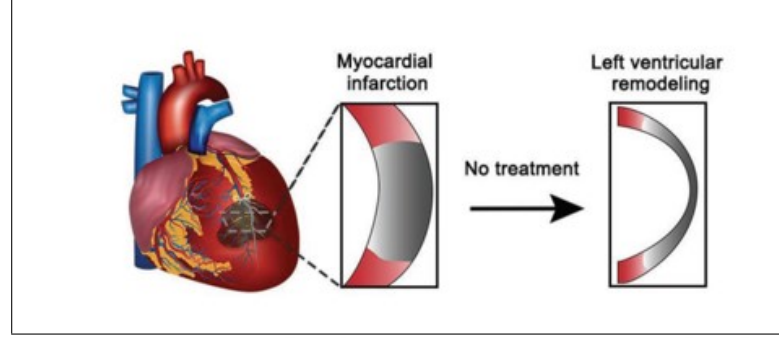


Figure 2.2 LV remodeling [48].

2.1.3 Cardiac Tissue Engineering

Cardiac tissue engineering involves effort to repair damaged or unfunctional myocardium tissue which is incapable self-healing. To date, several strategies such as whole heart implantation and cell therapies have been applied, however shortage of donors, immune rejection and low cell survival limits these strategies. In recent studies, tissue engineers have used advanced and last technology, 3D printing, to produce cardiac scaffolds to regenerate damaged myocardium. In order to improve cardiac scaffolds, there have been several efforts using various natural hydrogels like alginate, collagen, gelatin and hyaluronic acid, as well as synthetic polymers such as PLGA (poly(lactide-co-glycolide) PCL (polycaprolactone) [49]. Figure 2.3 shows classical manufacturing process, implantation, significance of mechanical support and desired therapeutic effects of a 3D-printed cardiac scaffold.

Izadifar et al. [18] indicated that collagen-alginate scaffolds reinforced with carbon nanotubes improved cell attachment and electric conductivity, but their range of Elastic modulus was 43-54 kPa which is at the lowest levels of the Young's modulus range given for cardiac patch in the literature and not sufficient to meet the maximum mechanical strength of the heart. There are a couple of studies that used gelatin for

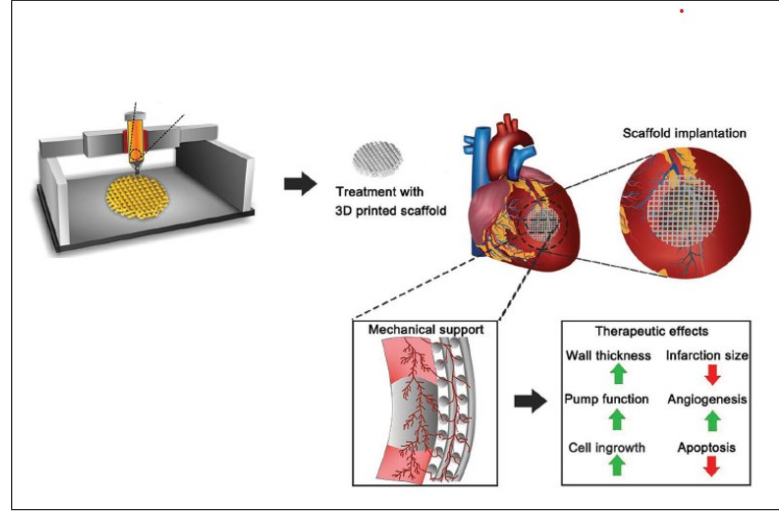


Figure 2.3 Schematic representation of cardiac patch implantation [48].

cardiac patches in the literature. Roche et al. [50] prepared the cardiac cells incorporated with gelatin-alginate hydrogels 3D bio-printed scaffolds. Although they could show the cell viability until the end of day 28, they did not study the mechanical properties of their patches. Tijore et al. [51] also fabricated micro-channeled gelatin-based and enzymatically crosslinked scaffolds by 3D extrusion bioprinting method. Figure 2.4 shows that their scaffolds highly uptake the water at the end of the 21 days due to their enzymatically crosslinking method. In addition, their mechanical result showed that Young's modulus of gelatin-based and enzymatically crosslinked scaffolds was almost at the lower limit of for human myocardium and insufficient to heart contraction, which requires a stiffness of 80 kPa [51]. These findings have provided novel insights into how to produce cardiac constructs with optimal properties as demonstrated in our research.

2.2 Bone Tissue Engineering Applications

2.2.1 Bone Structure and Remodeling

Bone tissue is a unique structure consisting of organic, inorganic and water components. The Organic side includes mostly collagen, whereas the inorganic side

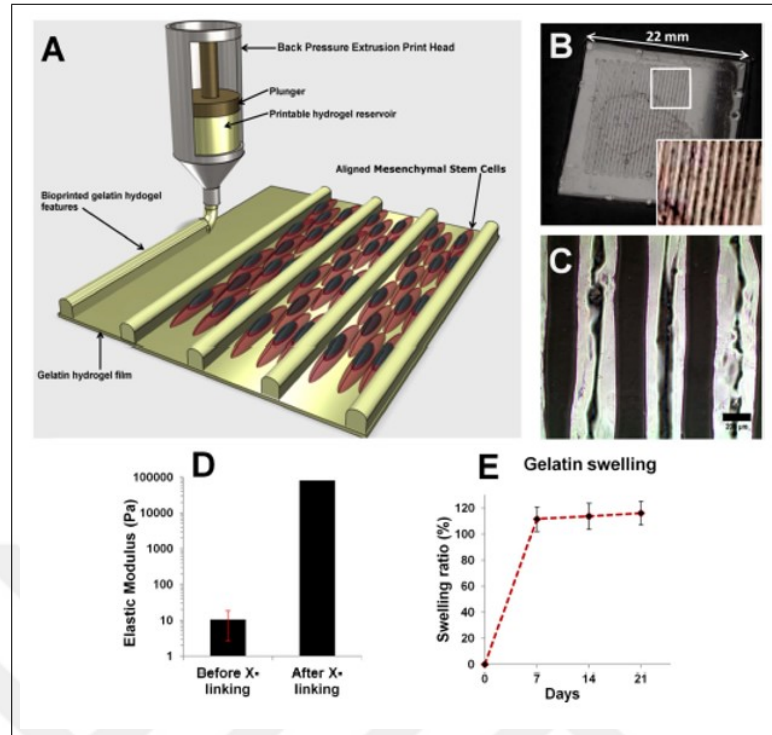


Figure 2.4 (a) Illustration of the 3D printing workflow and model architecture, (b) photograph of printed gelatin-based scaffolds with defined patterns, (c) bright-field microscopy image of the gelatin hydrogel microstructure, (d) comparison of elastic modulus between crosslinked and non-crosslinked gelatin samples; (e) water uptake profile of printed gelatin hydrogels [51].

is constituted of HA crystals. These crystals are located on collagen fibrils, which constitutes the basic structure of bone at the micron level, as shown in Figure 2.5. At the next hierarchical structure, collagen fibrils are arranged into fiber. Then parallel fibers are described as lamella. Following this structure, osteon is formed, which is a unit of bone [52].

2.2.2 Bone Losses

Critical-sized defects may arise due to trauma, tumor resection (especially in cases of bone cancer), large bone segments often need to be surgically removed, resulting in significant bone loss that cannot regenerate on its own. Similarly, metabolic bone diseases, infections, and certain genetic disorders can impair the natural healing capacity, making reconstruction particularly challenging. Therefore, effective bone regeneration strategies are crucial for restoring structural integrity and function in these

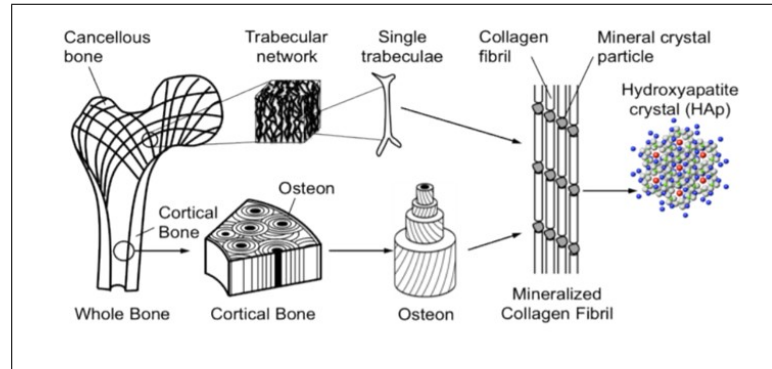


Figure 2.5 Hierarchical structure of bone [52].

complex clinical cases [53].

2.2.3 Bone Tissue Engineering

In order to create a design similar to bone structure, HA is widely used with various natural and synthetic polymeric solutions in bone tissue engineering. Although there are a lot of studies that developed gelatin-HA bulk composites in the literature, a few efforts exist to accomplish this via 3D-printed scaffolds for bone tissue defects. Fan and colleagues [54] showed that n-HA with alginate-gelatin hydrogels were printed at 25 °C, without crosslinker, and achieved high cell activity and cell growth, pore structure as well as cytotoxic property, but the increased amount of HA in the scaffolds improved the Young's modulus to certain extent. Moreover, a higher amount of HA resulted in lower scaffold deformation, which is demonstrated in Figure 2.6.

Another study including Gel-HA by Nosrati et al. [55], Young's modulus of 3D-printed gelatin-HA-GO were higher than ones without GO, which was far lower than the bone tissue's mechanical values. To investigate the effect of different percentages of crosslinker and drying type of the scaffolds, Gupta et al. [56] produced gelatin-carboxymethyl chitin sodium salt-hydroxyapatite scaffolds via 3D printing method. With the increase of EDC crosslinker, open surface pores and swelling rate were decreased. Scaffolds dried at 25 °C had higher swelling rate and mechanical properties than those produced by the lyophilization method.

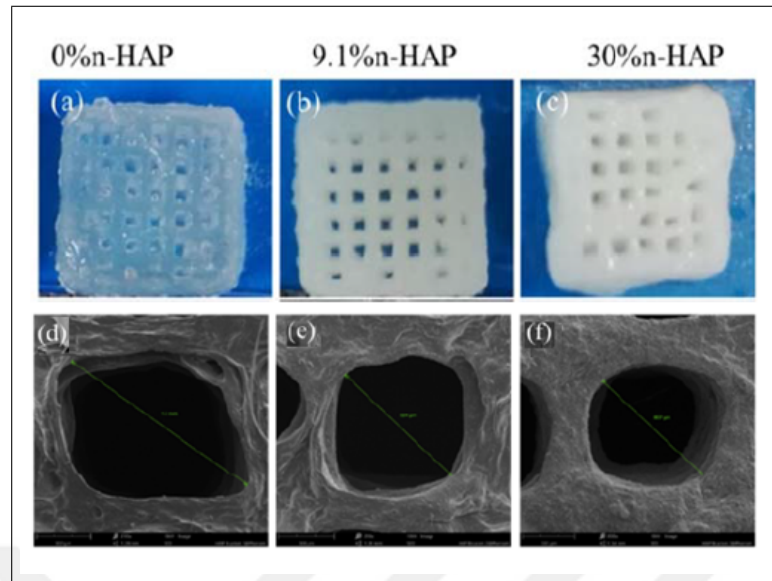


Figure 2.6 Morphology of hydrogels scaffolds [54].

Chen et al. [19] fabricated 3D-printed HA-based scaffolds to address the limited mechanical strength commonly observed in bone scaffolds. Utilizing gelatin, carboxymethyl cellulose (CMC), and chitosan, the team successfully fabricated porous scaffolds containing up to 70 wt% HA with optimized macrostructure ($\sim 600 \mu\text{m}$ pores). The scaffolds exhibited a compressive strength of up to 14.3 MPa, which is comparable to cancellous bone, indicating their potential for bone repair and regeneration applications. Garai and Sinha [57] synthesized biomimetic CMCâHA nanocomposites using an in situ process that avoids toxic crosslinkers and mimics natural matrix-mediated mineralization. The 3D-printed structures achieved mechanical properties—1.74–12 MPa in compressive strength and 157–330 MPa in modulusâthat fulfill the standards for cancellous bone grafts. In vitro cytotoxicity studies with rat bone marrow mesenchymal stem cells confirmed the nanocompositesâ biocompatibility, supporting their suitability as load-bearing synthetic graft materials. Gupta et al. [56] introduced a cryogenic 3D printing method to fabricate highly porous Gel/Carboxymethyl-Chitin/HA scaffolds with multiscale porosity, in Figure 2.7 The combination of cryoprinting and lyophilization enabled the formation of constructs with up to 90% microporosity, enhancing swelling capacity and significantly promoting the attachment, proliferation, and mineralization of both Saos-2 osteoblasts and human umbilical cord mesenchymal stem

cells. These soft, sponge-like scaffolds are particularly promising for patient-specific bone defects.

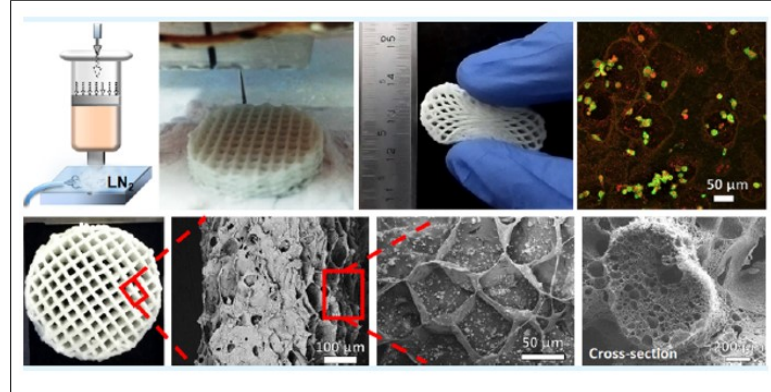


Figure 2.7 Fabrication, morphological characterization, and cytocompatibility of cryogenically 3D-printed Gel-based scaffolds [56].

Ravoor and Elsen [58] utilized robocasting to fabricate HA scaffolds reinforced with multiwalled carbon nanotubes (MWCNTs), using CMC as the polymeric binder. Vacuum sintering at 1000 °C effectively preserved MWCNTs and minimized oxidation. Among all groups, the 0.5 wt% MWCNT-HA scaffolds exhibited the highest compressive strength (3.36 ± 0.67 MPa) and demonstrated cytocompatibility over 35 days in vitro. These results highlight the potential of MWCNTs to improve the structural integrity of HA-based bioceramic scaffolds. Sarkar et al. [59] developed a self-assembled CMC-Gel-HA nanocomposite without using chemical crosslinkers, as shown in Figure 2.8. The resulting three-dimensional structures exhibited compressive strengths ranging from 40–86 MPa and elastic moduli between 0.4–1.2 GPa, closely resembling both cancellous and cortical bone. In vitro assays using MG-63 osteoblast-like cells confirmed high alkaline phosphatase activity and extracellular mineralization, indicating excellent osteogenic potential. The study highlights the promise of these nanocomposites for load-bearing orthopedic applications.

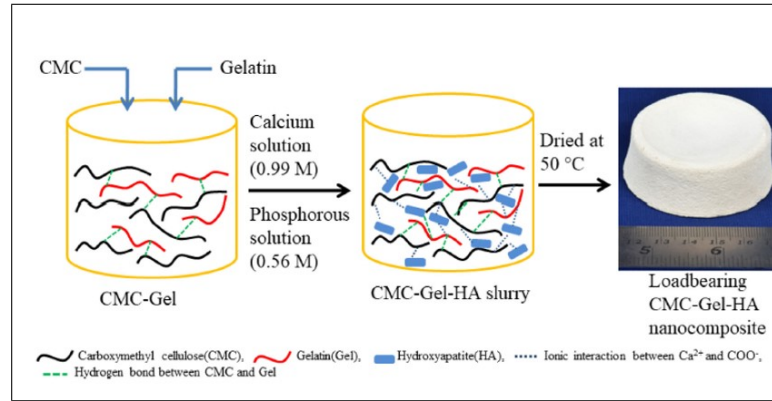


Figure 2.8 A schematic illustration depicting the fabrication process of robust three-dimensional CMC-Gel-HA nanocomposites [59].

2.3 Extrusion-based 3D Printing Technology

3D printing technology is also referred to as additive manufacturing (AM) which is a material manufactured layer-by-layer in desired shape. 3D printing is a producing process to build 3D objects from a digital file [17]. A digital 3D object is created by using computer aided design (CAD) and exported in stereolithography (STL) file format. Personalized structures can be generated through the use of Computed tomography (CT) or magnetic resonance imaging (MRI). To prepare the model for 3D printing, the STL file is converted into G-code (geometric code), which contains the specific instructions and parameters required to guide the printer during fabrication, as illustrated in Figure 2.9 [60],[61].

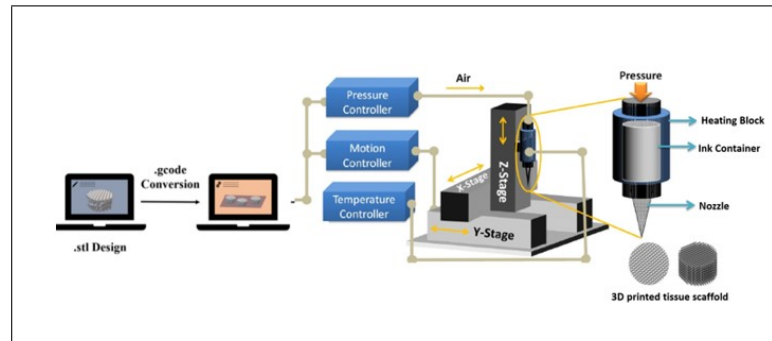


Figure 2.9 3D printing process to produce tissue scaffold [61].

There are three main types of 3D printing techniques: Inkjet Printing, Fused Deposition Modeling (FDM) and Laser-based printing. The working principle of FDM

involves extruding a molten polymer through a nozzle and depositing it layer by layer to form the desired shape. Extrusion printing is similar to FDM except for material. In extrusion-based 3D printing, hydrogel-based ink is used, and the crosslinking procedure is conducted for stability after printing. Extrusion systems is divided into three: screw-driven, piston- and pneumatic-, as shown in Figure 2.10 [62].

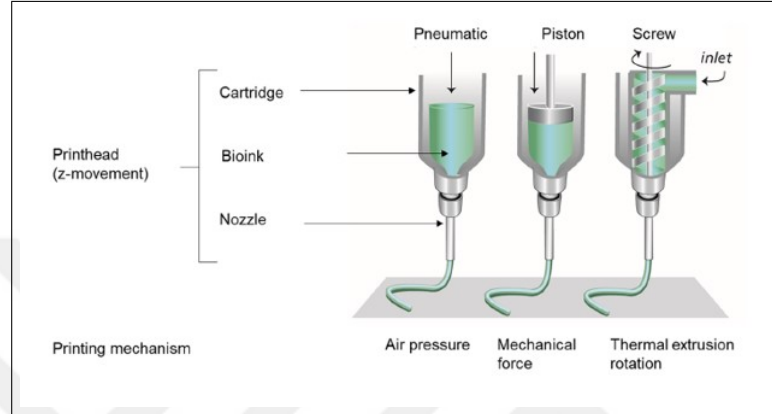


Figure 2.10 Extrusion based 3D printing technology [62].

In this study, air pressure system in hydrogel printing will be used in patient specific designs with irregular shape for bone tissue and cardiac patch for cardiac tissue. Figure 2.11 shows that bone fracture is scanned by CT and converted into the STL format to receive proper file format. This technology enable production of a special-designed scaffold with desired porosity [63],[64].

2.4 Gelatin

In the 3D printing tissue engineering, Gel, a denatured product of collagen is of high interest in printable polymer which is promotes cell attachment and proliferation and adhesion due to its structure containing Arg-Gly-Asp (rgd)-like sequence [20],[65]. Gel-based cardiac patches have the potential to integrate various desirable characteristics for cardiac tissue engineering [66],[67],[68]. Shabankhah et al. [69] reported that modifying the surface of 3D-printed poly(ϵ -caprolactone) (PCL)/graphene oxide (GO) composite (10% w/v, 0.02 gr, respectively) with Gel (3% w/v) provided a significant increase for both the electrical conductivity and H9C2 cardio myoblast cell

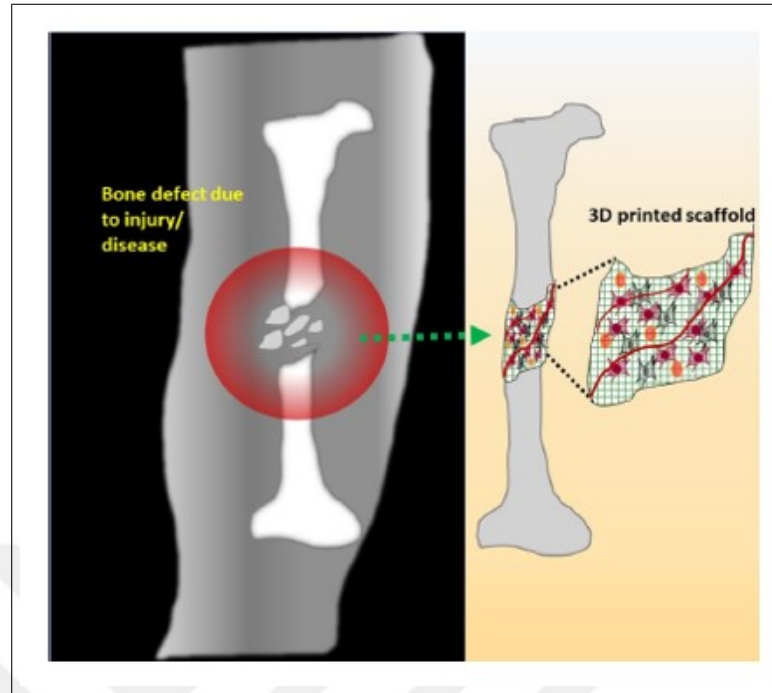


Figure 2.11 3D printing technology for treating bone tissue defects [64].

viability of the 3D-printed composites. Tijore et al. [51] fabricated micro-channeled microbial transglutaminase (mTgase) enzymatically crosslinked Gel (5%) scaffolds by 3D extrusion bioprinting method. These scaffolds demonstrated the capability to induce myocardial commitment and well-oriented growth of bone marrow-derived human mesenchymal stem cells (hMSCs), while also enhancing the function of native cardiomyocytes (CMs).

Ketabat et al. [70] have shown that when used with alginate, Gel is significantly more effective in promoting H9C2 cell proliferation compared to when it is used alone. However, the challenge of 3D printing in Gel-based polymeric solutions lies in creating a printable hydrogel with suitable viscosity that can be smoothly dispensed in a continuous line without spreading while also keeping its vertical structure [71]. Hence, a viscosity regulator and a crosslinking agent are required to maintain the desired consistency and flow characteristics of Gel-based hydrogels.

2.5 Carboxymethyl Cellulose

CMC is a water-soluble polysaccharide with hydrophilic hydroxyl and carboxyl groups [31]. CMC is gaining attention due to its viscosity promoter property, biocompatibility, biodegradability, and cost-effectiveness [72],[73]. Habib et al. [74] showed that by increasing the CMC concentration from 1 to 4% in the alginate hydrogel structure, each filament of 3D-printed scaffolds had a higher shape fidelity due to the excellent viscosity provider and thickening properties of CMC. It has also been demonstrated that CMC promotes epithelial cell migration and attachment to matrix proteins which enhance the wound healing [75]. Sedighim et al. [76] investigated the mechanical and biological performance of CMC (0.5%)-alginate (5%)-hydroxyethyl methacrylic acid (HEMA)-acrylic acid (AA) based hydrogels reinforced polyvinyl alcohol for cardiac tissue regeneration. Their scaffolds showed cell viability over 80% after 15 days. Here, effect of the CMC-Gel hydrogels on the viability of H9C2 cardiomyoblast cells was studied for the first time. Carbon nanostructures are used to induce electrical conductivity and biocompatibility requirements in cardiac patches. Velasco-Mallorquí et al. [77] showed that the electrical conductivity and C2C12 skeletal myoblasts cell viability have been enhanced by incorporating carbon nanotubes into Gel (2%)/CMC (1%) polymerized cryogel.

2.6 Hydroxyapatite

HA, a calcium phosphate material, is widely recognized for its compositional similarity to the mineral phase of natural bone, which makes it highly popular for bone tissue engineering [59]. Although it shows excellent biocompatibility, pure HA is inherently brittle and has low mechanical strength, limiting its application in load-bearing environments [56]. To address this, recent efforts have focused on developing HA-based composites by incorporating polymers such as chitosan, CMC, Gel [58]. These combinations enhance the mechanical properties as well as create more favorable the biological response for bone regeneration.

Chen et al. [19] utilized extrusion-based 3D printing to fabricate porous HA/Gel/CMC scaffolds. These scaffolds (with controlled macroporosity $\sim 600\text{ }\mu\text{m}$) at 70 wt% HA achieved compressive strengths up to 14.3 MPa, closely approximating that of cancellous bone, while maintaining high porosity necessary for tissue in-growth and vascularization. The study demonstrated that mechanics were strongly influenced by both HA concentration and print geometry. Moreover, the biocompatibility of the composites was confirmed, showing their suitability for bone repair application.

Similarly, Garai and Sinha [57] reported a biomimetic in situ synthesis of CMC/HA nanocomposites with compressive strengths ranging from 1.74 to 12 MPa, achieved through an in situ mineralization approach, regardless of the use of toxic crosslinkers. Their approach based electrostatic interactions between CMC and HA precursors to guide the nucleation and growth of HA nanoparticles within a polymer matrix, forming a mechanically robust and biocompatible network. These nanocomposites supported bone marrow stem cell adhesion and growth, and exhibited favorable behavior in simulated body fluid, indicating good bioactivity. Overall, such investigations confirm the potential of HA-polymer composites in bone engineering functional scaffolds capable of supporting bone healing in both structural and biological terms.

2.7 Graphene Oxide

Graphene oxide (GO) has -OH, COC, -C=O and -COOH functional groups which improves biocompatibility, adsorption and dispersibility [78]. In the study of Jiang et al. [79], electrically conductive composite scaffolds based on chitosan (CS) (20 g/L) were developed with GO (50, 100, 150, 600 mg/L) to mimic the electrical conductivity of cardiac muscle, supporting cardiac cell adhesion, extension, and cell-cell interactions, as well as enhancing the expression of specific cardiac proteins and genes even without external electrical stimulation. Park et al. [80] utilized GO to improve the therapeutic performance of mesenchymal stem cells (MSCs) following MI. Normally, high levels of harmful molecules (ROS) in the heart after a heart attack cause these cells to die. GO provided a supportive interface that improved MSC adhesion

and resistance to oxidative stress, thereby increasing cell engraftment and survival in the infarcted myocardium.



3. DEVELOPMENT OF 3D-PRINTED CARDIAC SCAFFOLDS

3.1 Preparation of Cardiac Hydrogel Inks

Gel (0.75 gr) was dissolved in 10 ml distilled water at 50 °C for 20 min. Then, CMC (0.75, 1 and 1.5 gr) powder was added to Gel solutions gradually. The polymers were mixed with magnetic stirrer at 50 °C until a homogenous solution was obtained. Then, the produced solution was loaded into the ink container. For the groups with GO, GO (w/w according to Gel concentration) was added to the Gel solution and sonicated (14 Watts) in ice bath for 2 hours. Then, CMC was added in the solution. Before printing, CMC/Gel based polymeric inks were centrifuged to remove bubbles at 4000 rpm for 4 min [81]. Thus, a homogenous polymeric solution was obtained leading to a smooth printing process. The polymeric inks with different compositions for cardiac patches are shown in Table 3.1.

Table 3.1
Compositions of polymeric inks used to prepare cardiac patches.

Group Name	CMC (w/v%)	Gelatin (w/v%)	GO (w/w%)
C7.5/G7.5	7.5	7.5	0
C7.5/G7.5/GO0.5	7.5	7.5	0.5
C7.5/G7.5/GO1	7.5	7.5	1
C10/G7.5	10	7.5	0
C10/G7.5/GO0.5	10	7.5	0.5
C10/G7.5/GO1	10	7.5	1
C10/G7.5/GO1.5	10	7.5	1.5
C15/G7.5	15	7.5	0
C15/G7.5/GO0.5	15	7.5	0.5
C15/G7.5/GO1	15	7.5	1

3.2 Rheological Analysis

Rheological studies were conducted using a stress-controlled rheometer (Anton Paar, MCR302, Graz, Austria) with a parallel plate geometry (diameter: 15 mm). Rheological measurements assist in optimizing the flow of polymeric inks with varying percentages of CMC. The oscillatory amplitude sweep test was performed to describe the linear viscoelastic region (LVR) of inks at 10 rad/s. Angular frequency sweep test was achieved with the frequency between 10^{-1} and 10^2 rad/s by applying 1% strain within the linear viscoelastic region. The temperature sweep test was performed from 25 °C to 80 °C. The dependence of ink viscosity on shear rate was analyzed at the range of 10^{-2} to 10^2 s⁻¹ shear rates to examine shear thinning properties. All rheological studies were conducted at 25 °C except for temperature sweep test.

3.3 3D Printing and Crosslinking of Cardiac Scaffolds

The cardiac scaffolds were fabricated by using Axolotl Biosystem A1 Bioprinted. The square shape of the 3D scaffolds was created by using Solidworks computer-aided design (CAD). The dimensions of the scaffolds were standardized as 10 mm × 10 mm × 2 mm. The printing speed was set as 4mm/s, while the needle type was chosen as 21G.

After the scaffolds were printed, as shown in Table 3.2, they were crosslinked by immersing in -(3-dimethylaminopropyl)-n'-ethylcarbodiimide hydrochloride (EDC): N-hydroxysuccinimide (NHS) solution with a ratio of 5:1 (in 90% ethanol) containing 100 mM:20 mM, 50 mM:10 mM, and 25 mM:5 mM for 1h at 4 °C, respectively [37]. To stop crosslinking procedure, the scaffolds were immersed in 0.1 M disodium hydrogen phosphate (Na₂HPO₄, Merck) solution for 2h [82]. Then, they were washed 3 times with phosphate buffer saline (PBS) solution and deionized (DI) water for following characterizations.

Table 3.2
Amounts of crosslinking concentrations.

Concentration Ratio	EDC (mM)	NHS (mM)
5:1	100	20
5:1	50	10
5:1	25	5

3.4 Characterizations of Cardiac Scaffolds

3.4.1 FTIR

To analyze interaction between Gel, CMC and GO of 3D-printed CMC/Gel based cardiac patches, FTIR (Nicolet FTIR Instruments, Thermofisher) was used. The FTIR spectrum was recorded from 4000 cm^{-1} to 550 cm^{-1} with 32 scans.

3.4.2 Size of Cardiac Scaffolds

Scanning electron microscopy (SEM) (FEI-Philips XL30) was used to analyze the cardiac patch morphology. Measurements of filament and pore sizes were taken from four different regions on SEM images [83].

3.4.3 Mechanical Analysis

The mechanical properties of the scaffolds were carried out on the samples. The mechanical properties under tension were assessed using a Universal Test Machine (Zwick/Roell Z 100) equipped with a 500-N load cell, and the crosshead speed was set at 5 mm/min. These specimens were prepared with dimensions of $10\text{ mm} \times 10\text{ mm} \times 2\text{ mm}$ and had an infill density of 30%. Young's Modulus was derived from the linear segment of the stress-strain curves [9],[36]. Five samples were repeated for each experimental group.

3.4.4 Electrical Conductivity

Electrical Conductivity of 3D-printed CMC/Gel based cardiac scaffolds was measured with four-probe working equipment (Gamry Instruments Reference 3000) from -0.01 V to 0.01 V at 1 s intervals. Samples were prepared with 30 mm × 4.5 mm × 12 mm dimensions with 40% infill density. Before the measurements, copper wires (0.54 mm diameter) were placed on the cardiac scaffold at equal intervals.

A small current I from a constant-current source was passed through the outer two probe tips and the voltage drop V was measured between the inner two probe tips. Below first equation (Eq. 3.1) include V (applied voltage), I (measure current), A (area of the electrodes), and L (distance between electrodes) shows the ρ (volume resistivity). Then, σ , electrical conductivity, was calculated as the second equation (Eq. 3.2), which is the inverse of the resistivity [84],[85]. Four replicates were examined for electrical conductivity test.

The resistivity was calculated given below Eq. 3.1:

$$\rho = \frac{V \times A}{I \times L} \quad (3.1)$$

Electrical conductivity was calculated in given Eq. 3.2:

$$\sigma = \frac{1}{\rho} \quad (3.2)$$

3.4.5 Biodegradation Tests

The swelling, degradation and pH behavior of 3D-printed cardiac scaffolds were characterized in PBS solution. The samples were printed with dimensions of $10\text{ mm} \times 10\text{ mm} \times 2\text{ mm}$ with 5 layers. The 3D-printed cardiac patches were placed in 12-well plates with 3 ml of PBS (pH:7.4) for each well at 37°C . PBS was refreshed after every three days.

The dry scaffolds were weighed (W_o) after crosslinking with EDC/NHS solution and then immersed in PBS solution. They were weighted (W_s) after 1, 7, 14, 21, and 28 days. For the degradation test, the measurements were taken after 1, 7, 14, 21 and 28 days [86]. When the samples were taken out from PBS solution, they were lyophilized for 24 hours and weighted (W_d). Three replicates were used for each study group. Below equations (Eq. 3.3 and Eq. 3.4) were used for % swelling and % degradation [87]. Moreover, pH measurements of 3D-printed cardiac patches were taken after 1, 7, 14, 21 and 28 days.

$$\% \text{ Weight loss} = \frac{W_o - W_d}{W_o} \times 100 \quad (3.3)$$

$$\% \text{ Water uptake} = \frac{W_s - W_o}{W_o} \times 100 \quad (3.4)$$

3.4.6 Water Contact Angle Test

The surface hydrophilicity of CMC/Gel/GO 3D-printed cardiac patches were evaluated by contact angle measurements by using KSV Instruments LTD CAM101 at room temperature. The deionized water was dropped on the surface of each sample in three parts, each image was captured after the first second [76].

3.4.7 Cell Culture Studies

H9C2 rat cardio myoblast cells (CRL1446-Sigma) were cultured in high-glucose Dulbecco's Modified Eagle Medium (DMEM, Sigma) supplemented with 10% fetal bovine serum (FBS, Biowest), 2 mM L-glutamine (Biowest 100X-200 mM) , and 1% penicillin-streptomycin (Biowest) under standard cell culture atmosphere conditions (37 °C, 5% CO₂ and 95% humidity). The media was changed every 2 days [88],[89].

Before the cell seeding, the scaffolds (10 mm × 10 mm × 2 mm) were sterilized with 70% ethanol and UV irradiation for 2h [68]. 5×10^4 cells were seeded on each well of 24 well-plate [70].

To analyze the relative % cell viability and proliferation, the Alamar Blue assay (AlamarBlue™ Cell Viability, Invitrogen) was conducted on 3 and 7 days. In order to distinguish between cells cultured on the scaffolds and those cultured around or on the well plate surface, the scaffolds were removed from their original plates to new ones [70]. On the analysis day, the culture medium was replaced by the test solution comprising of culture medium with 10% Alamar Blue solution. Then, scaffolds were incubated for 3.5 h. Following the completion of the incubation period, 100 μ l of solution (as three parallel) was exemplified to a 96 well-plate and optical densities of solutions were read using a microplate reader (Bio-Rad,iMark™) at 570 and 595 nm wavelengths [90],[91].

Tissue culture plate (TCP) on third day was considered as 100% to analyze and other values normalized accordingly.

Fluorescence imaging was performed to show the cell spreading and morphology of the cytoskeleton, for cultured H9C2 cells on the cardiac patches. After 3 days of incubation, the scaffolds were rinsed with 4% (w/v) paraformaldehyde (PFA) solution to fix the cells for 10 min, following this, permeabilized in 0.2% Triton X-100 for 20 min at room temperature [68]. Then, samples were immersed in 1% bovine serum albumin (BSA) to block unspecific bonding for 1 h. To stain actin filament (F-actin)

and nucleus, Phalloidin-iFluor 488 reagent (ab176753, Abcam) and 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) were used for 1h and 20 min in dark, respectively [92]. The scaffolds were observed using a fluorescence microscope (Carl Zeiss, Axio Vert. A1) with 10 $\times$ magnification. ImageJ software was employed for the analysis of the images. Live/dead assay was conducted by diluted acridine orange [AO]-propidium iodide [PI] solution mixture. The samples were gently shaken in this solution for 1 min. Then, solution was removed, and the samples were washed with twice PBS. Fluorescence imaging was utilized to observed live/dead cell [93].

3.5 Statistical Analysis

The data was analyzed by using a one-way analysis of variance (ANOVA). Significance was determined by p-values: * for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$, showing meaningful differences between the data.

3.6 Results and Discussion

The rheological behavior of the hydrogels was examined to investigate their printability and mechanical properties. Figure 3.1 (a), (b), and (c) show the amplitude sweep test, angular frequency test, and shear rate by shear viscosity, respectively.

Initially, an oscillatory amplitude-based strain-sweep test was conducted to determine the linear viscoelastic region (LVR), ensuring that the hydrogels maintained their structural integrity up to a strain value ranging from 0.01% to 100%, as depicted in Figure 3.1 (a). Higher storage modulus (G') was observed with increase in CMC, which show the enhanced shape fidelity of cardiac patches [83]. Figure 3.1 (a) also shows that the % strain required to disrupt the network structure of CMC/Gel hydrogels decreased with increasing CMC content. All hydrogel groups exhibited a substantial viscoelastic region. 1% strain was considered as suitable point for subsequent

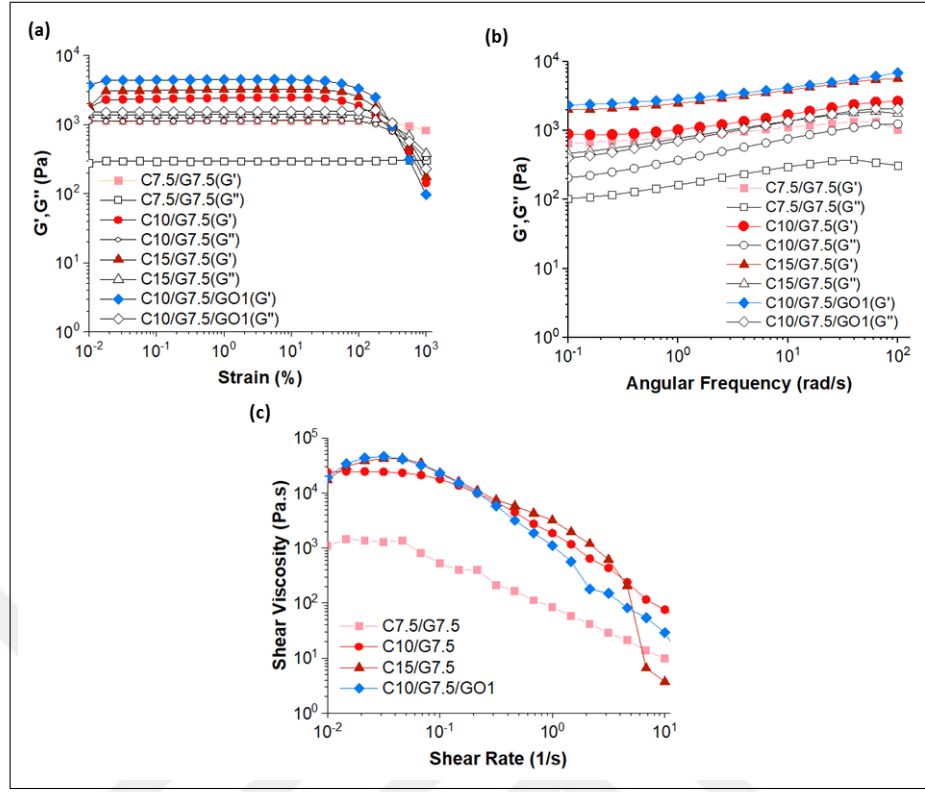


Figure 3.1 (a) Amplitude-dependent variation at frequency 10 rad/s and (b) frequency-dependent variation at 1% strain of G' and G'' changes, (c) shear-rate dependent variation of viscosity of C7.5/G7.5, C10/G7.5, C15/G7.5 and C10/G7.5/GO1 hydrogels at 25 °C.

frequency and temperature sweep tests [94].

According to Figure 3.1 (b), with an increase in the frequency, storage modulus had a dominant characteristic compared to loss modulus (G''), which showed a significant elastic behavior (solid-like) response for all hydrogels [95]. C10/G7.5/GO1 exhibited higher values for G' and G'' compared to the other groups, indicating a higher storage and viscous behavior, respectively [96]. Furthermore, for all study groups, G' and G'' showed a broad viscoelastic plateau along the entire studied frequency range, considered as an indication of stable inks [86].

The shear thinning property have an essential role in the printability of hydrogels [97]. As shown in Figure 3.1 (c), the shear viscosity for various hydrogels concentrations (7.5, 10, and 15 % CMC) was decreased with increase of shear rate, which indicated all hydrogels had the shear thinning behavior (non-Newtonian regime) considered suitable

for printing.

Figure 3.2 shows temperature-dependent changes on G' , G'' , complex viscosity, printing temperature, and printing pressure of CMC/Gel hydrogels.

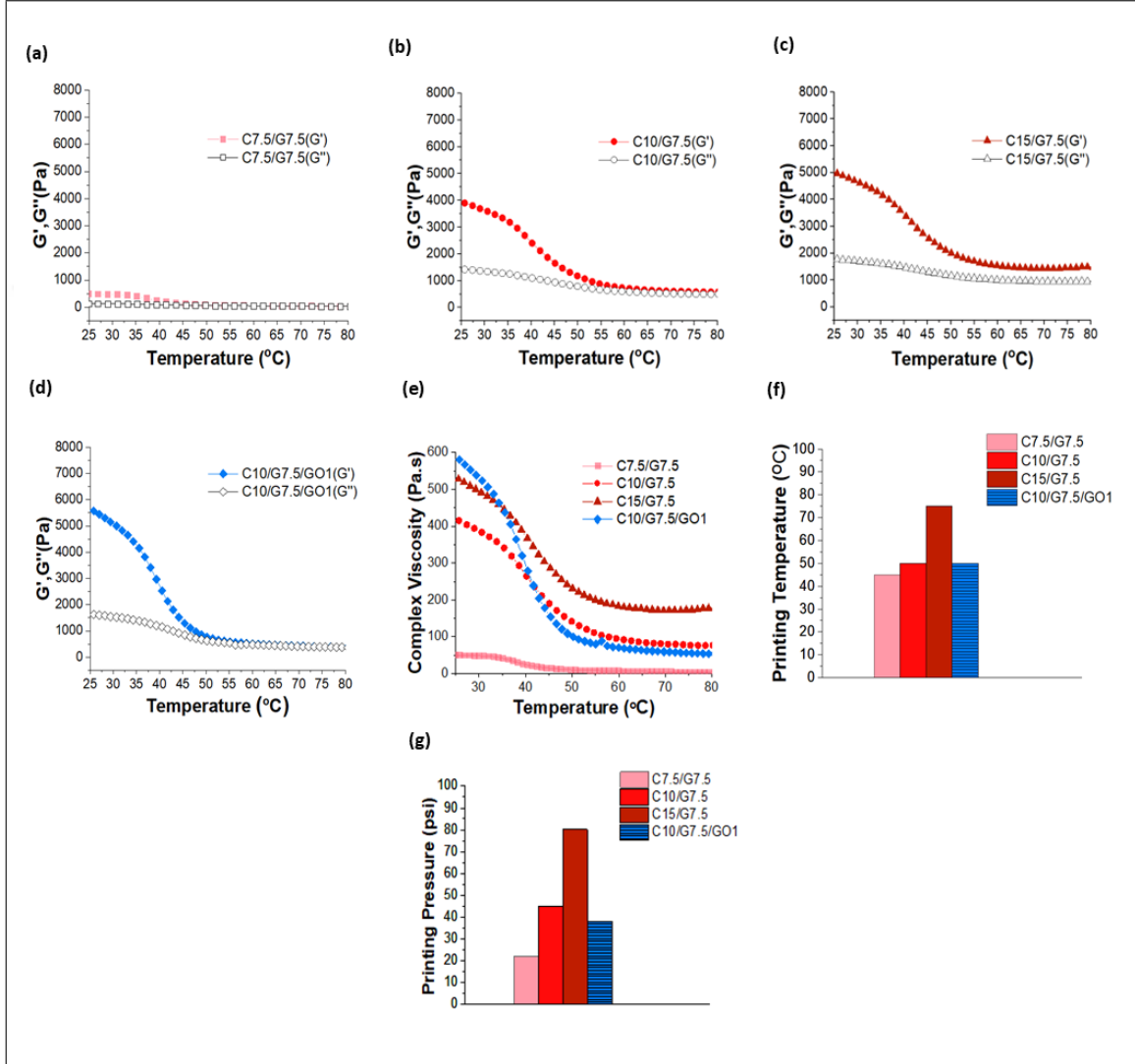


Figure 3.2 Temperature-dependent variation of G' and G'' of (a) C7.5/G7.5, (b) C10/G7.5, (c) C15/G7.5, and (d) C10/G7.5/GO1. (e) temperature dependent variation of complex viscosity for all groups at 1% strain and 10 rad/s angular frequency between 25 and 80 °C. (f) printing temperatures and (g) printing pressures of study groups.

The gelation point according to the temperature of hydrogels was assessed to ensure printing stability [98]. Figure 3.2 (a-d) shows that with increase in CMC concentration, gelation temperature gradually increased. As illustrated in Figure 3.2 (e), it is apparent that the viscosity of the group containing GO is higher than that of

the group characterized by the highest CMC concentration ratio (C15/G7.5), at room temperature. However, with increasing temperature, there is a substantial decline in viscosity observed for C10/G7.5/GO1 group, such that it falls below the viscosity value of the C10/G7.5 group.

The extrusion pressure was set within the range of 18 psi to 85 psi. The printing temperature was set within the range of 36 °C to 106 °C [49],[97]. The optimized printing parameters for all other groups, where mechanical testing has been completed, are given in Table 4.2.

Table 3.3
Printing parameters of inks.

Sample	Printing temp. (°C)	Pressure (psi)
C7.5/G7.5	45	18–21
C7.5/G7.5/GO0.5	36	28–31
C7.5/G7.5/GO1	40	26–28
C10/G7.5	50	40–48
C10/G7.5/GO0.5	50	35–43
C10/G7.5/GO1	50	35–40
C10/G7.5/GO1.5	50	45–47
C15/G7.5	70–80	75–85
C15/G7.5/GO0.5	80	77
C15/G7.5/GO1	106	76–85

Achieving the appropriate degree of flexibility and stiffness of cardiac patch is critical both to prevent deformation during surgical implantation and to maintain endurance to contractility of cardiac tissue [88]. A too high elastic modulus of a cardiac tissue may have the potential to be detrimental to the structural integrity of the heart. In this study, firstly, different concentrations of GO-incorporated CMC/Gel cardiac patches were produced, and the effect of EDC:NHS, CMC and GO concentrations on the mechanical properties were investigated, as shown in Figure 3.3.

Figure 3.3 (a) and (b) shows the EDC:NHS concentrations (25:5, 50:10, and 100:20) effect on the elastic modulus and % strain at break of different concentrations

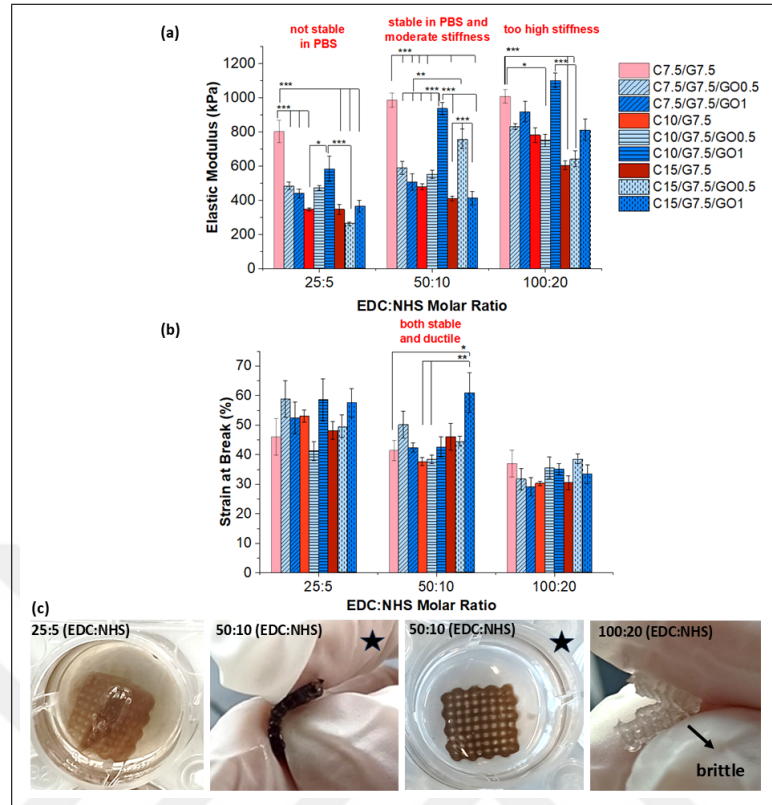


Figure 3.3 Mechanical characterization of prepared CMC/Gel based 3D-printed cardiac patches with different gel, CMC and GO concentration: (a) Elastic Modulus and (b) Strain at Break. (c) Representative images of specimens with different EDC:NHS concentrations and their flexibility. ★ shows the ideal mechanical behavior of the cardiac patches. (n = 5) one-way ANOVA, Tukey test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

of CMC/Gel and GO incorporated CMC/Gel 3D-printed cardiac patches. The results showed that the mechanical properties of CMC/Gel-based 3D-printed cardiac patches were adjusted by varying the concentration of EDC:NHS, CMC and GO to match the mechanical properties of human myocardium which has elastic modulus of 20-500 kPa. As shown in Figure 3.3 (a) and (b), as the EDC:NHS concentration increased, there is a clear trend of increase of elastic modulus and decrease of % strain at break, respectively. The expected outcome was consistent with the findings of Adhirajan et al.'s study[99]: a higher EDC:NHS concentration reduces free amine groups, resulting in a higher percentage of covalent crosslinking between amino and COOH groups of Gel groups.

A previous study has reported that increasing covalent bonding reduces extensibility and increases elastic modulus [68]. Moreover, a high cross-linking ratio (100:20),

which resulted in a fragile and brittle material, may not be suitable for achieving the desired stiffness in cardiac tissue engineering scaffolds as illustrated in Figure 3.3 (c).

Based on the three days degradation study in PBS, it is observed for C10/G7.5/GO1 in Figure 3.3 (c), 25:5 study groups did not have stability, on the other hand 50:10 and 100:20 groups demonstrated high stability due to sufficient covalent crosslinking.

Figure 3.4 (a) and (b) shows the effect of CMC and GO on the mechanical properties of cardiac patches, respectively.

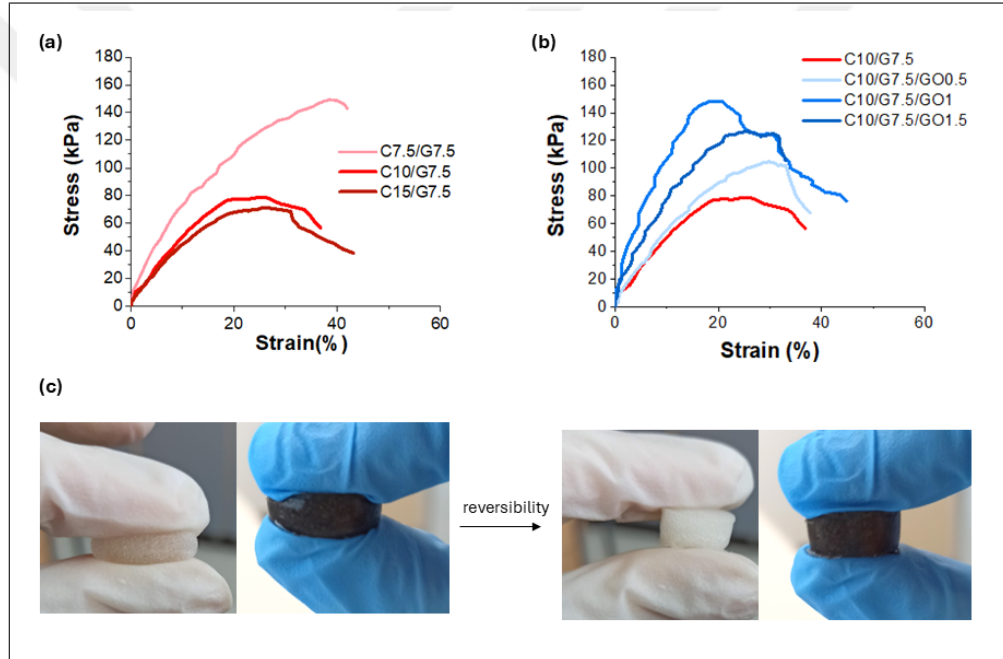


Figure 3.4 (a) CMC and (b) GO effects on the mechanical properties for cardiac patches prepared with 50:10 EDC: NHS ratio. (c) demonstration of reversible property of C10/G7.5 and C10/G7.5/GO1 (it has been produced in bulk to show their flexibility).

Figure 3.4 (a) illustrates stress-strain curves for the effect of concentration of CMC on CMC/Gel-based 3D-printed cardiac patches. As CMC concentration increased from 7.5% to 15%, the elastic modulus decreased from 986.76 kPa to 410.87 kPa. CMC is shown to increase % strain at break with increase of its wt % in the scaffolds however this effect was not significant as illustrated in Figure 3.4 (a). According to Jacquart et al. [100], their research revealed that incorporating CMC into the calcium phosphate cement matrix led to a significant enhancement of the ductility

of the scaffolds. This is also likely the effect of the reversible hydrogen bonds formed by CMC, which facilitate energy dissipation [101], as shown in Figure 3.4 (c). Strong bonds act as permanent crosslinks such as covalent bonds, providing stiffness, while weak bonds such as hydrogen bonding break and reform reversibly, dissipating energy leading to flexibility of the scaffolds [102].

In Figure 3.4 (b), the stress-strain curves of C10/G7.5, C10/G7.5/GO0.5, C10/G7.5/GO1, and C10/G7.5/GO1.5 were shown for the cardiac patches. From these curves, by the increase in GO concentration to 1% within C10/G7.5, elastic modulus is significantly increased due to the increase in hydrogen bonding between GO and both polymers [103]. For C10/G7.5 study groups, with addition of 1 wt% GO, Elastic modulus is increased by 96% and % strain at break is increased by 13%, as shown in Figure 3.5. The formation of uniform Gel/GO aqueous dispersions occurred because of the favorable reversible hydrogen bonding interactions between the functional groups on GO and the polar groups within the Gel macromolecular chains, which gives flexibility to the material as shown in Figure 3.4 (c) [103]. For 1.5% GO, agglomeration of GO occurred within CMC/Gel hydrogel, which led to decrease of the elastic modulus compared to C10/G7.5/GO1.

In our previous study, Ege et al. [104] investigated the effects of EDC:NHS, CMC, and GO concentrations on mechanical properties utilizing Extreme Gradient Boosting (XGB) machine learning model to this dataset. Feature importance of the independent parameters was evaluated for flexibility, UTS and elastic modulus. According to this study, EDC/NHS concentration was found to be the most influential factor on flexibility following with GO and then CMC. This finding supported our experimental studies. For modulus, EDC/NHS concentration was also the most influential factor which was found to be very critical. CMC and GO had almost equal contribution to these two mechanical parameters. On the other hand, for UTS, CMC had almost equal contribution with NHS/EDC concentration and the effect of GO was less significant. These results can also be observed from our illustrated experimental studies.

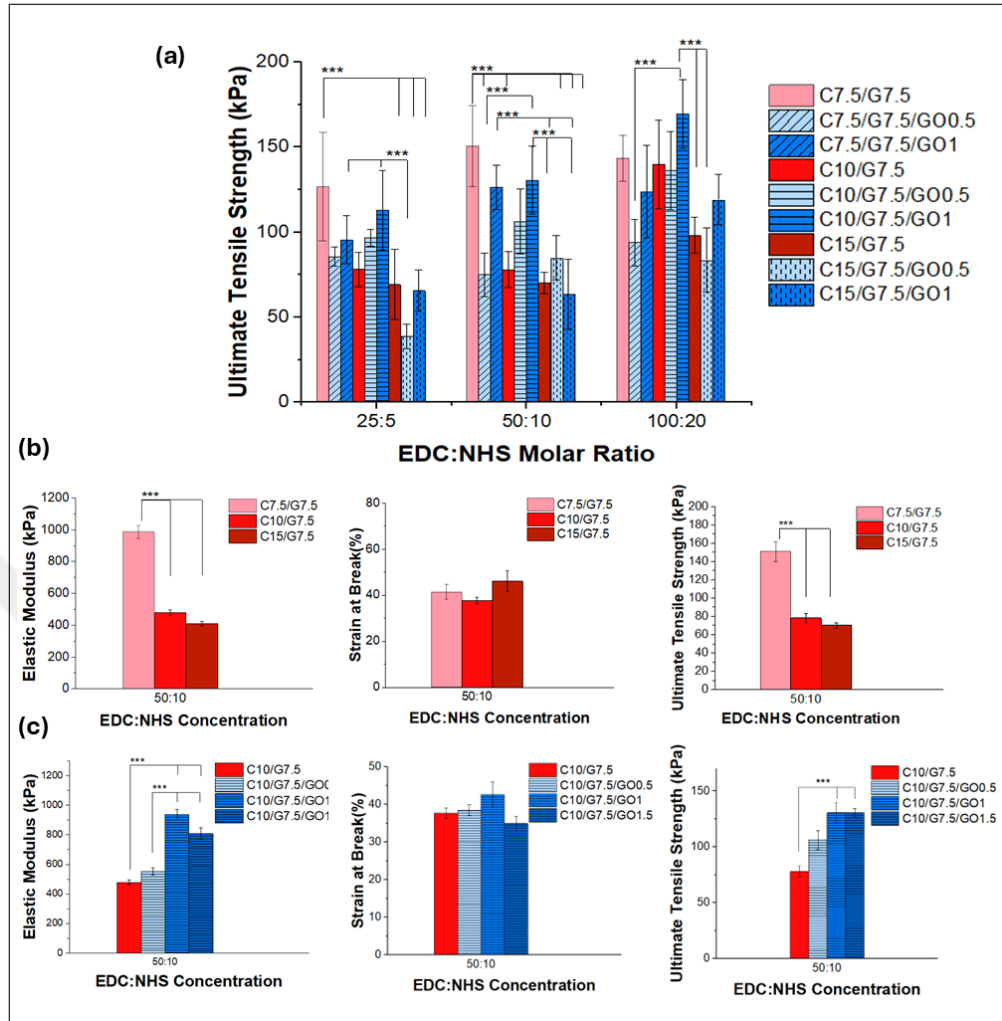


Figure 3.5 (a) UTS of CMC/Gel and CMC/Gel/GO cardiac patches, (b) elastic modulus, strain at break (%), and UTS of CMC/Gel cardiac patches. (c) elastic modulus, strain at break (%), and UTS of CMC/Gel/GO cardiac patches. (n=5) one-way ANOVA, Tukey test * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 3.6 (a-d) shows the SEM images of cardiac patches printed with 30% infill density and 5 adjacent layer. The pore and filament sizes of the cardiac patches were determined by analyzing the SEM images. The incorporation of a higher concentration of CMC into CMC/Gel resulted in a significant decrease in pore size and an increase in filament size (** $p < 0.001$). Likewise, the addition of 1% GO to CMC/Gel similarly led to smaller pores and larger filaments (** $p < 0.001$). This phenomenon attributed to results of rheological study. At high concentrations of CMC, printing temperature and pressure values were increased to ensure printability. This allows more amount of ink to come out per unit of time at a consistent printing speed, leading to ink accumulation within the filament. As a result, a higher concentration of CMC and incorporation of

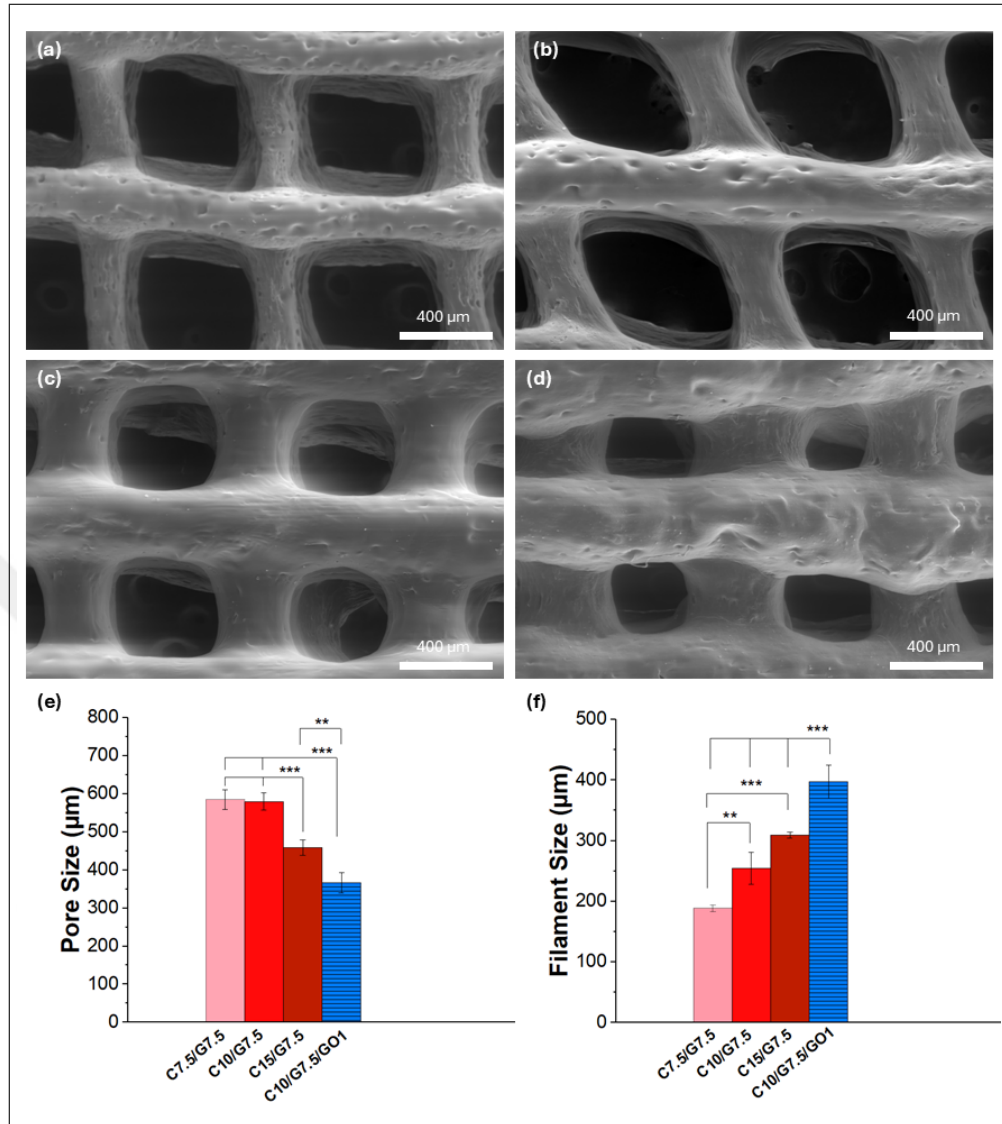


Figure 3.6 SEM images of (a) C7.5/G7.5, (b) C10/G7.5, (c) C15/G7.5 and (d) C10/G7.5/GO1, (e) pore size, and (f) filament size of cardiac patches. (n = 4) (scale bar: 400 μm).

GO decreases pore size while increasing filament size [105].

The spectra of all CMC/Gel based cardiac patches showed the same characteristic peaks. To confirm the interaction between CMC, Gel and GO within the cardiac patches, FTIR spectroscopy was used to examine changes in functional groups among the different patches. The observed shifts in bands from 3261 cm^{-1} to 3278 cm^{-1} indicate stretching vibrations of N-H and O-H bonds between CMC and Gel, as shown in Figure 3.7 (a). The band at 2983 cm^{-1} indicated aliphatic C-H stretching [106]. Additionally, the peak at 1636 cm^{-1} is assigned to the -COO^{-1} stretching vibration of

carboxymethyl groups and C=O stretching of amide band, providing further evidence of electrostatic interactions (ionic interactions) between CMC and Gel due to the negatively charge (carboxyl groups) of CMC and positively charge (amino groups) of Gel [106],[107],[108]. This peak also overlapped the band of amide bonds (amide I) to show the stable amide bond for all study groups [109]. The peak at around 1044 cm^{-1} confirms C-O-C bond stretching vibrations [107]. EDC plays a role in facilitating the reaction between molecules that possess free carboxylic and amine groups, leading to the formation of covalently amide bonds, as shown in Figure 3.7 (b) [35]. The increase in CMC and GO results in a higher number of ionic bonds between CMC and Gel and hydrogen bonding among CMC, Gel and GO (blue and green points, respectively). Furthermore, Figure 3.7 (c) shows representative images of 3D-printed cardiac patches.

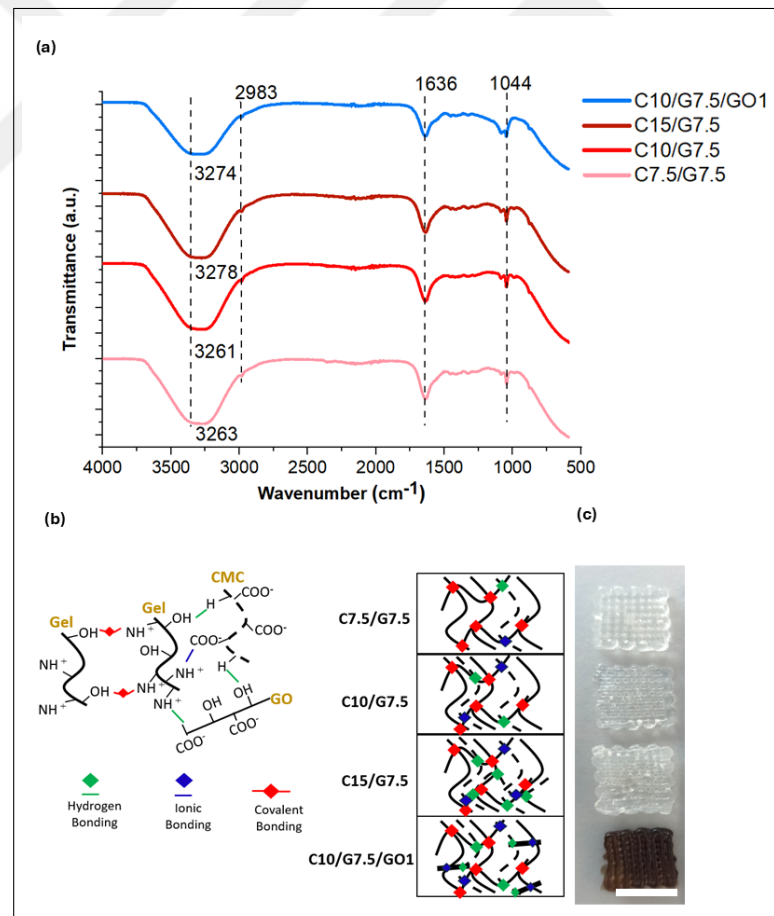


Figure 3.7 (a) FTIR spectrum, (b) crosslinking mechanism among CMC/Gel-GO, and (c) representative images and 3D-printed cardiac patches (scale bar: 10 mm).

The surface wettability of 3D-printed CMC/Gel based cardiac patches is assessed quantitatively using contact angle measurements, which plays a crucial role in cell growth and adhesion [110]. The water contact angle results of CMC/Gel-based 3D-printed cardiac patches were shown in Figure 3.8 (a) and (b). Electrical conductivity was assessed using the experimental setup shown in Figure 3.8 (c), with the results presented in Figure 3.8(d).

According to Figure 3.8 (a), C10/G7.5 showed the highest value of contact angle among all study groups. A dramatic decrease of contact angle ($34^\circ \pm 1^\circ$) was observed for C15/G7.5, suggesting the non-homogenous mixing and remaining of abundant unbound OH groups of CMC. C10/G7.5/GO1 had the most hydrophilic surface with a contact angle of $15^\circ \pm 3^\circ$ among all groups, the difference was found to be statistically significant. This is likely due to the presence of abundant hydrophilic functional groups of GO [111].

Following a myocardial infarction, the fibrous tissue often lacks electrical conductivity [112]. To address this issue, a conductive cardiac patch could support the heart contractions and help spread electrical signals in the damaged area [89]. Shabankhah et al. [69] showed that Gel coating and GO incorporation significantly increased the electrical conductivity of Poly(ϵ -caprolactone) (PCL)-based cardiac patches, which is from 2.75×10^{-4} to 1.49×10^{-2} S/m.

An increase in the CMC from 7.5% to 10% resulted to a significant decrease in conductivity of cardiac patches from 70.3×10^{-4} to 45.2×10^{-4} , as illustrated in Figure 3.8 (d). This is probably due to the a lower Gel content within the group.

Water absorption is another important property of tissue scaffolds, which affects the cell growth, nutrient transfer, and gas exchange [113]. As shown in Figure 3.9 (a), incorporation of 15% CMC within cardiac patches, resulted in no significant increase in swelling ratio. Similarly, as the loading GO increased, more water molecules were retained within the composite hydrogels which also increased swelling [103]. C7.5/G7.5 may not be able to swell due to its high degree of degradation, which is showing a linear

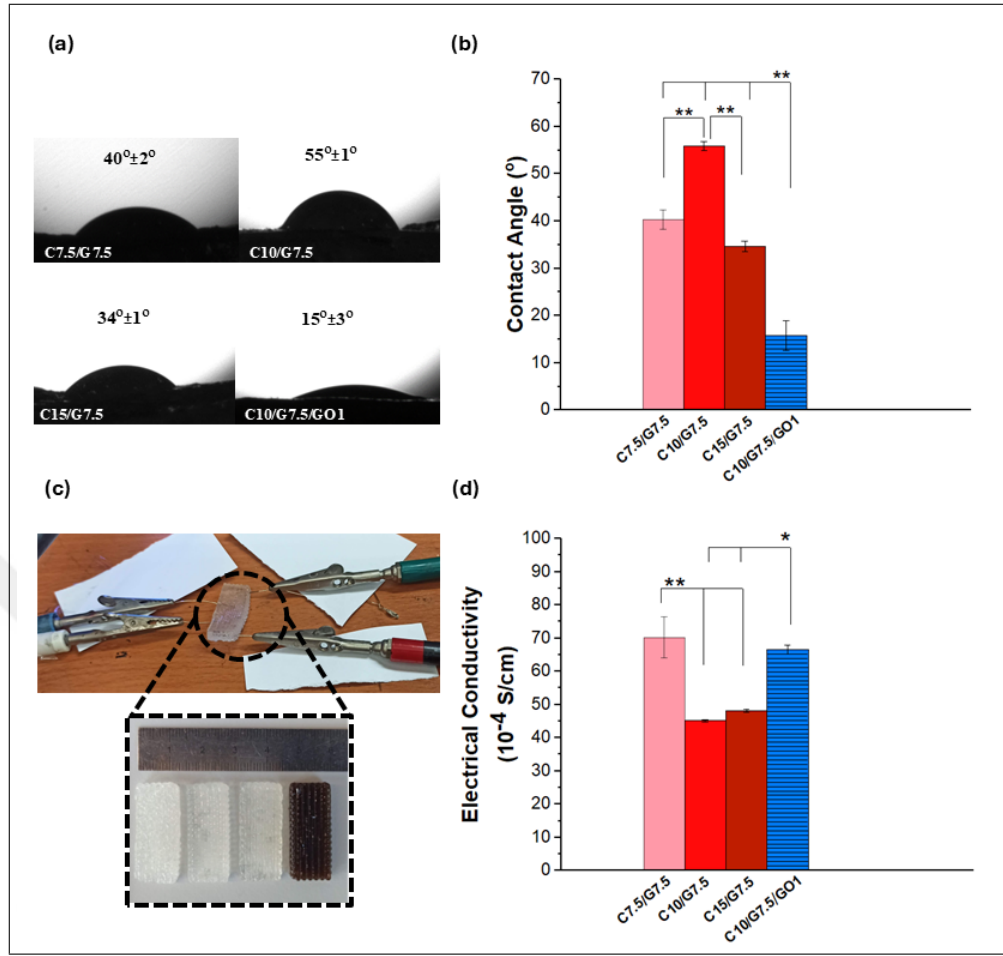


Figure 3.8 (a) Illustration of contact angle measurement of the samples, (b) contact angle values, (c) experimental setup for electrical conductivity and (d) electrical conductivity values. $n=3$, one-way ANOVA, Tukey test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

increasing trend in Figure 3.9 (b). pH measurements of all groups were close to the normal pH range of body fluids which is 7.3-7.45, as illustrated in Figure 3.9 (c) [114]. Moreover, it is shown in Figure 3.9 (d) that there is more noticeable degradation in the C15/G7.5 group after 28 days.

The relative (%) cell viability of H9C2 cardiomyoblast cells cultured on the 3D-printed cardiac patches was analyzed by Alamar Blue assay for 3 and 7 days. As shown in Figure 3.10 (a), significantly higher relative % cell viability was observed as the content of CMC increased in the CMC/Gel scaffolds on day 3 ($p < 0.01$). This is possibly due to support of cell viability and adhesion with the addition of CMC as reported in previous studies [76].

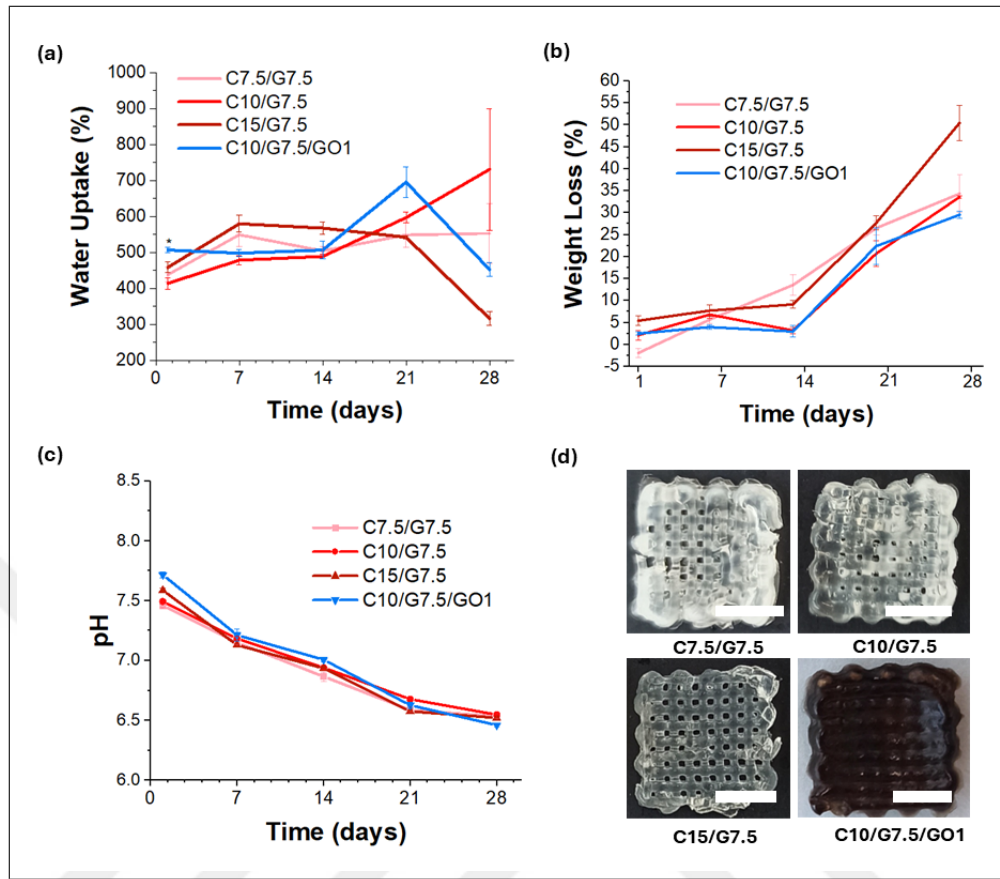


Figure 3.9 (a) % water uptake , (b) % weight loss, and (c) pH measurements for cardiac patches, (d) images of samples after 28 days incubation. $n=3$, one-way ANOVA, Tukey test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Cytoskeletal organization and cell nucleus was shown by F-actin and DAPI staining after 3 days incubation is shown in Figure 3.10 (b-e). According to the F-actin and DAPI staining results, there is high degree of attachment of H9C2 cells observed on all study groups. Representative fluorescence images for live/dead were presented in Figure 3.10 (f-i). The majority of cells showed in green-stained (live) cytoplasm while small portion of them was observed in red-staining (dead) [89].

By day 7, all groups exhibited a slight increase, maintaining the same trend observed on day 3. Statistical differences ($p < 0.01$) between C7.5/G7.5 and C15/G7.5, and C10/G7.5 and C10G7.5/GO1 confirm that increase of wt% of CMC and incorporation of GO is supportive for H9C2 cell viability. The positive effect of GO is consistent with results of Ghasemi et al.[93] which demonstrated that the addition of GO to polyethylene terephthalate (PET) samples enhanced the H9C2 cell adhesion

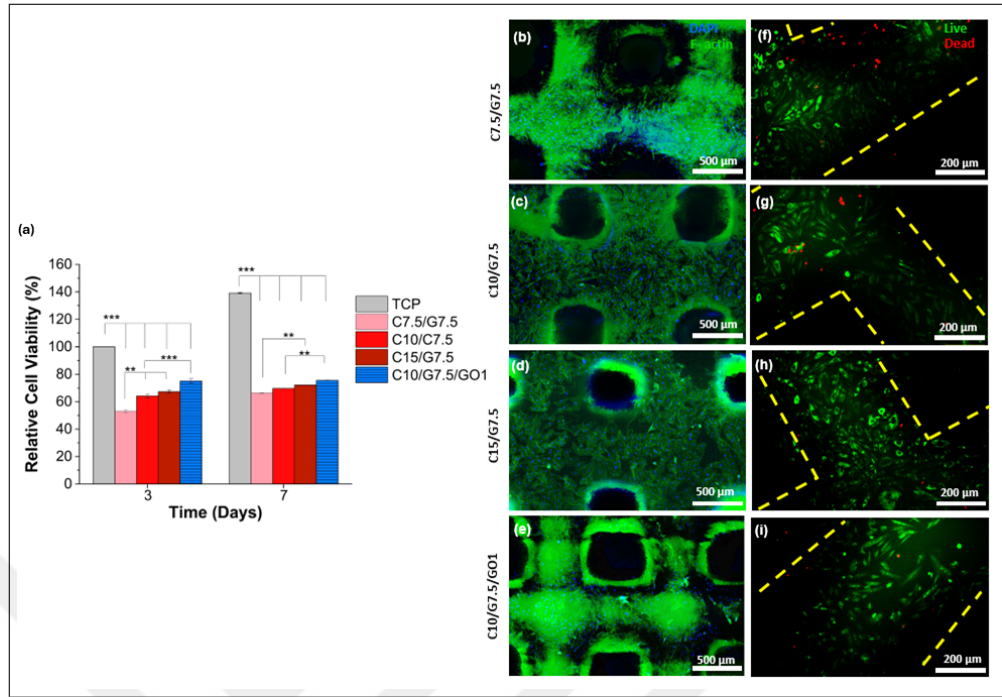


Figure 3.10 (a) Relative cell viability (%) of H9C2 cardio myoblast cells on the CMC/Gel cardiac patches on day 3 and 7. (b-c-d-e) Fluorescence images and (f-g-h-i) Live/dead stained H9C2 cells cultured on 3D-printed cardiac patches on day 3. The dotted yellow lines indicate the locations of the filaments. n=9, one-way ANOVA, Tukey test, * p<0.05, ** p<0.01, *** p<0.001

and spreading.

However, compared to TCP for day 7, the relative % cell viability is significantly lower. This implies that the cytocompatibility of the samples requires to be improved. This can be achieved by incorporation of growth factors such as vascular endothelial growth factor (VEGF) [115], transforming growth factor(TGF- β) [116], and fibroblast growth factor (FGF) [117] into the hydrogels.

In the future studies, connexin-43 (cx-43) [118] and cardiac troponin T (cTnT) [119] will be performed to assess the cell-to-cell communication and cardiomyocyte maturation. Additional analyses will focus on the expression of α -actinin protein [120] to analyze contraction of myocardium. One possible future direction includes examining the efficacy of gold nanoparticles [121] on these cardiac patches due to their electrical conductivity. Additionally, Gel could be stabilized to obtain higher myocardial regeneration and long-term construct stability using the enzymatic action of microbial

transglutaminase (mTgase) [122] as a natural crosslinker. Future study will also concern the incorporation of alginate [123], cardiomyocytes or induced pluripotent stem cells (iPSCs) [124] to CMC/Gel cardiac tissue platform to further improve its mechanical and biological properties.

In conclusion, CMC/Gel/GO cardiac patches were developed with tunable compositions, in this chapter. CMC and GO enhanced viscosity and flexibility, while optimal EDC/NHS crosslinking (50:10 mM) provided balanced mechanical strength. The scaffolds showed promising electrical and degradation properties, though further work is needed to improve cytocompatibility. Overall, the findings support their potential for cardiac tissue engineering.

4. DEVELOPMENT OF 3D-PRINTED BONE SCAFFOLDS

4.1 Preparation of Bone Hydrogel Inks

First, Gel solution (7.5 % and 15 % w/v) was prepared using a magnetic stirrer at 50 °C. For the final solution, HA (10 wt% and 20 wt %) and CMC (7.5 % w/v) were completely dissolved in the Gel solution at 50 °C, respectively. All hydrogels were centrifuged at 1000 rpm for 15 min to remove the bubbles. Table 4.1 shows the composition of produced scaffolds. HA was determined as the total wt%.

Table 4.1
Ink contents.

Formulation	HA (%)	Gel (%)	CMC (%)
G7.5/C7.5	–	7.5 %	7.5 %
HA10/G7.5/C7.5	10 %	7.5 %	7.5 %
HA20/G7.5/C7.5	20 %	7.5 %	7.5 %
G15/C7.5	–	15 %	7.5 %
HA10/G15/C7.5	10 %	15 %	7.5 %
HA20/G15/C7.5	20 %	15 %	7.5 %

4.2 Rheological Analysis

Rheological tests were performed using stress-controlled rheometer (Anton Paar MCR302, Graz, Austria) with 15 mm parallel plate geometry. The linear viscoelastic region (LVR) was determined via amplitude sweep test at 10 rad/ s. Then, the frequency test was conducted from 0.1 to 100 rad/s, applying 1% strain. Storage modulus was measured under 1% strain for all study groups. Temperature-dependent behavior was assessed from 25 °C to 80 °C. Additionally, shear rate-dependent viscosity was evaluated between a shear rate of 0.01 and 100 s⁻¹ [125].

4.3 Printing Characterizations

4.3.1 Printability Test

In the printability test, the shape fidelity of the filaments at different pressure values (16-31 psi) was determined. A single layer has been printed on the platform for each composition of the hydrogel inks.

4.3.2 Filament Collapse Test

Filament collapse test was conducted with a linear array of six pillars. The distance between the pillars was 16 mm, 8 mm, 4 mm, 2 mm, and 1 mm [126]. The images were immediately captured by camera once the printing was performed in 60 seconds. With optimized printing pressure and printing temperature, the ideal printing speed was determined by the filament collapse test.

4.3.3 Printing Accuracy Test

Printing accuracy was assessed by quantitatively comparing the average areas of squares (2D-grids) in the designed printing model with the areas of the squares in printed hydrogel scaffolds. From the image of bone scaffolds after printing, the areas were calculated by Image J. The average grid area of the designed model was calculated as $0.232 \mu\text{m}^2$ (A_i), whereas the grid area of any printed scaffold is represented by A . Then, % printing accuracy was determined by below Eq. 4.1 [127]:

$$\text{Printing accuracy (\%)} = \left[1 - \left| \frac{A_i - A}{A} \right| \right] \times 100 \quad (4.1)$$

4.4 3D Printing and Crosslinking of Bone Scaffold

The Axolotl Biosystems A1 Bioprinter was used to fabricate Gel/CMC and nano-HA/Gel/CMC bone scaffolds using a 21-gauge needle. The printing speed was set to 6 mm/s. The scaffolds were printed with a square-shaped porous structure, with varying sizes tailored for each specific characterization. Table 4.2 shows the printing parameters for each study group.

Table 4.2
Printing temperature and pressure values of different hydrogel formulations.

Sample	Printing Temperature (°C)	Printing Pressure (psi)
G7.5/C7.5	40	18–20
HA10/G7.5/C7.5	40	19–23
HA20/G7.5/C7.5	40	23–30
G15/C7.5	70	17–20
HA10/G15/C7.5	70	23–30
HA20/G15/C7.5	70	26–36

After the scaffolds were printed, they were crosslinked with EDC:NHS solution (100 mM:20 mM in 90% ethanol). After 16 h, crosslinked scaffolds were kept in di-Sodium hydrogen phosphate (Na_2HPO_4) solution for 2 h. Then, they were washed 3 times with distilled water and PBS solution for following studies.

4.5 Characterizations of Bone Scaffolds

4.5.1 Fourier Transform Infrared (FTIR) Spectroscopy

FTIR spectrum was recorded in the range of $400\text{--}4000\text{ cm}^{-1}$ with 4 cm^{-1} resolution and 24 scans.

4.5.2 Mechanical Test

The compression mechanical testing of HA/Gel/CMC scaffolds was conducted using a Universal Testing Machine (Zwick/Roell Z100) equipped with a 500-N load cell at 25 °C. Crosshead speed was 0.5 min⁻¹ [128]. Before testing, the samples (7.5 mm × 7.5 mm × 2.5 mm) were freeze-dried using a lyophilizer [129]. Compressive modulus was obtained from the linear region of the stress-strain curves [130]. Ultimate compressive strength was determined by calculating maximum strength before scaffold failure. Five samples were used for each study group.

4.5.3 Contact Angle

The surface wettability of bone scaffolds was evaluated by using KSV Instruments CAM101. The water contact angle (WCA) was measured after 5s.

4.5.4 Biodegradation Studies

Following the crosslinking procedure, the scaffolds (10 mm × 10 mm × 2 mm with 5 layers) were dried and weighted (Wo). The bone scaffolds were immersed in 3 mL PBS within 12 well-plates and incubated at 37 °C to determine the behavior of % water uptake and % weight loss. PBS was refreshed every 3 days. After specified time intervals (1, 7, 14, 21 and 28 days), the specimens were removed from PBS and weighed (Ws) to calculate % water uptake, as shown in Eq. 4.2. Then, the samples were dried and again weighted (Wd) to calculate the % weight loss, as shown in Eq. 4.3. Additionally, pH measurements of the 3D-printed bone scaffolds were recorded at the same time intervals. All drying processes were carried out using lyophilization, which involved freeze-drying the samples for 24 hours. Each sample group was subjected to three replicates.

$$\% \text{ water uptake} = \left(\frac{W_s - W_o}{W_o} \right) \times 100 \% \quad (4.2)$$

$$\% \text{ weight loss} = \left(\frac{W_o - W_d}{W_o} \right) \times 100 \% \quad (4.3)$$

4.5.5 Bioactivity Test

To show the biomineralization capability, the bone scaffolds were immersed in 50 ml falcon tubes and incubated in stimulated body fluid (SBF= solution (pH: 7.4) at 37 °C. SBF solution was prepared according to study of Kokubo and Takadama [131]. After 28 days, the scaffolds were lyophilized for 24 h. Apatite deposition on surface was characterized by FTIR and scanning electron microscopy (SEM, FEI-Philips XL30). Furthermore, surface composition was determined by energy dispersive spectrometer (EDS). Phase composition of nano-HA/Gel/CMC after incubation for 28 days was examined by using X-ray diffractometer (Rigaku D/Max2200-pc). Diffraction data was presented by scanning of 2θ angles between 15° and 80° with step size 0.02°.

4.5.6 Cell Culture Studies

For the in vitro cell culture studies, MC3T3-E1 pre-osteoblasts (ATCC® CRL-2593™) were cultured in MEM- α (Biowest) supplemented with 10% fetal bovine serum (FBS, Biowest) and 1% penicillin-streptomycin (Biowest) at 37 °C in 5% CO₂ and 95% humidity [132]. Cells at passage numbers between 3 and 5 were utilized to evaluate the biocompatibility of the scaffolds. The scaffolds (10 mm $\times$ 10 mm $\times$ 2 mm) were first seeded with 50 000 cell/well onto a 24-well plate, after being sterilized by UV radiation and immersion in 70% ethanol. Medium was refreshed every other day.

Cell proliferation of MC3T3-E1 pre-osteoblasts was measured using the Alamar Blue assay after 1, 3, 5 and 7 days of culture. The scaffolds were transferred to a new plate to distinguish between cells cultured on the scaffolds and those on the well plate surface. On each of these days, the 3D-printed scaffolds were incubated with culture medium containing 10% Alamar Blue solution. After 4 hours of incubation, absorbance was measured at 570 nm and 595 nm [83]. The positive control, which consisted of cells cultured without scaffolds (TCP), was set as 100% for cell viability measurements. The cell nucleus was stained with DAPI for 20 min in the dark.

The calcium deposition of 3D-printed scaffolds by MC3T3-E1 pre-osteoblasts was assessed by Alizarin Red S (ARS) staining after 14 days of culture by qualitative and quantitative analysis by microplate reader. One day after seeding, differentiation medium (containing 50 $\mu\text{g}/\text{mL}$ ascorbic acid, 10 mM β -glycerophosphate, and 100 nM dexamethasone in complete medium) was applied and refreshed every 2 days for 14 days. The cells were fixed with 4% paraformaldehyde at 25 °C for 15 minutes. After fixation, the scaffolds were washed three times with deionized water (diH_2O). Furthermore, 40 mM Alizarin red staining solution (pH 4.2) was added to each well, and the plates were gently shaken at room temperature for 30 min in dark. To remove excess dye, the cells were washed five times with diH_2O . The stained samples were then examined using an optical microscope (HIROX KH-8700).

To obtain quantitative results, the plates were incubated with 10% acetic acid solution for 30 min. The acetic acid solution containing the cells from each well was then transferred to microcentrifuge tubes. The tubes were vortexed for 30 sec, heated at 80 °C for 10 min, and then cooled on ice for 5 min. After centrifugation at 20 000 g for 15 min, the supernatants were transferred to new tubes and neutralized with 10% ammonium hydroxide. A 75 μL aliquot of the elute was transferred to a 96-well plate, and the absorbance was measured at 405 nm using a microplate reader (Bio-Rad Mark). A standard curve for Alizarin red S was prepared by diluting a 40 mM solution and plotting absorbance against concentration. The Alizarin red S concentration was then calculated using the trend line equation [133]. Nine samples were repeated for each study group.

4.6 Statistical Analysis

Data analysis was conducted using one-way ANOVA, with statistical significance denoted by p-values: * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$), indicating significant variation between groups.

4.7 Results and Discussion

In bone tissue engineering applications using extrusion-based 3D printing, the successful fabrication of constructs with desirable shape accuracy and mechanical stability is closely linked to the rheological behavior of the hydrogel inks. The flow behavior and consistency of these inks are critical for producing well-defined filaments with high shape fidelity [58]. To evaluate these characteristics, key rheological parameters such as viscosity, shear stress, and viscoelastic moduli were measured under varying conditions of strain amplitude, frequency, and temperature. G' and G'' , components of the dynamic modulus, indicate the solid-like (elastic) and liquid-like (viscous) responses of a material, respectively [134].

Figure 4.1 (a) presents the results of amplitude sweep tests performed on Gel/CMC and HA/Gel/CMC hydrogels to identify their linear viscoelastic region (LVR). The samples showed stable viscoelastic behavior between 0.01% and 10% strain, maintaining both elastic and viscous responses. When strain exceeded 100%, plastic deformation was observed in the hydrogels. To ensure the preservation of structural integrity for further testing, 1% strain was chosen as the standard. As shown in Figure 4.1 (b), G15/C7.5 exhibited a significantly higher storage modulus (G') than G7.5/C7.5 ($p < 0.05$), indicating that increasing the gelatin content reinforces the elasticity of the network. In G15/C7.5-based compositions, the incorporation of 10% HA slightly reduced G' , while 20% HA significantly increased it, indicating that mechanical strength is improved at higher HA levels. Notably, the HA20/G15/C7.5 formulation exhibited the highest storage modulus among all tested groups (* $p < 0.001$). Figure 4.1 (c) illus-

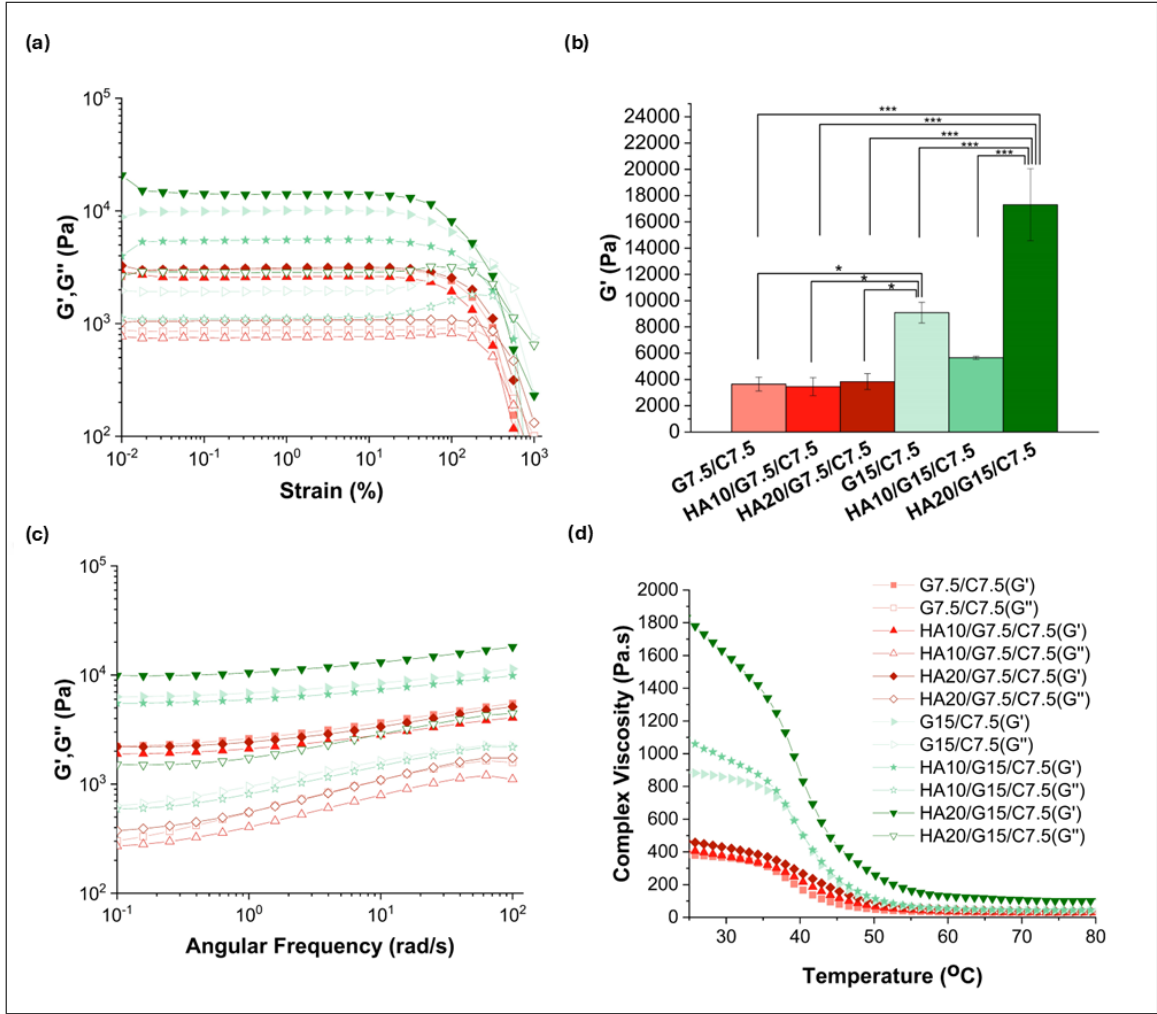


Figure 4.1 (a) Changes in storage modulus (G') and loss modulus (G'') as a function of strain (%). (b) storage modulus values measured at a constant strain of 1%. (c) variation of G' and G'' with respect to angular frequency (rad/s). (d) Complex viscosity as a function of temperature ($^{\circ}\text{C}$). (n=3).

trates the frequency-dependent viscoelastic behavior with the storage and loss moduli for both Gel/CMC and HA/Gel/CMC hydrogels. All formulations demonstrated dominant solid-like behavior at 25°C , as evidenced by the storage modulus G' consistently remained higher than G'' across the frequency range.

As shown in Figure 4.1(d), temperature sweep tests revealed that hydrogels containing 7.5% gelatin showed minimal changes for complex viscosity around 25°C , regardless of HA content.

However, in G15/C7.5-based formulations, complex viscosity enhanced with in-

creasing HA concentration. Due to the disruption of hydrogen bonding at higher temperatures, complex viscosity across all samples decreased with values eventually falling below 400 Pa·s [135].

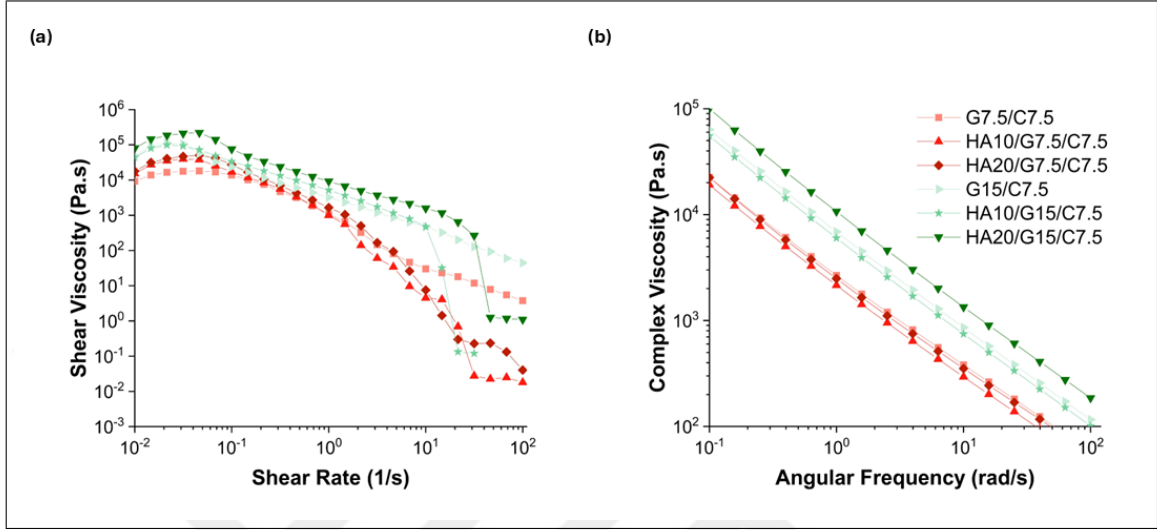


Figure 4.2 (a) The relationship between shear viscosity and shear rate (1/s). (b) frequency-dependent variation of complex viscosity with respect to angular frequency.

Figure 4.2 (a) illustrates that shear viscosity increases as the concentrations of gelatin and hydroxyapatite rise. All hydrogel formulations showed typical shear-thinning behavior, as evidenced by the decrease in viscosity with increasing shear rate [136],[137]. This characteristic rheological feature is particularly important for enabling smooth material flow through the nozzle during the 3D printing process [138],[139]. Additionally, Figure 4.2 (b) shows that complex viscosity decreases with higher angular frequencies, indicating a weakening in hydrogen bonding. This disruption contributes to a loss of structural integrity in the gelatin matrix and resulting in decrease of gel strength [137].

As shown in Figure 4.3, the gelation temperatures for the low-gelatin groups (7.5% Gel) were recorded as 48.5°C for G7.5/C7.5, 53°C for HA10/G7.5/C7.5, and 56°C for HA20/G7.5/C7.5. In samples with 15% gelatin, were identified as 54°C, 56°C, and 63°C for G15/C7.5, HA10/G15/C7.5, and HA20/G15/C7.5, respectively. A rise in gelation temperature was observed in all groups with increasing HA content across all formulations. This trend can be primarily explained to the viscosity

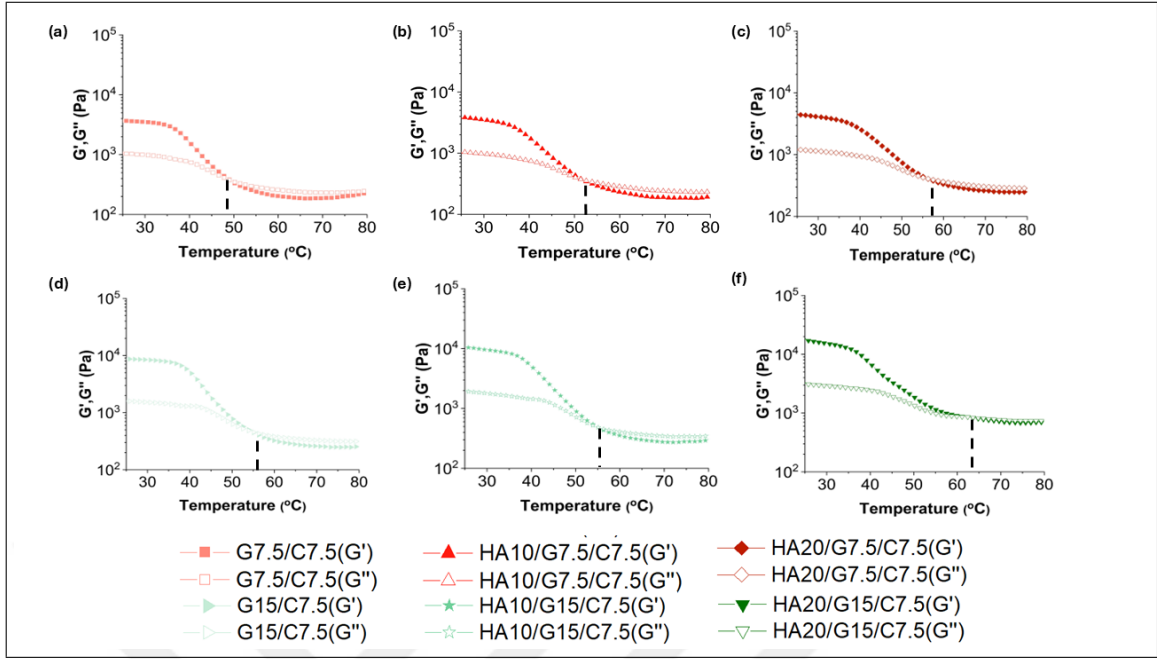


Figure 4.3 Temperature-dependent variations in storage modulus (G') and loss modulus (G'') observed during the temperature sweep tests for hydrogel formulations (a) G7.5/C7.5, (b) HA10/G7.5/C7.5, (c) HA20/G7.5/C7.5, (d) G15/C7.5, (e) HA10/G15/C7.5, (f) HA20/G15/C7.5 hydrogel inks.

increase caused by HA incorporation [140]. In addition, potential ionic interactions between HA, CMC, and gelatin may promote the gel network, further contributing to the elevated gelation temperatures. Similarly, increasing the gelatin concentration also resulted in higher gelation temperatures. In 15% Gel formulations, the viscosity at the gelation point was substantially higher, likely due to the formation of more extensive hydrogen bonding networks compared to 7.5% Gel groups. This enhanced bonding hinders material extrusion at the gelation temperature.

As shown in Figure 4.4 (a–f), strut formation was assessed at constant temperatures (40°C for 7.5% Gel and 70°C for 15% Gel) under varying the printing pressure from 16 psi to 32 psi. The comparisons of these images clearly highlight differences in printability among the hydrogel formulations. Figure 4.4(g) provides a visual representation of both flow behavior and viscosity by showing how each hydrogel ink spreads on a spatula.

For the ideal extrusion conditions, the printed struts maintained superior shape

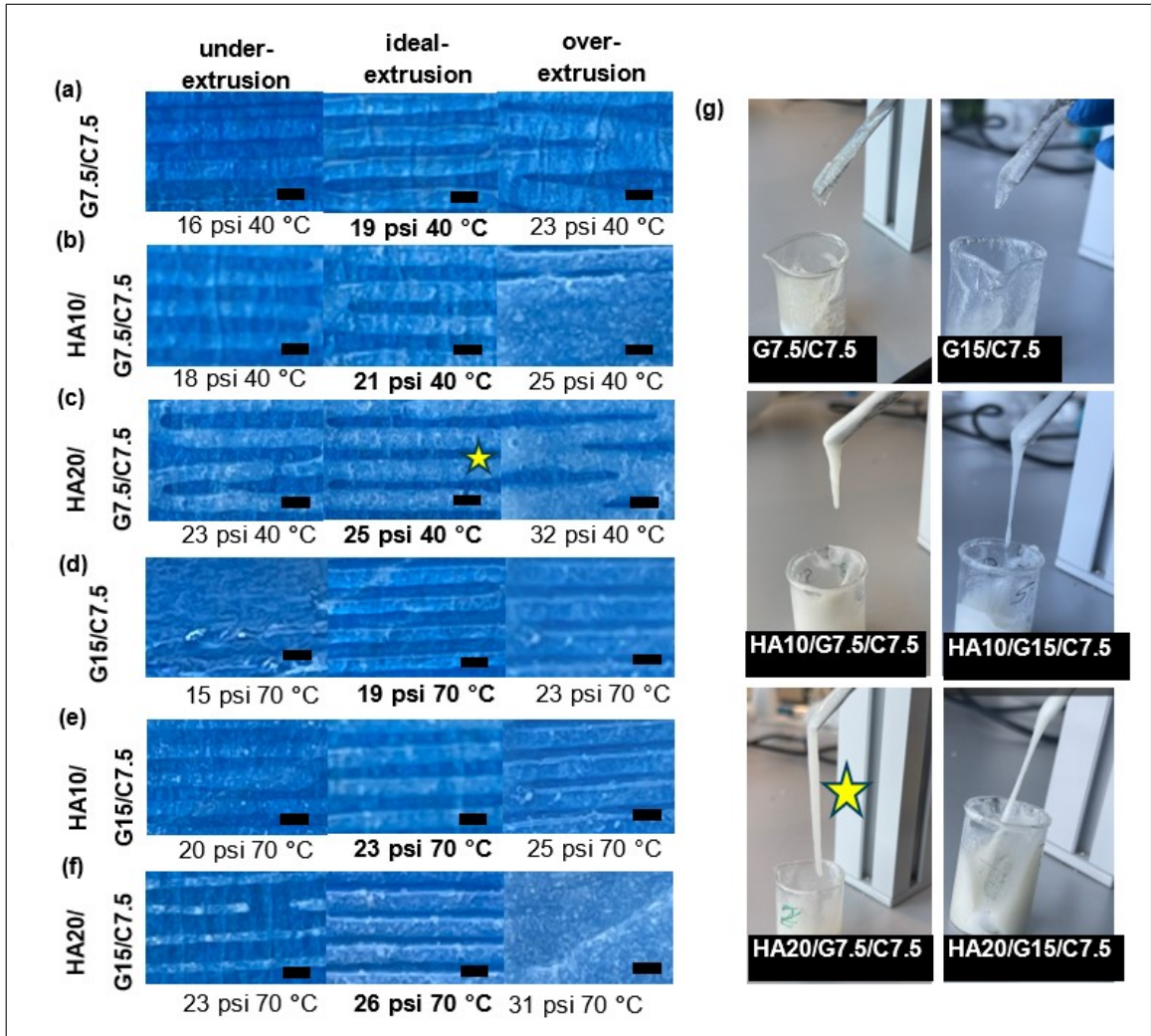


Figure 4.4 (a-f) Evaluation of printability under varying temperatures and pneumatic pressures, on filament formation and (g) flow characteristics and viscosity trends across all groups. (scale bar: 1 mm) (yellow star indicates optimized hydrogel ink).

fidelity compared to both under-extruded and over-extruded samples. In the 7.5% Gel-based formulations, increasing the pressure led to a decline in structural accuracy. Specifically, over-extrusion was observed at 23 psi for G7.5/C7.5, at 25 psi for HA20/G7.5/C7.5 and at 32 psi for HA20/G7.5/C7.5. The addition of HA enhanced the viscosity of the hydrogel inks, thus requiring higher pressures for proper extrusion [141]. This rise in required printing pressure with increasing HA content is primarily attributed to enhance hydrogen bonding and ionic interactions introduced by HA [142].

For the hydrogels containing 15% Gel, printing temperature was set at 70°C. Below this temperature, even the printer's maximum pressure was insufficient for ex-

trusion. However, the high temperatures required for printing could hinder the suitability of these inks for use in bioprinting with live cells. Consequently, the 7.5% Gel groups were found to be more favorable for biofabrication purposes. Among these, the HA20/G7.5/C7.5 formulation was selected as the most promising candidate due to its better consistency and lower required printing temperature, as depicted in Figure 4.4 (g).

Figure 4.5 (a-b) presents the outcomes of filament collapse testing for the hydrogel inks. This test aimed to evaluate whether the struts could remain stable without any supporting structure. Visual analysis showed that increasing the printing speed resulted in sagging of the suspended filaments across all hydrogel groups. The deflection was especially noticeable at the longest span between supporting pillars, which made it possible to evaluate collapse behavior across various speeds. Based on these results, a printing speed of 6 mm/s was chosen as optimal, as it maintained filament shape without support.

Figure 4.6 shows the designed scaffolds, pore size, filament size and printing accuracy of 3D-printed bone scaffolds.

Figure 4.6 (a) shows the modeling of scaffolds produced by 3D printer. Pore geometry in 3D-printed bone scaffolds is a crucial property in printing process, influencing bone regeneration and vascularization. For this, 300-900 μm of porosity is considered to be optimal [143]. Figure 4.6 (b) shows that adding 20% HA significantly reduced pore size ($p < 0.001$). Moreover, increasing Gel significantly reduced the pore size in groups where HA was 10%. Filament size also significantly increased from 542 μm to 857 μm in Gel 7.5% groups while HA ratio increased from 0 to 20% ($p < 0.001$), as shown in Figure 4.6 (c). For the groups with 15% gelatin, it significantly increased from 821 μm to 925 μm ($p < 0.01$). When HA particles are added to a polymer matrix, they interact with each other and with the polymer chains. The incorporation of 20% nano-HA led to a decrease in printing accuracy, as shown in Figure 4.6 (d). As evidenced by many studies, nano-HA causes swelling of scaffolds, which reduces shape fidelity [144]. Therefore, 10% nano-HA is observed to be optimal for shape fidelity in

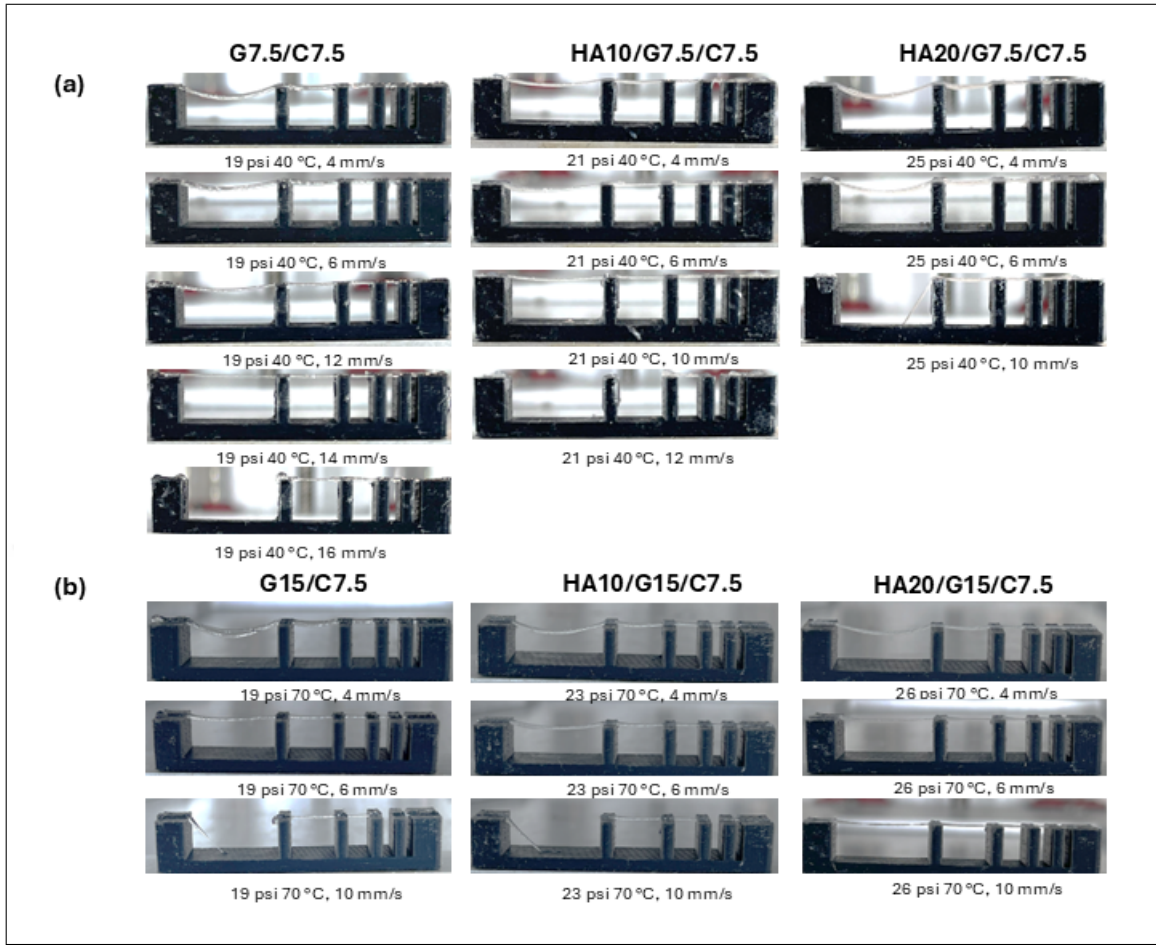


Figure 4.5 The filament collapse evaluation for (a) 7.5% and (b) 15% Gel hydrogel inks.

comparison to 20% nano-HA groups.

Figure 4.7 shows the FTIR spectrum prior to the crosslinking process and potential interactions of 3D-printed bone scaffold process. Gel exhibits amide I peak at 1635 cm^{-1} , which corresponds to C=O stretching, Amide II peak ranging from $1543\text{--}1589\text{ cm}^{-1}$ indicating N-H bending vibrations, and amide III peak at $1242\text{--}1265\text{ cm}^{-1}$ which attributed to the triple helix structure [145]. Furthermore, CMC is characterized by prominent absorption band between $1600\text{--}1415\text{ cm}^{-1}$, related to COOH^- groups of CMC [146]. Peaks in between $3289\text{--}3302\text{ cm}^{-1}$ attributed the O-H groups for both Gel and CMC [132]. nano-HA peak at 1543 cm^{-1} overlaps with amide II.

The spherical morphology and distribution of nano-HA (nanopowder, particle

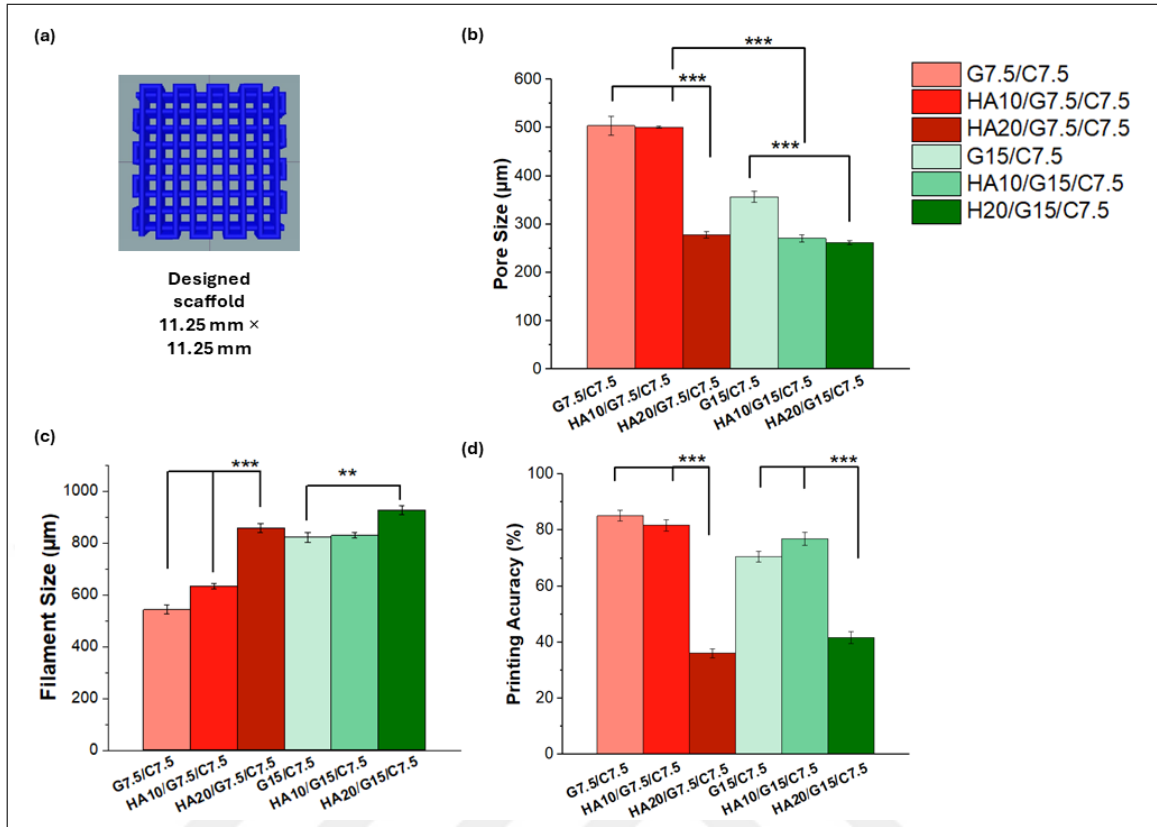


Figure 4.6 Analysis of shape fidelity (a) image of designed bone scaffold pore (b) pore size (c) filament size and (d) printing accuracy (%), (mean±standard deviation error, n=10, one-way ANOVA, Tukey test, * p<0.05, ** p<0.01, *** p<0.001).

size 200 nm (BET), 97.0) was shown in Figure 4.8 (a). A spherical morphology enhances the material's surface area, facilitating cell adhesion and interactions with the biological tissue [147].

Figure 4.8 (b) illustrates the preparation of Gel/CMC and HA/Gel/CMC hydrogels, their 3D printing process, and scaffolds appearance highlighting structural differences. The filaments were smoothly printed without spreading over each other.

Figure 4.9 shows the mechanical performance of 3D-printed bone scaffolds. The influence of nano-HA and Gel in nano-HA/Gel/CMC bone scaffolds was investigated by compression tests.

Representative stress-strain curves of bone scaffolds are presented in Figure 4.9 (a). Figure 4.9 (b) shows the compressive modulus of bone scaffolds. Addition of 10

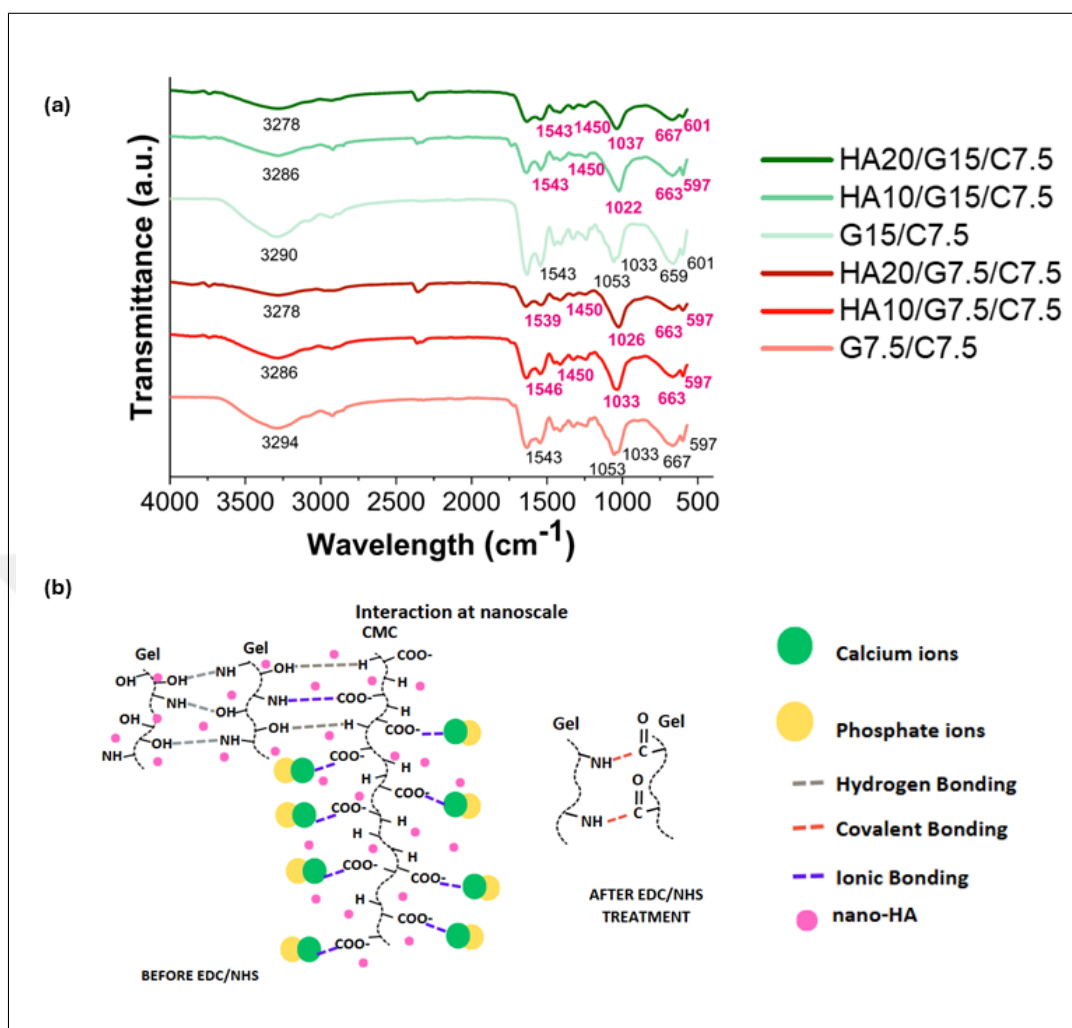


Figure 4.7 Interactions of nano-HA and polymeric components. (a) FTIR spectrum and (b) possible molecular interactions among nano-HA, Gel and CMC.

wt% nano-HA to Gel/CMC hydrogel improved the compressive modulus and reached value of 8.3 MPa and 6.1 MPa for HA10/G7.5/C7.5 and HA10/G15/C7.5, respectively. The effectiveness of carbodiimide can be reduced by the presence of an inorganic phase in the polymer matrix [129]. The compressive modulus of nano-HA/Gel scaffolds is similar to the compressive modulus of trabecular bone therefore they are considered to be suitable for non-load bearing bone tissue regeneration due to their cell-adhesive properties [135].

According to Figure 4.9(c), HA10/G15/C7.5 shows the highest compressive strength value. 10% nano-HA probably had a homogenous dispersion within the hydrogel leading to improved mechanical performance. On the other hand, 20% nano-HA

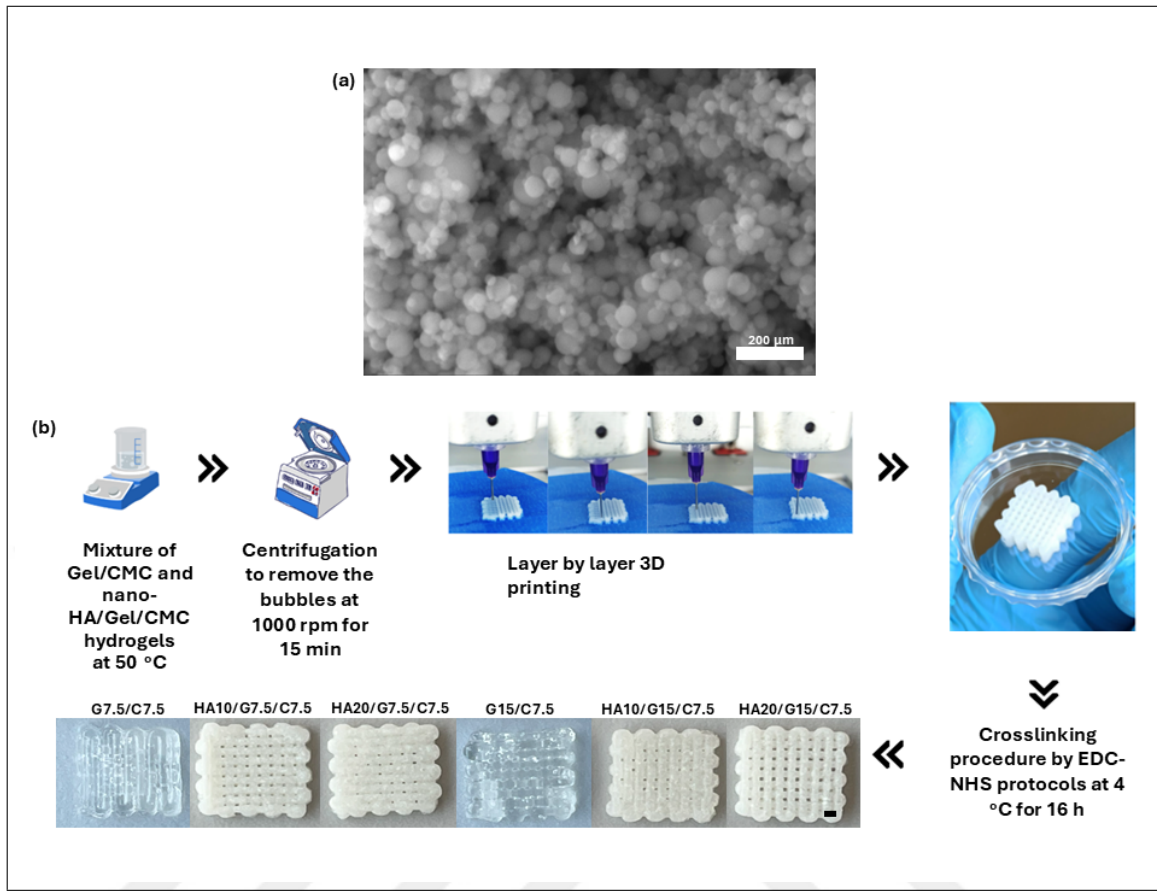


Figure 4.8 (a) SEM images of spherical nano-HA ($\times 300\,000$ magnification), (b) schematic illustration of the preparation of HA/Gel/CMC scaffolds using extrusion-based 3D printing.

probably had agglomeration which resulted in deterioration of mechanical performance. Additionally, ductility of 10% nano-HA was superior and 20% nano-HA was relatively brittle which further support agglomeration of 20% nano-HA samples [148]. The compressive strength of natural trabecular bone is typically ranging from 0.1 to 16 MPa, whereas the range of nano-HA/Gel/CMC scaffolds was ~ 0.8 –2.5 MPa [149]. The highest compressive strength was found as 2.6 MPa for HA10/G15/C7.5.

The ability of scaffolds to uptake water and degrade is a vital characteristic for bone tissue regeneration [150]. Figure 4.10 shows water contact angle measurements, the profile of water uptake capacity, % degradation and pH measurements of 3D-printed bone scaffolds.

The hydrophilicity was assessed for 5s after formation of the water droplet, as

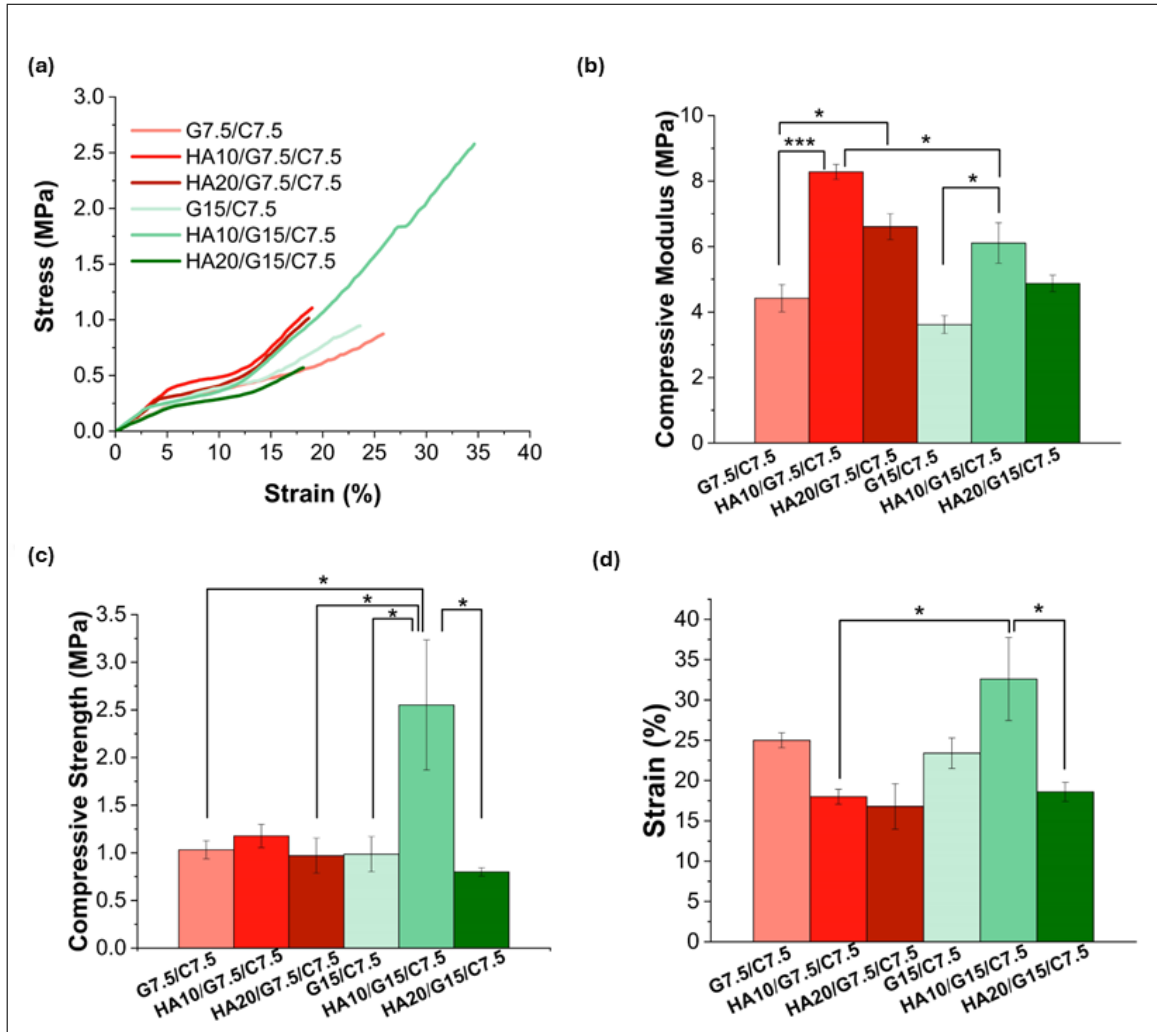


Figure 4.9 Mechanical analysis under compression stress. (a) stress-strain curves, (b) compressive modulus, (c) compressive strength and (d) % strain values of 3D-printed bone scaffolds. (mean $\pm$ standard error, $n = 5$, one-way ANOVA, Tukey test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

depicted Figure 4.10 (a). Increasing the Gel content from 7.5% to 15% decreased the hydrophilicity in every group where HA was fixed, which is due to the presence of more covalent bonds arising from EDC crosslinking process, as shown in Figure 4.10 (b). The addition of nano-HA decreased wettability at 5 s for the groups with 7.5% Gel. In contrast, the groups containing 15% gelatin improved the surface wettability with increase in HA. The measured water contact angles were 49° , 32° , and 28° for G15/C7.5, HA10/G15/C7.5, and HA20/G15/C7.5, respectively.

The lower concentration (7.5%) of Gel may affect the surface hydrophilicity differently. In comparison to control groups without HA, the contact angle increased in

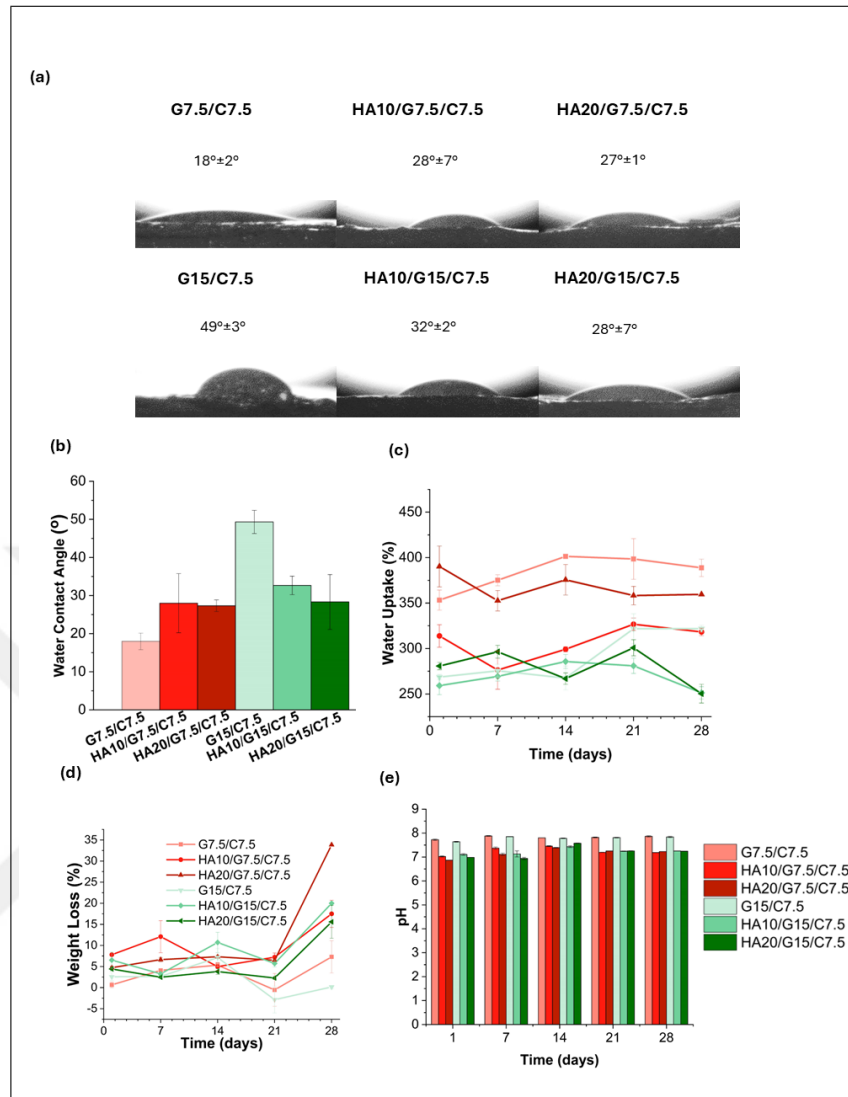


Figure 4.10 (a) Illustration and (b) measurements of WCA. (c) % water uptake, (b) % weight loss, and (c) pH measurements of the 3D-printed HA/Gel/CMC bone scaffolds after specific incubation periods.

groups where HA was added [151]. Deng et al. [152] also reported that increasing nano-HA from 0 to 50% (w/w) in high concentration of Gel (30% w/v) led to decrease of the WCA. In another study, since most of the Gel groups were crosslinked by EDC/NHS (100 mM:20 mM) and with the carboxyl groups of CMC further HA addition increased the concentration of unbound OH⁻ and Ca²⁺, thereby enhancing the hydrophilic properties of the scaffold surface [153].

Figure A.1 shows the wet-state images of the 3D-printed nano-HA/Gel/CMC bone scaffolds after specific incubation period to further support the water uptake

behavior. Figure 4.10(c) shows that G7.5/C7.5 had the highest % water uptake in all study groups for all time points except for day 1. It has been shown that the water uptake rate decreases when the HA concentration is constant and as the Gel ratio increases from 7.5% to 15%. This is probably because the increased crosslinking density with further Gel addition reduced the amount of water retained within the scaffolds, which reduced %water uptake [154]. In the study groups with 10% nano-HA addition, % water uptake generally decreased compared to the control group (G7.5/C7.5 and G15/C7.5). This is possibly due to higher viscosity with the addition of nano-HA which reduces swelling [155]. Qui et al.[136] also show that increase of wt% of HA particles from 0% to 40% within the HA/methacrylated-CMC samples decreased the % swelling ratio due to the enhanced crosslinking density and internal interactions between the organic and inorganic structure. However, with the addition of 20% HA, % water uptake has been increased. This is due to possible in-homogenous blending of HA within the polymeric matrix [148].

Figure 4.10(d) shows the % weight loss profile of bone scaffolds. There was no significant weight loss % up to day 21. However, weight loss % increased for all groups after day 21. HA20/G7.5/C7.5 exhibited the highest degradation ($\sim 35\%$) by day 28, whereas G15/C7.5 had the lowest weight loss %, attributed its higher content of covalent bonding contributed greater stability.

pH measurements of bone scaffolds in PBS ranged from 7.72 to 7.82, indicating the compatibility of bone scaffolds with the physiological environment during the 28 days, as shown in Figure 4.10(e) [156].

Apatite formation, fundamental to bone mineralization, is essential for bone regeneration, which improves the mechanical properties of scaffolds, supports cell growth and accelerates the bone healing process [157]. To illustrate the apatite formation, SEM, FTIR and XRD were performed on bone scaffolds incubated in SBF for 28 days.

Figure 4.11(a-f) illustrates apatite formation after 28 days of SBF incubation by using SEM. HA addition provides ideal microenvironment for osteoblast adhesion,

bone tissue regeneration and differentiation due to its active sites [136]. G7.5/C7.5 and G15/C7.5 had a smoother surface, while the groups with HA indicate a rough surface [158]. Other study groups had a spherical-like aggregate formation, which indicated apatite precipitation [159]. When comparing groups with the highest HA concentration, the group with the highest gelatin concentration (HA20/G15/C7.5) led to more apatite formation. This suggests that a higher Gel concentration enhances HA formation.

Figure 4.11(g) indicates the FTIR spectrum of nano-HA/Gel/CMC bone scaffolds that were immersed in SBF for 28 days. The peaks at $1631\text{--}1635\text{ cm}^{-1}$, 1426 cm^{-1} , and 1329 cm^{-1} correspond to the stretching vibrations of the carboxyl group ($-\text{COOH}$) in CMC [136]. The FTIR spectrum of CMC showed two bands at 1600 cm^{-1} and 1415 cm^{-1} , which correspond to the symmetric and asymmetric vibrations of the carboxylate group (COO^-) [146]. CMC and Gel peaks were also appeared in Figure 4.11(g) as they were also shown in Figure 4.7(a).

Specific HA peaks were observed at 1026–1037 cm^{-1} corresponding to the asymmetric stretching vibrations (v3) of phosphate groups (PO_4^{3-}). Additionally, peaks at 1450–1546 cm^{-1} attributed to carbonate groups (CO_3^{2-}). HA peaks were highlighted in pink text in Figure 6 (g). The shift of OH stretching vibration to a lower frequency, and from 3294–3278 cm^{-1} in 7.5% Gel groups, confirms the formation of hydrogen bonding between NH and OH, and H and OH [57],[125]. This improves that hydrogen bonding, electrostatic interaction and ionic interactions between COO^- groups of CMC and Ca^{2+} ions, PO_4^{3-} in HA influence nucleation and growth of HA nanoparticles [57].

Figure 4.11(h) shows XRD patterns of nano-HA/Gel/CMC scaffolds to evaluate their crystallinity. HA peaks were shown as 25.98° (002) and 31.94° (211) [160]. Other characteristics peaks of HA were observed at 32.2° (112), 33° (300), 34.1° (202), 39.6° (310), 46.8° (222), 49.6° (231) and 53.3° (004) (JCPDS card no:09-0432 peaks). No diffraction peak was associated with the presence of Gel and CMC, which are amorphous materials. Moreover, Gel/CMC had no apatite peaks in the XRD spectrum. This is possibly because the observed apatite bands in FTIR for Gel/CMC were in amorphous phase therefore they were not present in their XRD spectrum. Additionally, HA10/G15/C7.5 and HA10/G7.5/C7.5 had less apatite peaks due to the lower amount of HA present. However, most of the diffraction peaks of HA were confirmed in HA20/G15/C7.5 and HA20/G7.5/C7.5 groups.

Figure 4.12 shows the elemental mapping of 3D-printed nano-HA/Gel/CMC bone scaffolds. Since G7.5/C7.5 and G15/C7.5 study groups had no apatite formation or inherent HA, they had no distribution of calcium or phosphate ions. On the other hand, there were abundant Ca and P ions for nano-HA containing groups.

EDS analysis demonstrates that the scaffolds are mainly composed of carbon (C), oxygen (O), phosphate (P) and calcium (Ca), as shown in Figure B.1 The Ca/P ratios of the HA10/G7.5/C7.5, HA20/G7.5/C7.5, HA10/G15/C7.5, and HA20/G15/C7.5 were 1.51, 1.78, 1.66, and 1.72, respectively. These values closely resemble Ca/P ratio of nano-HA [135].

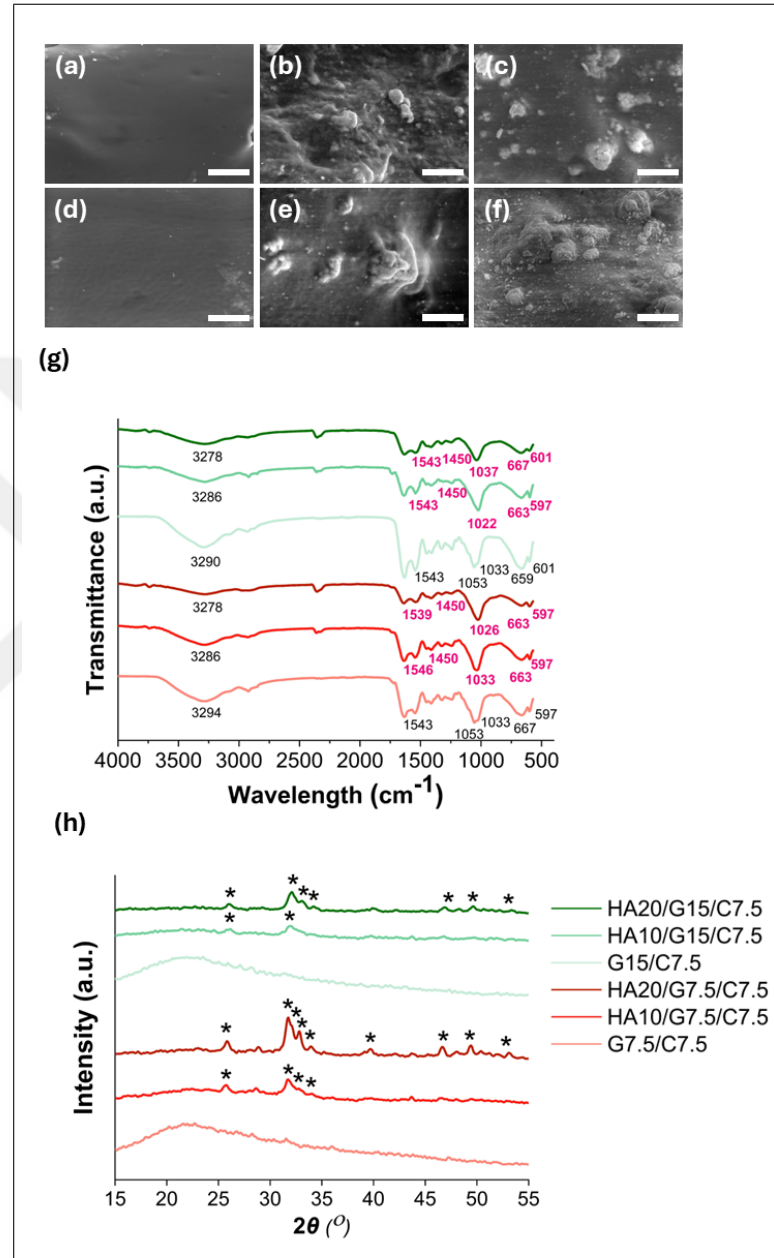


Figure 4.11 SEM images of the surface morphology of 3D-printed scaffolds after 28 days of SBF incubation: (a) G7.5/C7.5, (b) HA10/G7.5/C7.5, (c) HA20/G7.5/C7.5, (d) G15/C7.5, (e) HA10/G15/C7.5, (f) HA20/G15/C7.5 scaffolds (scale bar: 40 μm , magnification 2000 $\times$). (g) FTIR spectrum and (h) XRD spectrum of 3D-printed bone scaffolds for day 28.

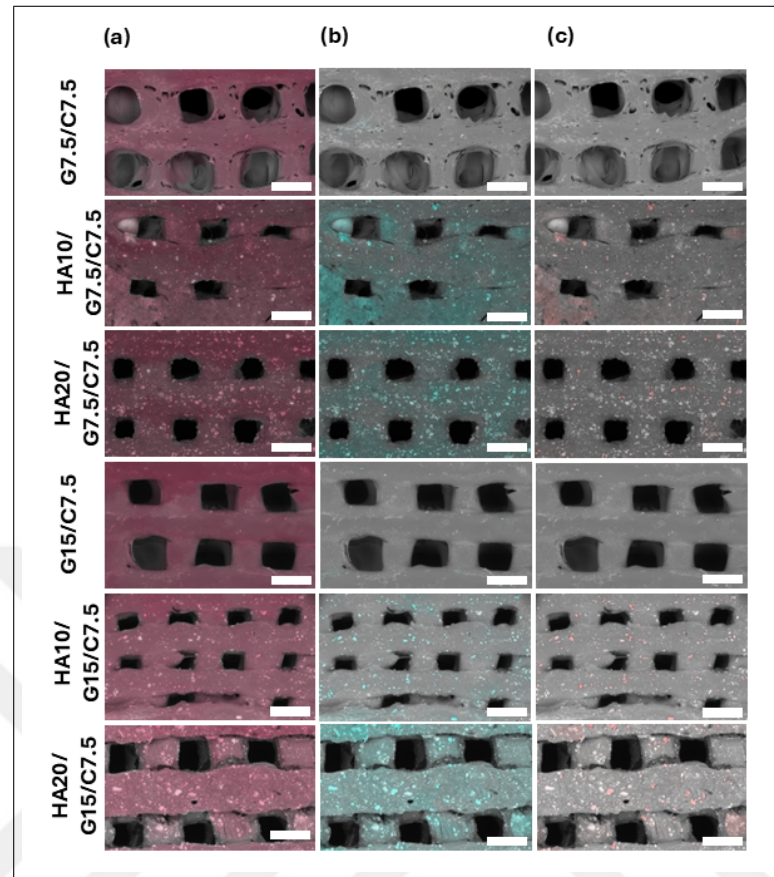


Figure 4.12 SEM/EDS micrographs with elemental mapping after 28 days of immersion in SBF. (a) Carbon (C) mapping, (b) Calcium (Ca) mapping, and (c) Phosphorous (P) mapping. (scale bar: 500 μm , magnification:150 $\times$).

The cell proliferation of pre-osteoblasts on the 3D-printed nano-HA/Gel/CMC bone scaffolds was studied using Alamar Blue assay. Cellular response is critical to determine the cytocompatibility of the materials. Figure 4.13(a-b) shows the relative % cell viability and DAPI staining of nano-HA/Gel/CMC 3D-printed scaffolds. According to Figure 4.13(a), the cell proliferation was higher in groups without HA than those with HA for all time points. This behavior probably can be due to early differentiation of cells which was confirmed with Alizarin red staining [161].

Figure 4.13(c) shows the level of the Alizarin red staining of the 3D-printed nano-HA/Gel/CMC bone scaffolds. After 14 days of culturing, the cells were stained with the Alizarin red s which was utilized to determine calcium mineralization on the 3D-printed structure quantitatively and qualitatively. G7.5/C7.5 exhibited higher mineralization compared to G15/C7.5. At 7.5% of Gel concentration, higher water uptake (as shown

in Figure 4.10c)) probably promoted higher mineralization. In contrast, a 15% Gel concentration formed a denser structure, restricting ion diffusion. This can lead to reduced calcium binding and nucleation sites, which reduced mineralization [162].

For 10% of HA, it was clear that mineralization was higher in groups where Gel is 15%. On the other hand, as can be seen from Figure 4.13(c), HA20/G7.5/C7.5 and HA20/G15/C7.5 groups had similar mineralization density.

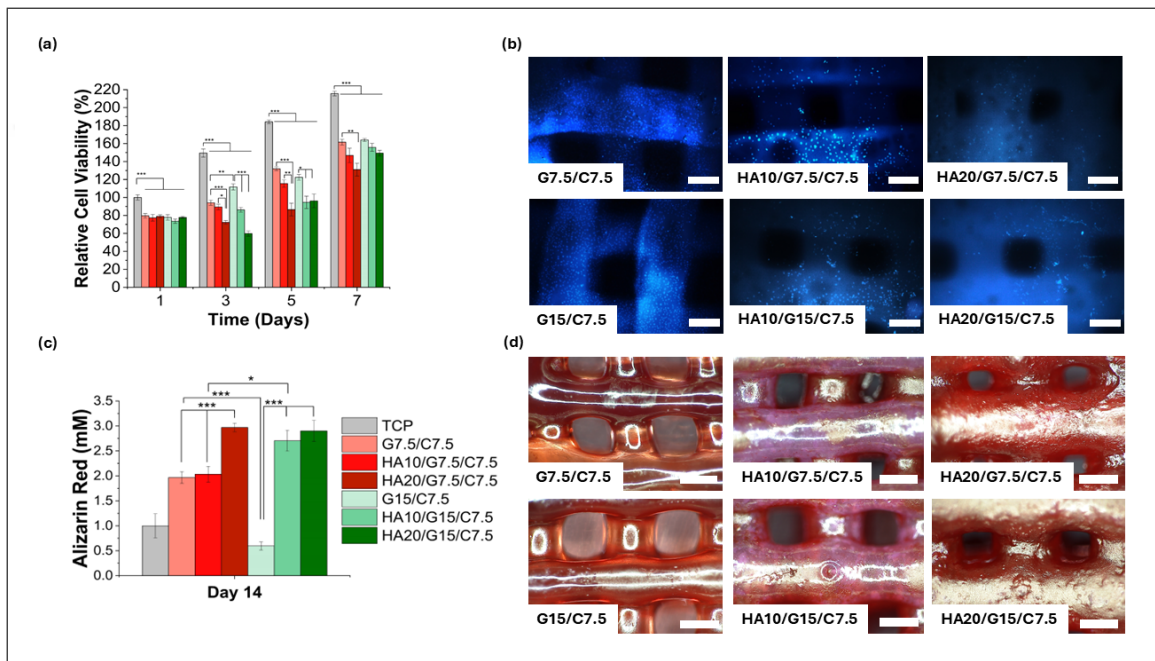


Figure 4.13 (a) Relative cell viability (%) of MC3T3-E1 cells on 3D-printed HA/Gel/CMC bone scaffolds and (b) DAPI staining. (c) Quantitative analysis and (d) representative images of Alizarin Red S Staining for 14th day of incubation of 3D-printed bone scaffolds. (scale bar= 500 μ m, magnification= 60 $\times$) (mean $\pm$ standard error, $n = 9$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

Overall, HA10/G15/C7.5 and HA20/G15/C7.5 had 3 times higher mineralization compared to G15/C7.5. Therefore, it can be concluded that scaffolds with higher nano-HA content could significantly promote the mineralization of MC3T3-E1 pre-osteoblasts [163]. In Figure 4.13 (d), Alizarin staining is also illustrated, which reflects visuals for the discussed mineralized scaffolds.

Further optimization and investigation are essential to ensure their effective integration with surrounding bone tissue. For future studies, 15% of nano-HA may be studied to optimize the mechanical characteristics of Gel/CMC hydrogels with nano-

HA in more detail. Additionally, surfactants such as Pluronic acid may be used to prevent agglomeration of nano-HA in the prepared scaffolds. Moreover, the key osteogenic markers including runt-related transcription factor 2 (Runx2), alkaline phosphate (ALP), collagen type I (Col1) and osteocalcin (OCN), can be analyzed. Finally, in-vivo studies are required to fully assess the potential of the scaffolds for practical bone tissue engineering applications.

In conclusion, this chapter demonstrated the successful 3D printing of nano-HA/Gel/CMC bone scaffolds. While 10% nano-HA yielded optimal mechanical and structural properties, 20% nano-HA enhanced bioactivity. These scaffolds facilitated cellular proliferation and exhibited potential in bone tissue engineering.

5. CONCLUSION

In the first experimental chapter, CMC/Gel/GO cardiac patches were developed with different concentrations of CMC (7.5-15%) and GO (0.5-1.5%). Rheological studies revealed that the CMC increased viscosity which improved printability of the scaffolds. The mechanical properties were highly effected by EDC/NHS concentration. A high concentration (100:20 mM) of EDC/NHS led to brittleness of the scaffolds due to very high degree of covalent bonding. EDC/NHS with a concentration of 50:10 was found to be most suitable concentration for optimized mechanical properties. CMC and GO were found to be also very influential for achieving flexibility due to introduction of hydrogen bonding. Although, considerably high flexibility was achieved, the flexibility of scaffolds can be further improved by incorporating of alginate to these scaffolds for production of cardiac patches. The electrical properties and degradation behavior of the cardiac patches were also suitable for production of cardiac scaffolds. However, future research is required to improve their cytocompatibility. These results indicate the potential of these scaffolds for cardiac tissue engineering applications.

In the second experimental chapter, by using nano-HA and Gel, nano-HA/Gel/CMC bone scaffolds were successfully fabricated via extrusion-based 3D printing technology. Study groups with 10 wt% concentration of nano-HA showed optimal results in terms of shape fidelity and mechanical properties, whereas those of above 10% led to agglomeration. MC3T3-E1 pre-osteoblasts were attached and proliferated on days 1, 3, 5 and 7 on Gel/CMC and nano-HA/Gel/CMC bone scaffolds. Furthermore, alizarin red and SBF studies with SEM, FTIR and XRD revealed that 20 wt% of nano-HA improved bioactivity of the bone scaffolds. Overall, these scaffolds are promising for bone tissue engineering applications.

Collectively, the findings of this thesis emphasize the versatility of extrusion-based 3D printing in developing tissue-specific scaffolds through strategic modulation of material composition and crosslinking chemistry. Concerning soft tissue engineering

approaches, CMC/Gel/GO-based cardiac patches demonstrated favorable rheological behavior, mechanical tunability, and electrical conductivity suitable for myocardial applications. Moreover, nano-HA/Gel/CMC scaffolds exhibited enhanced structural integrity, mineralization capacity, and osteoconductivity, aligning with the requirements of bone tissue regeneration. By addressing the distinct demands of both soft and hard tissues, this research establishes a promising foundation to develop scaffolds that are both functionally dynamic and biomimetic, designed specifically for regenerative medicine.



APPENDIX A. The Wet-state Images of the 3D-printed nano-HA/Gel/CMC Bone Scaffolds

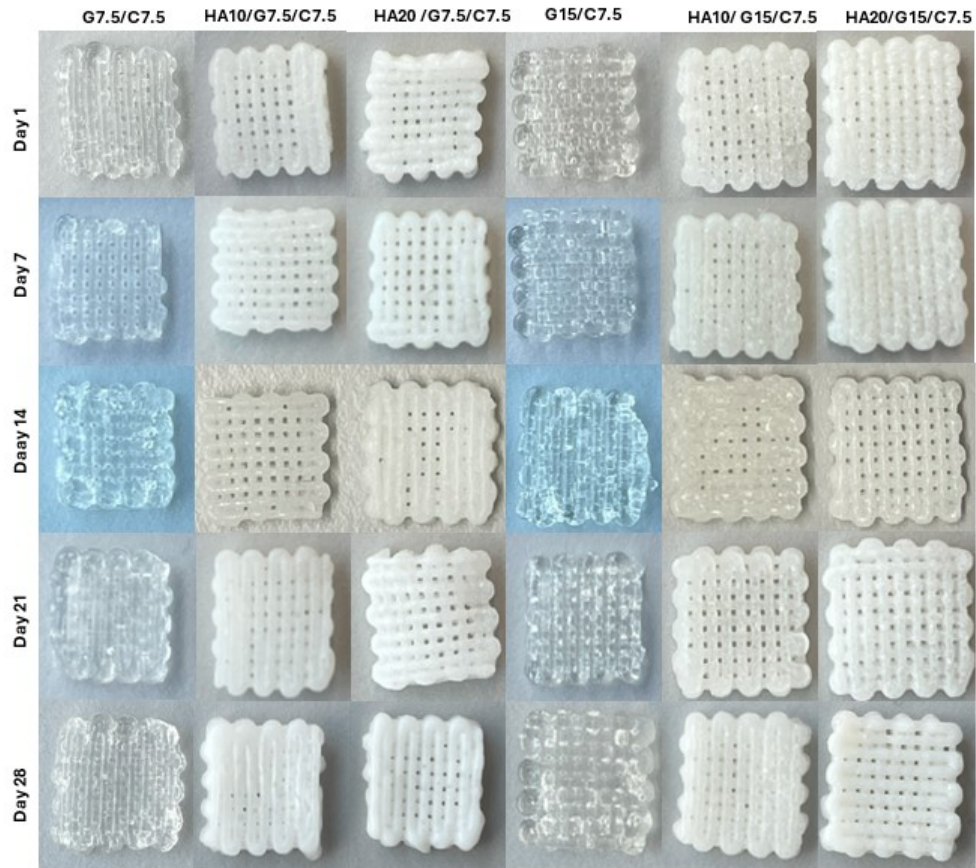


Figure A.1 Wet state of bone scaffolds. (scale bar: 10 mm).

APPENDIX B. EDS Analysis

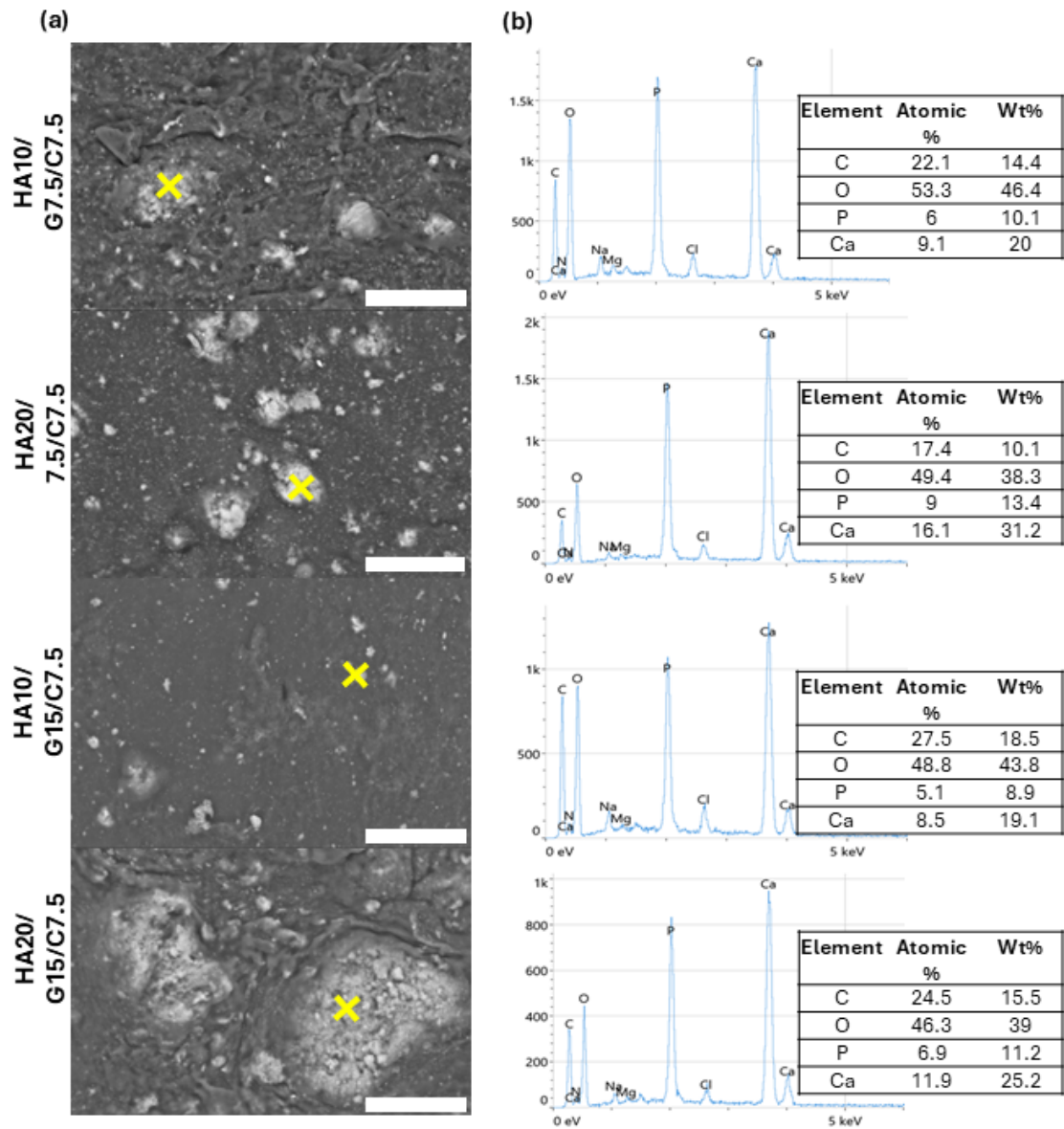


Figure B.1 EDC analysis of bone scaffolds (scale bar: 50 μ m).

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