



**TÜRKİYE CUMHURİYETİ
ADANA ALPARSLAN TÜRKEŞ SCIENCE AND TECHNOLOGY
UNIVERSITY**

**GRADUATE SCHOOL
BIOENGINEERING DEPARTMENT**

**CHITOSAN SCAFFOLDS CONTAINING BORON COMPOUND FOR
BONE TISSUE ENGINEERING**

Oula ALKHALIFA ALDHAM

M.Sc.



**TÜRKİYE CUMHURİYETİ
ADANA ALPARSLAN TÜRKER SCIENCE AND TECHNOLOGY
UNIVERSITY**

**GRADUATE SCHOOL
BIOENGINEERING DEPARTMENT**

**CHITOSAN SCAFFOLDS CONTAINING BORON COMPOUND FOR
BONE TISSUE ENGINEERING**

Oula ALKHALIFA ALDHAM

M.Sc.

**THESIS ADVISOR
Assoc. Prof. Merve ÇAPKIN YURTSEVER**

ADANA, 2025

DECLARATION OF CONFORMITY

In this thesis study, which was prepared following the thesis writing rules of Adana Alparslan Türkeş Science and Technology University Institute of Graduate School, I declare that I provide all the information, documents, evaluations and results in accordance with scientific ethics and moral codes without resorting to any means or assistance that would be contrary to scientific ethics and traditions. I also declare that I refer to all of the articles I used in this study with appropriate references and accept all moral and legal consequences if a situation is found contrary to my statement regarding my work.

....../....../20..

[Signature]

Name SURNAME

ÖZET

KEMİK DOKU MÜHENDİSLİĞİ İÇİN BOR BİLEŞİĞİ İÇEREN KİTOSAN İSKELELER

Oula ALKHALIFA ALDHAM

Yüksek Lisans, Biyomühendislik Anabilim Dalı

Danışman: Doç. Dr. Merve ÇAPKIN YURTSEVER

Ağustos 2025, 85sayfa

Bu çalışmada, sodyum pentaborat pentahidrat (SPP) içeren kitosan (Ch) ve tıbbi kullanıma uygun (MG) kitosan ile üretilen iskeleler dondurarak kurutma yöntemi ile elde edilmiş, karakterizasyon çalışmaları yapılmış ve kemik doku mühendisliği uygulamalarındaki potansiyellerini belirlemek amacı ile hücre kültürü çalışmaları gerçekleştirilmiştir. SPP %1 (v/v) asetik asitte çözünmüş kitosan polimer çözeltisine, %0.125, %0.25, %0.5, %0.75 ve %1 (w/w) oranlarında eklenmiştir. 3 boyutlu gözenek yapısı elde edebilmek için 2 gün -20 °C’de dondurulan polimer -80 °C dondurarak kurutma cihazında kurutulmuş buradan elde edilen iskeleler etanol veya NaOH ile stabilize edilip tekrar dondurarak kurutma cihazında kurutulmuştur. Tez çalışmasında üç farklı deney grubu ile çalışılmıştır: hücre kültür ortamında çözürülen iskelesiz serbest SPP, %0,125 ila %1 (a/a) konsantrasyonlarda SPP içeren kitosan iskeleler ve %0,5 ve %0,75 (a/a) SPP içeren tıbbi sınıf kitosan iskeleler.

Kitosan (Ch) polimeri ile yapılan çalışmalarda taramalı Elektron Mikroskopu (SEM) ve ImageJ analizleri, artan SPP konsantrasyonunun ortalama gözenek boyutunu azalttığını göstermiştir. En küçük gözenek yapısı %1 SPP içeren iskelede gözlemlenmiştir. Su tutma testleri, tüm iskelelerin 30 dakika içinde şişme dengesine ulaştığını, ancak SPP konsantrasyonu arttıkça şişme kapasitesinin azaldığını ortaya koymuştur. En yüksek su tutma kapasitesi saf kitosan iskelede, en düşük ise %1 SPP içeren iskelede ölçülmüştür. Dört haftalık biyobozunma testleri, yüksek SPP içeriğine sahip iskelelerde daha fazla ağırlık kaybı olduğunu göstermiştir. Ch – SPP iskelelerin antimikrobiyal özellikleri incelendiğinde *Aspergillus niger* and *Candida albicans*’a karşı doza bağlı olarak antifungal etki gösterdiği belirlenmiştir. MC3T3-E1 fare osteoblast hücreleri iskeleler üzerinde kültüre edildiğinde

hücre canlılığının tüm gruplarda ilerleyen günlerde azaldığı görülmüştür. Hücre üremesinin Ch – SPP iskeleler tarafından desteklenmediği düşünülerek hem kitosan çeşidi hem de stabilizasyon yönteminin hücre çoğalması üzerine etkisi incelenmiştir. Sonuçlar, tıbbi kullanıma uygun kitosan (MG) ile üretilen ve NaOH ile stabilize edilen iskelelerin MC3T3-E1 hücrelerinin çoğalmasını desteklediğini göstermiştir. Bu nedenle devam eden çalışmalarda tıbbi kullanıma uygun kitosan ile %0.5, %0.75 (w/w) oranında SPP içeren iskeleler üretilmiş, karakterize edilmiş ve hücre kültürü çalışmaları gerçekleştirilmiştir. Resazurin canlılık analizi sonuçları MCET3-E1 hücrelerinin iskeleler üzerine tutunduğunu ve çoğalmalarını desteklediğini göstermiştir. Metabolik aktivite son günlerde düşüş gösterse de, DAPI boyama sonuçları, özellikle %0.5 ve %0.75 SPP gruplarında 10. günde artan hücre çekirdeği yoğunluğunu ortaya koymuş, bu da hücre çoğalmasının devam ettiğini göstermiştir. SEM analizi sonuçlarında da özellikle MG Ch - 0.5 SPP grubunda hücrelerin yoğun bir şekilde çoğaldığı ve yayıldığı görülmüştür.

Sonuç olarak, SPP ile zenginleştirilmiş tıbbi kullanıma uygun kitosan iskelelerin antifungal özellik gösterdiği, MC3T3-E1 hücrelerinin çoğalmasını destekleyerek biyouyumlu olduğu belirlenmiştir. Tüm formülasyonlar arasında, NaOH ile stabilize edilmiş tıbbi kullanıma uygun kitosan içinde %0.5 (w/w) SPP içeren iskele, kemik doku mühendisliği için en umut verici yapı olarak öne çıkmaktadır.

Anahtar Kelimeler: Kitosan, SPP, MC3T3-E, Kemik doku mühendisliği, İskele

ABSTRACT

CHITOSAN SCAFFOLDS CONTAINING BORON COMPOUND FOR BONE TISSUE ENGINEERING

Oula ALKHALIFA ALDHAM

M.Sc., Department of Bioengineering

Supervisor: Doç. Dr. Merve ÇAPKIN YURTSEVER

August 2025, 85 pages

In this study, scaffolds produced with sodium pentaborate pentahydrate (SPP) containing chitosan (Ch) and medical grade (MG) chitosan were obtained by freeze drying method, characterization studies were performed and cell culture studies were carried out to determine their potential in bone tissue engineering applications. SPP was added to the chitosan polymer solution dissolved in 1% (v/v) acetic acid at ratios of 0.125%, 0.25%, 0.5%, 0.75% and 1% (w/w). In order to obtain a 3-dimensional pore structure, the polymer was frozen at -20 °C for 2 days and freeze dried at -80 °C in and they were stabilized by ethanol or NaOH and freeze dried again. Three different experimental groups were studied in the thesis: free SPP dissolved in cell culture medium, chitosan scaffolds containing SPP at concentrations ranging from 0.125% to 1% (w/w), and medical-grade chitosan scaffolds containing 0.5% and 0.75% (w/w) SPP. Scanning Electron Microscopy (SEM) and ImageJ analysis of the studies with chitosan (Ch) polymer showed that increasing SPP concentration decreased the average pore size. The smallest pore structure was observed in the scaffold containing 1% SPP. Water retention tests revealed that all scaffolds reached swelling equilibrium within 30 minutes, but the swelling capacity decreased as the SPP concentration increased. The highest water uptake capacity was measured in the pure chitosan scaffold and the lowest in the scaffold containing

1% SPP. Four-week biodegradation tests showed greater weight loss in scaffolds with higher SPP content. When the antimicrobial properties of Ch – SPP scaffolds were examined, it was determined that Ch - SPP scaffolds showed a dose-dependent antifungal effect against *Aspergillus niger* and *Candida albicans*. When MC3T3-E1 mouse osteoblast cells were cultured on the scaffolds, it was observed that cell viability decreased in all groups in the following days. Considering that cell proliferation was not supported by Ch-SPP scaffolds, the effect of both chitosan type and stabilization method on the proliferation of MC3T3-E1 cells was examined. The results showed that the scaffolds produced with medical grade chitosan (MG) and stabilized with NaOH promoted the proliferation of MC3T3-E1 cells. Therefore, in the ongoing studies, scaffolds containing 0.5%, 0.75% (w/w) SPP with medical grade chitosan were produced, characterized and cell culture studies were carried out. Resazurin viability assay results showed that MCET3-E1 cells adhered to the scaffolds and supported their proliferation. Although metabolic activity decreased in the last days, DAPI staining results revealed increased cell nuclei density at day 10, especially in the MG Ch - 0.5 SPP and MG Ch - 0.75 SPP groups, indicating that cell proliferation supported. SEM analysis results also showed that the cells proliferated and spread intensively, especially in the MG Ch - 0.5 SPP group.

In conclusion, SPP-enriched medical grade chitosan scaffolds were found to be antifungal and biocompatible by supporting the proliferation of MC3T3-E1 cells. Among all formulations, the scaffold containing 0.5% (w/w) SPP in NaOH-stabilized medical grade chitosan stands out as the most promising scaffold for bone tissue engineering.

ACKNOWLEDGEMENTS

The authors thank to the Scientific Research Projects Coordination Unit of Adana Alparslan Türkeş Science and Technology University (BAP) with the project number 24303003.

First and foremost, I would like to express my sincere gratitude to my advisor, Assoc. Dr. Merve Çapkın Yurtsever, for her valuable guidance, continuous support, and encouragement throughout the preparation of this thesis. Her knowledge, experience, and positive attitude have greatly contributed to my academic and personal development.

I owe my deepest appreciation to my mother for her endless love, patience, and sacrifices, which have always been a source of strength and inspiration for me. My heartfelt thanks also go to my father, whose wisdom, encouragement, and constant support have guided me in every step of my academic journey.

I am also truly grateful to my siblings, who have always supported and motivated me with their love, care, and encouragement during this process.

Above all, I am profoundly grateful to my husband, whose love, understanding, and encouragement have been my greatest source of strength. His patience and constant support have played an essential role in helping me overcome difficulties and complete this thesis.

TABLE OF CONTENTS

CERTIFICATION OF APPROVAL	Error! Bookmark not defined.
ÖZET	ii
ABSTRACT	iv
TABLE OF CONTENTS	vii
LIST OF FIGURES	x
LIST OF TABLES	xii
LIST OF ABBREVIATIONS	xiii
1. INTRODUCTION	1
2. LITERATURE REVIEW	4
2.1. Bone	4
2.2. Treatment Strategies for Bone Healing	5
2.3. Tissue Engineering	6
2.4. Bone Tissue Engineering	8
2.5. Scaffolds	9
2.6. Biomaterials	10
2.6.1. Natural Biomaterials	10
2.6.2. Synthetic Biomaterials	11
2.6.3. Bioscaffold Fabrication Techniques	12
2.7. Biofabrication Technique - Freeze-Drying	13
2.8. Cellular Approach	15
2.9. MC3T3 cell line	19
2.10. Effects of boron compounds on bone tissue engineering	20
2.12. Effects of small molecules on bone tissue engineering	24
3. MATERIALS AND METHODS	26
3.1. Production of Chitosan Scaffolds	27
3.2. Characterization of scaffolds	29
3.2.1. SEM Analysis	29
3.2.2. FTIR Analysis	30
3.2.3. Water Uptake Analysis	30
3.2.4. Mechanical Analysis	30

3.2.5. Biodegradation Studies	31
3.2.6. Antimicrobial Activity Evaluation	31
3.3. Cell Culture Studies	32
3.3.1. MC3T3-E1 Cell Culture – SPP Studies (Free SPP in cell culture medium)	32
3.3.2. Osteogenic Differentiation of MC3T3-E1 Cells in the Presence of SPP	32
3.3.3. Resazurin Analysis	33
3.3.4. ALP Staining	33
3.4. Cell Culture Studies on Ch/SPP Scaffolds	34
3.4.1. Preliminary Studies using L929 Mouse Fibroblast Cell Line	34
3.4.2. Cell Culture Studies Using MC3T3-E1 Cells	35
3.4.2.1. Viability MC3T3-E1 Cells on Ch/SPP Scaffolds / Resazurin Analysis	35
3.4.2.2. Morphology of MC3T3-E1 Cells on Ch/SPP Scaffolds	35
3.4.2.3. DAPI/Factin Fluorescence Staining of MC3T3-E1 Cells on MG Ch/SPP Scaffolds	36
3.5. Cell Culture Studies on Medical Grade Ch/SPP Scaffolds and Using Different Stabilization Techniques for Scaffolds	36
3.6. Statistical Analysis	37
4. RESULTS AND DISCUSSION	37
4.1. Characteristics of Scaffolds	37
4.1.1. Morphology of Scaffold	37
4.1.2. Biodegradation	40
4.1.3. Water uptake	43
4.1.4. FTIR Analysis	46
4.1.5. Mechanical Analysis	47
4.1.6. Antimicrobial Activity	48
4.2. MC3T3-E1 Cell Line – SPP Studies	49
4.3. L929 Cell Line / Ch-SPP Scaffold Studies (Preliminary Studies)	55
4.4. MC3T3-E1 Cell Line / Ch-SPP Scaffold Studies	55
4.4.1. Resazurin Analysis	55
4.5. Comparison of Stabilization Methods for Ch and Medical Grade Ch for MC3T3-E1 Cell Attachment and Proliferation	59
4.6. Effect of Sodium Pentaborate Pentahydrate Concentration in Medical-Grade Chitosan Scaffolds on MC3T3-E1 Cell Viability	60

4.6.1. Resazurin Analysis	60
4.6.2. SEM Analysis	61
4.6.3. DAPI/F-actin Fluorescence Staining of MC3T3-E1 Cells on MG Ch-SPP Scaffolds	62
4.6.4. Water Uptake Analysis	65
4.6.5. FTIR Analysis	67
5. DISCUSSION	68
6. CONCLUSION	76
7. FUTURE WORKS	76
References	77
ÖZGEÇMİŞ	Error! Bookmark not defined.
Curriculum Vitae	Error! Bookmark not defined.
Education	Error! Bookmark not defined.

LIST OF FIGURES

Figure 2.1 Bone's structure is hierarchical, including macrostructure to subnanostructure	4
Figure 2.2 Large bone defect: a complicated concern.....	5
Figure 2.3 Tissue-engineered cartilage on a mouse model.....	7
Figure 2.4 Crucial scaffold characteristics influencing cell behavior.....	10
Figure 2.5 Stem cells used in bone tissue engineering.....	21
Figure 2.6 Culture procedure for ESC osteogenic differentiation.....	23
Figure 2.7 Bone marrow MSC differentiation strategy	25
Figure 3.1. Schematic of the main analyses of this thesis.....	26
Figure 3.2 Schematic Representation of Scaffold Preparation Process (RW Method.....	30
Figure 3.3 Preparation of chitosan scaffolds.....	31
Figure 4.1 SEM imaging results	40
Figure 4.2 Pore diameters calculated using ImageJ software.....	41
Figure 4.3 Visual degradation of scaffolds	42
Figure 4.4 Degradation percentage of scaffolds	43
Figure 4.5 Representation of precentage mass loss	43
Figure 4.6 Macroscopic swelling behavior before and after PBS immersio.....	45
Figure 4.7 Water uptake profiles of chitosan and chitosan–SPP scaffolds during a 60-minute immersion in PBS.....	46
Figure 4.8 Equilibrium water sorption capacity (Q) of chitosan and chitosan–SPP scaffolds with varying SPP concentrations.....	46
Figure 4.9 Possible interaction between SPP and chitosan (chemical structure images from PubChem, July 2025).....	47
Figure 4.10 FTIR analysis spectra of chitosan polymer, SPP, Ch, and Ch–SPP scaffolds.....	47
Figure.4.11 Mechanical analysis figure	48
Figure 4.12 Antifungal activity of Ch–SPP composite scaffolds demonstrated by an agar diffusion test.....	49
Figure 4.13 The effect of SPP on viability of MC3T3-E1 cells at 24 h culture.....	50
Figure 4.14. Invert phase contrast images of MC3T3-E1 cells with or without SPP at 0.125, 0.25, and 0.5 mg/mL concentrations in both culture and osteogenic differentiation media (20X).....	52

Figure 4.15. Resazurin analysis results of osteogenically induced and control MC3T3-E1 cells with / without SPP at days 1, 4, 6, and 13.....	53
Figure 4.16. Resazurin analysis results at day 13.....	54
Figure 4.17 ALP staining results of osteogenically induced MC3T3-E1 cells in control medium without SPP after 14 days (20X).....	54
Figure 4.18 Resazurin analysis results of L929 cells on Ch–SPP scaffolds.....	55
Figure 4.19 Resazurin analysis of MC3T3-E1 cells on Ch–SPP scaffolds on Days 7 and 10.....	56
Figure 4.20 SEM images of MC3T3-E1 cells cultured on Ch–SPP scaffolds (Day 1)....	57
Figure 4.21 Inverted phase contrast images (10X) of MC3T3-E1 cells underneath the scaffolds after removal (Day 7).....	58
Figure 4.22 Resazurin assay results for Ch and medical-grade Ch scaffolds stabilized with ethanol or NaOH (Days 1, 4, and 7).....	59
Figure 4.23 Macroscopic images of MG Ch–SPP scaffolds after resazurin analysis.....	61
Figure 4.24 Resazurin analysis of MC3T3-E1 cells cultured on MG Ch–SPP scaffolds (Days 1, 4, 7, and 10).....	61
Figure 4.25 SEM images of MC3T3-E1 cells on MG Ch–SPP scaffolds.....	62
Figure 4.26 Fluorescence microscopy images (10× magnifications) of DAPI stained MC3T3-E1 cells on the MG Ch – SPP scaffolds at day 1 and day 10.....	64
Figure 4.27 Fluorescence microscopy images (20x) of DAPI stained MC3T3-E1 cells cultured on MG Ch – SPP scaffolds on day 10.....	65
Figure 4.28 Fluorescence microscopy images (10x) of DAPI stained MC3T3-E1 cells cultured on MG Ch – 0.75 SPP scaffolds on day 10.....	66
Figure 4.29 Macroscopic images of MG Ch scaffolds before and after swelling in PBS for 60 min at room temperature.	67
Figure 4.30 Water uptake profiles of MG Ch - SPP scaffolds during a 60-minute immersion in PBS at RT.....	67
Figure 4.31 Comparison of water uptake capacity of MG Ch-SPP scaffolds after immersion in PBS for 60 min.....	68
Figure 4.32 FTIR analysis spectra of medical grade chitosan polymer, SPP, MG Ch and MG Ch-SPP scaffolds.....	69

LIST OF TABLES

Table 2.1. Advantage and disadvantage of FD.....	16
Table 2.2. Summary of key studies involving sodium pentaborate pentahydrate.....	24
Table 3.1 Sample names.....	28
Table 3.2 cell culture Sample names.....	33
Table 3.3 Comparision of chitosan scaffold types in the study.....	37



LIST OF ABBREVIATIONS

%	: Percentage
°C	: Degrees Celsius
ALP	: Alkaline Phosphatase
BTE	: Bone tissue engineering
Ch	: Chitosan
DBM	: Demineralized Bone Matrix
FBS	: Fetal Bovine Serum
FTIR	: Fourier Transform Infrared Spectroscopy
h	: Hour
HAp	: Hydroxyapatite
MC3T3-E1	: Mouse Pre-Osteoblast Cell Line
min	: Minute
MTT	: Colorimetric assay for assessing cell metabolic activity
PBS	: Phosphate-buffered saline
PCL	: Polycaprolactone
PDA	: Potato Dextrose Agar
PEEK	: Polyether Ether Ketone
PEG	: Polyethylene Glycol
PLA	: Polylactic Acid
PLGA	: Poly(lactide-co-glycolide)
PMMA	: Polymethyl Methacrylate
Q	: Equilibrium water uptake
RGD	: Arginine–Glycine–Aspartate
RT	: Room Temperature
SEM	: Scanning Electron Microscope
SPP	: Sodium Pentaborate Pentahydrate
UV	: Ultraviolet
w/v	: Weight per Volume
w/w	: Weight per Weight
α-MEM	: Alpha Minimum Essential Medium
MG Ch-SPP	: Medical grade chitosan with sodium pentaborate pentahydrate



1. INTRODUCTION

Skeletal system consists of bone tissue, bone marrow, and ligaments and is tasked with structural support, movement, and the production of blood cells . Bone tissue is tasked with more than one function like mechanical strength, calcium-phosphate homeostasis, and hematopoiesis and is also the site of attachment for muscles and tendons (Yıldırım, 2024) . While minor fractures heal spontaneously, chronic stress, diabetic states, infection, and age can compromise healing, and surgery or regenerative therapy might be necessary. Bone grafting technologies and tissue engineering approaches try to replicate the natural remodeling process of the body for enhancing bone regeneration (Oryan et al., 2013) . Tissue engineering (TE) offers a potential alternative to traditional bone grafts using the development of biocompatible scaffolds that stimulate bone regeneration and enhance healing rate and quality. This approach overcomes problems associated with autografts, allografts, and xenografts, such as immune rejection, infection risk, and anatomical limitations. Modern bone engineering involves the integration of osteogenic cells, osteoinductive factors, and osteoconductive scaffolds and is now capable of reconstructing large bone defects with more positive outcomes (Daldiken, 2024). Tissue engineering aims to develop scaffolds that give cells support for differentiation, growth, and integration for tissue repair. The scaffolds, which emulate the extracellular matrix (ECM), have structural support, regulate biochemical cues, and aid vascularization. An ideal scaffold must be mechanically stable, biodegradable, and biocompatible, possessing a controlled rate of degradation suitable for tissue regeneration. Biocompatibility is required for scaffolds to support tissue growth without triggering immune reactions. Bioactive scaffolds enhance cell interaction, migration, and differentiation with fewer undesirable reactions. Current developments are aimed at immune-inert biomaterials that modulate immune reactions with less activation of natural killer (NK) cells and inflammation (Echeverria Molina et al., 2021). Scaffolds made from natural biomaterials such as collagen, chitosan, hydrogels, and demineralized bone matrix (DBM) are the pillars of bone tissue engineering because they have the potential to replicate the tissue's extracellular matrix (ECM) morphology. They are biocompatible to accommodate cell attachment, growth, and differentiation, as well as biochemical signaling (Verrier et al., 2016) . Chitosan, a natural polysaccharide from chitin, shows promise as a biomaterial for various biomedical applications due to its biocompatibility, biodegradability, antibacterial activity, and ease of

modification (Hutmacher, 2001). According to opposing research, chitosan's ability to heal is unclear due to its low biodegradability, limited osteoconductivity (in the context of bone healing), and the presence of fibrous connective tissue, inflammatory cells, and scaffold remnants instead of the formation of new tissue. However, they demonstrated that enhancing chitosan with more biocompatible and biodegradable biomaterials, such as Gel, and with human platelet-derived promotive growth factors could improve the repair of radial fractures in rats (Oryan & Sahviah, 2017). Boron is an essential trace element that has been linked to bone health and development. Studies have shown that boron (for example, in the form of boric acid) can enhance mineralization and stimulate the expression of bone-related proteins in osteoblast cells, suggesting a supportive role in bone formation. Boron compounds have also demonstrated beneficial effects in other biological contexts, such as promoting wound healing and influencing cell proliferation. Sodium pentaborate pentahydrate (SPP), chemical formula $\text{Na}_2[\text{B}_4\text{O}_5(\text{OH})_4] \cdot 5\text{H}_2\text{O}$, is a particularly interesting boron-rich synthetic compound that has recently gained attention in biomedical research. Due to its high boron content, SPP has been explored for diverse applications. For instance, it has been added to shrimp feed formulations to reduce pathogen loads and has been used as an ingredient in certain enzymatic detergents. More relevantly, SPP has been incorporated into topical therapeutic formulations aimed at accelerating tissue repair, such as skin wound healing creams or gels. In the medical field, SPP's remarkable wound-healing and antimicrobial properties have been documented.

Multiple studies have demonstrated that SPP can significantly improve the healing of skin wounds. For example, (Demirci, 2016) reported that a Pluronic-based hydrogel enriched with SPP accelerated burn wound healing in a rat model. The SPP-loaded hydrogel enhanced key aspects of repair, including increased collagen deposition, angiogenesis (new blood vessel formation), and keratinocyte migration to re-epithelialize the wound. Treated wounds also showed upregulated expression of repair-associated proteins such as α -smooth muscle actin (α -SMA), transforming growth factor- β 1 (TGF- β 1), vimentin, and vascular endothelial growth factor (VEGF), indicating robust tissue regeneration; in addition, the SPP hydrogel exhibited broad-spectrum antimicrobial activity, helping to prevent infection at the wound site. Similarly (Doğan et al., 2014) found that a hydrogel containing SPP (NaB) markedly improved the closure of full-thickness skin wounds. In their study, SPP treatment promoted greater human dermal fibroblast migration and significantly increased wound contraction and tissue remodeling compared to control treatments. These findings suggest that boron-

containing compounds like SPP can positively influence the cellular processes involved in tissue repair and regeneration, while also providing protection against microbial contamination in wounds (Cebeci & Fikretin, 2022). Based on this background, we hypothesized that integrating a boron-rich compound (SPP) into a chitosan scaffold could yield a composite biomaterial with enhanced properties for bone tissue engineering. In the present study, porous chitosan scaffolds were fabricated with different concentrations of sodium pentaborate pentahydrate . We then systematically characterized the scaffolds' physicochemical properties and biological performance. Key evaluations included scaffold morphology and porosity (to ensure a structure conducive to cell infiltration), chemical structure confirmation of the boron additive, swelling behavior and enzymatic degradability (to assess how the scaffold would perform and resorb in physiological conditions), and mechanical stability. We also examined the antimicrobial efficacy of the SPP-incorporated scaffolds, given SPP's known antimicrobial properties, and tested their cytocompatibility using the MC3T3-E1 pre-osteoblast cell line. Cell viability assays and preliminary markers of osteogenic differentiation were measured to determine if the presence of SPP in the scaffold has any cytotoxic effects or osteoinductive benefits for bone-forming cells. The overall objective of this work was to evaluate whether SPP-enriched chitosan scaffolds could serve as effective, bioactive scaffolds for bone regeneration. The results of this study are expected to contribute to the development of improved composite scaffolds that harness the benefits of both natural polymers and boron-based additives for bone tissue repair.

2. LITERATURE REVIEW

2.1. Bone

Skeletal system consists of bone tissue, bone marrow, and ligaments and is tasked with structural support, movement, and the production of blood cells (Yıldırım, 2024) . Bone tissue is tasked with more than one function like mechanical strength, calcium-phosphate homeostasis, and hematopoiesis and is also the site of attachment for muscles and tendons (Yıldırım, 2024). Comprising organic (collagen) and inorganic (hydroxyapatite) composition, bone is strengthened and stiffened through hierarchical structure (Figure 2.1). At the microscopic scale, bone possesses Haversian canals, tubular tubes with blood vessels and nerves, surrounded by osteons in layers of lamella . The layers resist fracture and mechanical flexibility and ensure durability from mechanical stress (Kocagöz, 2024). Mineralized collagen at the subnanoscale contain hydroxyapatite nanocrystals (~500 nm in collagen fibrils) and collagen fibers at the nanoscale, with ~5 µm diameters, in which mineral deposit and increase in tensile strength occur (Kocagöz, 2024). Healing of bone occurs in a organized manner and this begins with the formation of hematoma that possesses the potential for attracting neutrophils and macrophages, which initiate the inflammation (Ek & Prof, 2013). Fibroblasts migrate to the site, inducing cellular proliferation and differentiation, while mesenchymal stem cells differentiate into osteoblasts and form a fibrous bone callus . Subsequently, periosteal and endosteal osteoblasts migrate into the area and create primary (immature) bone tissue, which is subsequently remodeled into mature bone (Tunçay, 2013). While minor fractures heal spontaneously, chronic stress, diabetic states, infection, and age can compromise healing, and surgery or regenerative therapy might be necessary . Bone grafting technologies and tissue engineering approaches try to replicate the natural remodeling process of the body for enhancing bone regeneration (Oryan et al., 2013) . Understanding hierarchical bone structure at macroscopic down to molecular level is key to developing biomaterials for bone tissue engineering (Kocagöz, 2024).

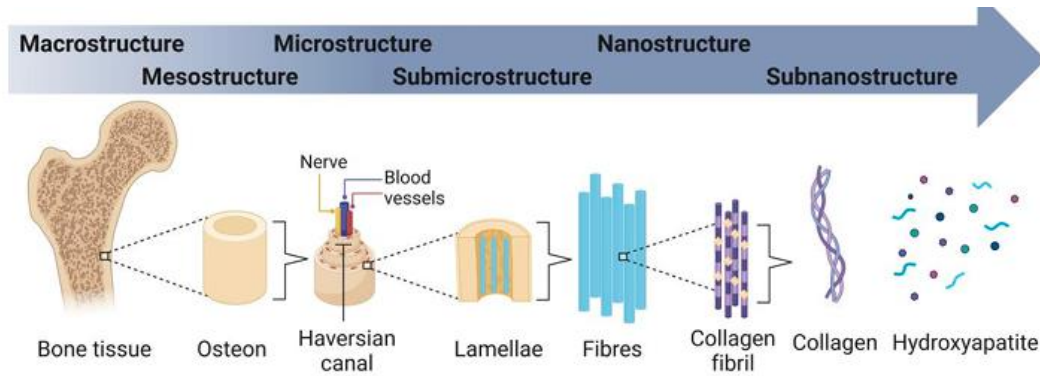


Figure 2.1. Bone's structure is hierarchical, that includes macrostructure to subnanostructure (Kocagöz, 2024).

2.2. Treatment Strategies for Bone Healing

There have been numerous therapeutic techniques developed that accelerate the healing process and correct bone defects (Figure 2.2). They consist of a variety of glycosaminoglycans, growth factors, stem cells, auto-, allo-, or xenografts, and tissue-engineered synthetic and biologic scaffolds (Oryan et al., 2013). Bone Graft Materials and Applications :Bone graft materials are predominantly employed as biocompatible structural supports and tissue engineering technology optimizes their potential to regenerate. In dentistry and orthopaedics, they rectify bone defects, i.e., periodontal illness. Autografts are highly osteoinductive in the formation of new bone but are not resorbable to allow structure maintenance. No graft material exists for all needs in the clinic. All grafts are osteoconductive as they induce bone formation but only autografts are osteoinductive and osteogenic since they possess living progenitor cells for bone growth (Miron, 2023). Autogenous and allogenic bone grafts remain active therapeutic options for osteogenic and osteoconductive stimulation of critical bone defect. Autografts, on the other hand, have some drawbacks such as donor site morbidity, pain, expense, and lack of availability, and there is late biomechanical failure (Verrier et al., 2016). Allografts, while having the advantage in the case of larger bone defects, risk the transmission of infectious diseases (e.g., hepatitis, HIV) and immune system rejection. Ethical concerns limit their use even more . Likewise, xenografts cause inflammatory responses, resorb too quickly, and are added with risks of zoonotic transmission of diseases like rabies and Epstein-Barr virus (Oryan et al., 2013). Bone defect healing continues to be a challenging process with good synchronization of scaffolds, cells, growth

factors, and mechanical stimulation. Tissue engineering provides an alternative for the limitations of grafting procedures through stimulating better bone regeneration through the utilization of sophisticated biomaterials and regenerative methods (Verrier et al., 2016).

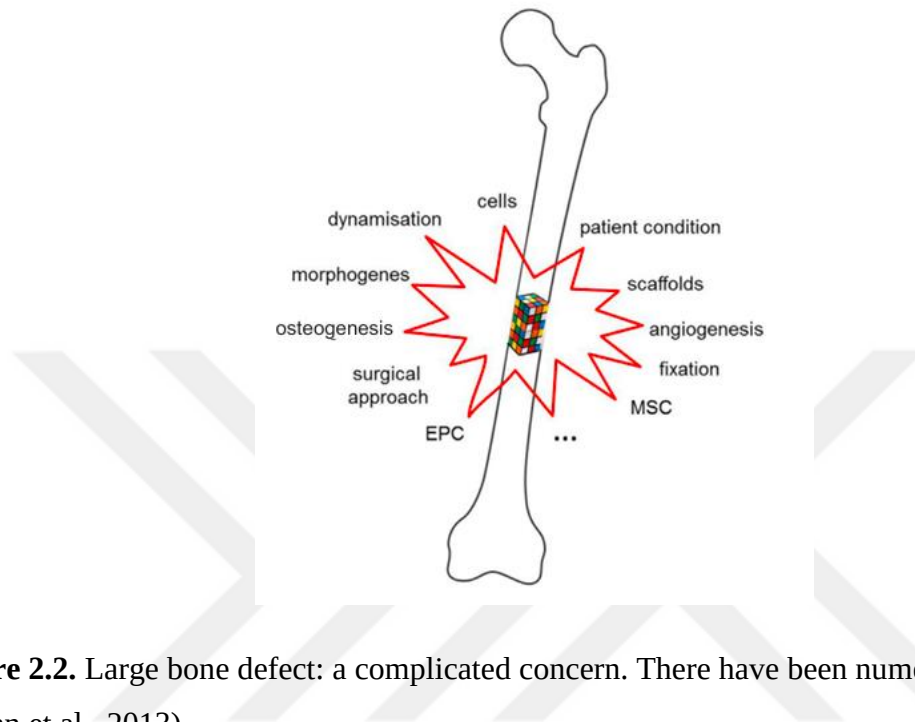


Figure 2.2. Large bone defect: a complicated concern. There have been numerous therapeutic (Oryan et al., 2013).

2.3.Tissue Engineering

Tissue engineering (TE) offers a potential alternative to traditional bone grafts using the development of biocompatible scaffolds that stimulate bone regeneration and enhance healing rate and quality. This approach overcomes problems associated with autografts, allografts, and xenografts, such as immune rejection, infection risk, and anatomical limitations. Modern bone engineering involves the integration of osteogenic cells, osteoinductive factors, and osteoconductive scaffolds and is now capable of reconstructing large bone defects with more positive outcomes (Daldiken, 2024). Tissue engineering history dates back to the 1970s when Dr. W.T. Green tried to regenerate cartilage by seeding chondrocytes onto bone spicules in nude mice. Although these initial attempts did not succeed, they indicated the possibility of biocompatible scaffolds for the growth of tissue. The significant break-through came in the 1980s, when synthetic, biodegradable scaffolds were discovered by Dr. Joseph Vacanti and Dr. Robert Langer, presenting opportunities for controlled cell delivery systems (Vacanti,

2006). TE is an inter-disciplinary method that mimics the natural repair processes of the body and develops innovative solutions to repair and regrow tissues (Yıldırım, 2024) . The BBC news report of the "auriculosaurus"—an ear designed from tissue on a mouse—demonstrated the principle of seeding cells onto scaffolds (Figure 2.3). These techniques have now been applied to dermal fibroblast culture on collagen fiber scaffolds and have made significant advances in the construction of artificial skin (Vacanti, 2006) . Bone fractures do not necessarily heal, even with advanced orthopedic care, and the quest for an understanding of fracture healing biology cannot cease. Tissue engineering methods utilize biomaterials, cells, and bioactive factors for stimulating bone regeneration and tissue integration (Yıldırım, 2024). The cells utilized in TE can be obtained from the patient (autografts), from other people (allografts), or from animals (xenografts), based on the clinical requirement . In combination with biocompatible biomaterials, TE scaffolds act as structural matrices and delivery vehicles for the proliferation and differentiation of cells. This technique has vast potential to counteract the effects of aging, heal injuries, and treat degenerative diseases, pushing the boundaries of modern medicine (Yıldırım, 2024) .



Figure 2.3 An image of the infamous mouse with the human ear shows newly created cartilage that has been tissue-engineered to resemble a human ear. (Vacanti, 2006) .

2.4. Bone Tissue Engineering

Bone tissue engineering uses scaffolds, seeded cells, and bioactive growth factors to facilitate bone repair and regeneration, offering a sophisticated solution in regenerative medicine. The surface physical properties of scaffolds, hydrophilicity, hydrophobicity, and biochemical composition play a crucial role in cell attachment. Some biological domains on scaffold surfaces, arginine–glycine–aspartate (RGD) sequences, are strongly proven to promote cell attachment. Studies have demonstrated that the optimum pore size for efficient bone regeneration lies between 20–1500 μm , with approximately 300 μm being particularly effective in osteogenesis because it has the capability of facilitating cell migration, vascularization, and bone ingrowth. However, pore sizes below 100 μm restrict the diffusion of nutrients and may cause necrosis, while pores greater than 750 μm may affect the mechanical integrity and effectiveness of regeneration (Xue et al., 2022). Tissue engineering provides a promising alternative to bone healing, particularly where fractures are surgical. By applying biomaterials, cells, and bioactive factors, the process enhances bone regeneration while restricting reliance on donor tissue. One of the most important advantages is the potential for a single operation, as properly designed scaffolds with optimal mechanical properties minimize the risk of implant failure and facilitate easy integration with native bone. Studies indicate the use of mesenchymal stem cells (MSCs) in bone tissue engineering, as they improve healing and decrease recovery time, ultimately improving patients' quality of life. In addition, application of bioactive molecules and growth factors (GFs) from endogenous origins has been explored to further enhance functional tissue development (Manzini et al., 2021). Tissue engineering of bone aims to mimic the structure and function of native bone tissue for more effective and physiologically relevant treatment. Bone cells like osteoblasts, osteoclasts, and osteocytes communicate within hybrid matrix to remodel and maintain tissue. To induce bone regeneration, researchers are creating biodegradable polymeric biomaterials as temporary scaffolds that mimic the natural ECM very closely. These matrices are used as a scaffold for bone regeneration, gradually degrading and being absorbed by the body as functional tissue is created (Maia et al., 2022).

2.5.Scaffolds

Tissue engineering aims to develop scaffolds that give cells support for differentiation, growth, and integration for tissue repair. The scaffolds, which emulate the extracellular matrix (ECM), have structural support, regulate biochemical cues, and aid vascularization. An ideal scaffold must be mechanically stable, biodegradable, and biocompatible, possessing a controlled rate of degradation suitable for tissue regeneration. Biocompatibility is required for scaffolds to support tissue growth without triggering immune reactions. Bioactive scaffolds enhance cell interaction, migration, and differentiation with fewer undesirable reactions. Current developments are aimed at immune-inert biomaterials that modulate immune reactions with less activation of natural killer (NK) cells and inflammation (Echeverria Molina et al., 2021). Critical scaffold properties for morphology and structure influencing cell behavior showed in Figure 2.4. Scaffolds must be porous, three-dimensional in structure to facilitate diffusion of nutrients and cell infiltration. Proper porosity and pore size improve oxygen exchange, vascularization, and mechanical stability. Surface modification, e.g., nanotechnology-based approaches, improves protein adsorption and osteoblast function, overall improving scaffold integration (Henkel et al., 2013) . A scaffold must balance mechanical support and degradation rates to remain stable while tissue remodeling takes place. Load-bearing applications require scaffolds that have early structural support with subsequent progressive degradation to facilitate new tissue formation. Bone remodeling is different—cancellous bone is regenerated in 3–6 months, whereas cortical bone is regenerated in more than 12 months. When degradation of the scaffold is coordinated with this time, it prevents early failure of mechanical support (Eltom et al., 2019) . Scaffolds are fabricated from natural polymers, synthetic polymers, bioceramics, or hybrid composites. Natural polymers like collagen and chitosan possess good biocompatibility but possess limited degradation times. Synthetic polymers like polylactic acid (PLA) and polycaprolactone (PCL) possess controlled degradation and better mechanical performance. The hybrid materials possess both these characteristics combined, more ensuring integration and duration (Echeverria Molina et al., 2021) . Fabrication techniques include traditional methods like solvent casting, gas foaming, and electrospinning and newer techniques like 3D printing and bioprinting. The new technologies allow precise scaffold customization, which improves functionality and reproducibility in clinical use (Henkel et al., 2013). Developments in biomaterials, nanotechnology, and biofabrication continue to advance scaffold performance.

Scaffold-free and biomimetic strategies are under development as exciting opportunities, which promote natural tissue growth without synthetic scaffolds. Upcoming research attempts to advance scaffold geometry to more effectively mimic natural tissue, to yield improved clinical performance for regenerative medicine procedures (Eltom et al., 2019).

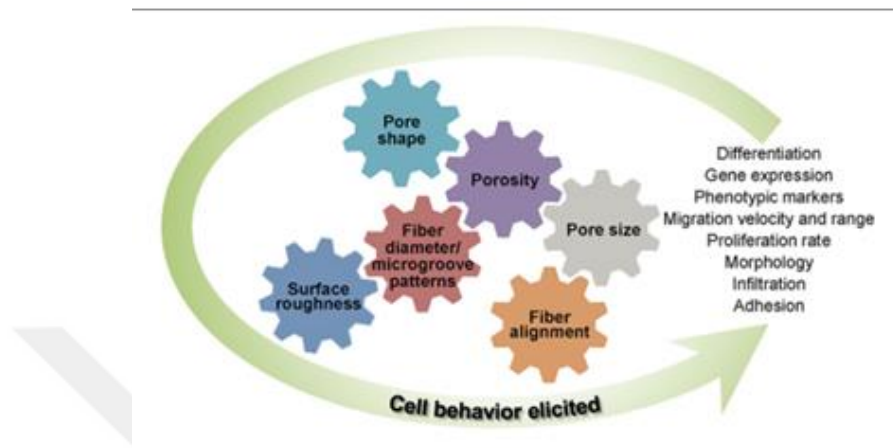


Figure 2.4 Crucial scaffold characteristics for structure and morphology that influence cell behavior (Henkel et al., 2013) .

2.6.Biomaterials

Biomaterials are the focal point of bone tissue engineering as they facilitate the delivery of osteogenic factors with controlled release and sustained biological activity and without loss of bioactivity. The ideal biomaterial would be biodegradable, non-toxic, and metabolized in vivo without affecting cellular function. Biomaterials can either be organic or inorganic, although the two can be hybridized to enhance their use (Manzini et al., 2021) .

2.6.1.Natural Biomaterials

Scaffolds made from natural biomaterials such as collagen, chitosan, hydrogels, and demineralized bone matrix (DBM) are the pillars of bone tissue engineering because they have the potential to replicate the tissue's extracellular matrix (ECM) morphology. They are biocompatible to accommodate cell attachment, growth, and differentiation, as well as biochemical signaling. Porous and spongy collagen scaffolds are applied in bone regeneration, while also as carriers of genes or growth factors. Chitosan, derived from chitin, has

osteoinductive properties, whereas hydrogels like alginate and fibrin are utilized to prepare injectable scaffolds for the repair of bone. While useful, these biomaterials have poor mechanical properties, and hybrid systems or composites can improve them (Verrier et al., 2016) . Bio-Oss, a depurinated matrix of bovine bone, is osteoblast and angiogenic favorable but slow resorbing with secondary integration complications. Autologous dentin-derived grafts (AutoBT) are osteoconductive and immune compatible but are tainted with clinical acceptability since preparation takes time (Xue et al., 2022) . Alginate, a brown alga component, gels by ionotropic gelation, while mechanical stability depends upon guluronate to mannuronate unit ratio. It is tunable for degradation and release control of growth factors, i.e., SDF-1 and BMP-2, for optimal osteogenesis. Silk fibroin scaffolds are HAp- and bone-mineralizable and used in tissue engineering. Chitosan is mechanically weak but can be strengthened by crosslinking through blending with HAp and genipin treatment. Decellularized matrices tissue-structured and vascularized are of great promise towards the regeneration of bones but must be modified so that their mechanical properties are improved (Maia et al., 2022) .

2.6.2. Synthetic Biomaterials

Scaffolds made of synthetic materials provide greater control of mechanical strength, porosity, and degradation rate and hence are best suited for healing large bone defects. Hydroxyapatite, β -tricalcium phosphate, bioactive glasses, and biodegradable polymers like poly(lactide-co-glycolide) (PLGA) and poly(ϵ -caprolactone) (PCL) are some preeminent synthetic biomaterials. These biomaterials are subjected to novel fabrication methods like 3D printing, electrospinning, and injection molding to create constructs that support osteogenesis. Poly(ethylene glycol) (PEG)- or hyaluronic acid-based synthetic hydrogels allow for the incorporation of bioactive molecules to create controlled drug release. Hybrid materials made up of synthetic polymers and synthetic or native ECM molecules or biominerals are among the most recent advances, aimed at creating enhanced bioactivity and mechanical properties. Regulatory concerns in the form of preclinical examinations are, nevertheless, a restraint for early clinical translation for more recent synthetic compounds (Verrier et al., 2016) . Synthetic polymers like PLA, PGA, and PLGA are undergoing considerable investigation for bone tissue engineering. Polymethyl methacrylate (PMMA) is also widely employed bone cement with strong hardening and strengthening but long-term constraining due to

cytotoxicity and nonbiodegradability. Follow-up: newcomers introduced the arrival of polyetherketoneketone (PEKK or PEEK), a biocompatible polymer with an excellent human bone elastic modulus, with the best thermal stability, corrosion resistance, and mechanical performance. Biodegradability and biocompatibility issues remain and are still modified by surface coating and bioactive additives towards improved performance. Over 20 new bone graft substitutes made up of highly hydroxyapatite and deproteinized mineral matrices have been created in the past decade alone, which shows the enhanced potential of biomaterials for bone regeneration (Xue et al., 2022). PLGA, PLA, and PCL possess improved mechanical properties and can even be engineered with cell-adhesion peptides like RGD. PLGA is, however, osteochondral regeneration tunable, while PLA and PCL, for load-bearing, must be chemically altered in order to increase cell adhesion (Maia et al., 2022).

2.6.3. Bioscaffold Fabrication Techniques

Gas foaming is a scaffold fabrication method that avoids high temperatures and organic solvents, preventing damage to surrounding tissues and bioactive compounds. It uses high-pressure carbon dioxide (800 psi) to create highly porous scaffolds. The dissolved gas destabilizes, leading to pore nucleation, polymer expansion, and density reduction. Porogens like sugar, salts, and wax control porosity. The polymer (e.g., PLGA) fuses around the porogen, forming a continuous matrix. After saturation with carbon dioxide, the porogen is removed, resulting in a highly interconnected pore structure (Liu et al., 2010).

Electrospinning the process of electrospinning A stream of an electrically charged polymer in a solution or viscous state is pulled into fibre by electrostatic forces during the electrospinning process. The four primary components of a basic electrospinning equipment are a metallic needle to facilitate the flow of electricity into the polymeric solution, a metallic collector for fibre collection, a syringe pump, and a power source. The spinneret and fibre collector are usually connected to opposite-ended electrical terminals to form a scaffold. Nanoscale fibre production is made possible by the material being dragged out and deposited onto a collector due to the potential difference between terminals. Electrospinning is usually used to create collagen and gelatine nanofibers with high porosities and large surface areas (Collins et al., 2021)

3D printing is one of the most popular methods for creating scaffolds. Extrusion, inkjet, and laser-assisted printing are some of the several techniques that jointly make up 3D printing . These methods allow for the layer-by-layer creation of increasingly complex and reproducible scaffolds. Furthermore, because various biomaterials, growth hormones, and cells may be printed simultaneously (3D bioprinting) with exact control over their distribution, the resulting scaffolds can accurately replicate the environmental aspects of bone tissue. Therefore, it is feasible to create a scaffold tailored to a patient in a minimal amount of time and expense. Choosing the best printing technique and the most suitable biomaterial (ink) are crucial for producing a proper 3D printed scaffold (Maia et al., 2022).

A new technology called bioprinting makes 3D printing even more useful by allowing things including biological elements or cells to be produced collaboratively. This novel method offers potential uses in tissue engineering for bone restoration, and a variety of biomaterials, including hydrogels, can be employed. They contain a lot of water, have a porous structure that promotes the flow of gases and nutrients, and have intriguing viscoelastic qualities for construction. In addition, they are soft and flexible. Because they resemble biological tissues, hydrogels like alginate offer a possible substitute for tissue engineering. The synergy of this kind of biomaterial with therapeutic choices including proteins, GFs, medication delivery, and cells can still be encouraged at the same time (Manzini et al., 2021).

2.7. Biofabrication Technique - Freeze-Drying

Freeze-drying (FD) is a very common technique used to produce bioactive scaffolds with porous structure. It is nonetheless mainly limited to the production of scaffolds in simple flat 3D geometries (Brougham et al., 2017) . FD involves drying the material first and subsequent sublimation of the solvent. Before this process, the sample must be frozen and emulsified so that pore formation is maintained throughout the process. The frozen samples are then placed in a system, wherein it uses vacuum and low temperature in removing a sequence of solvents without exposing the structure to breakdown or collapse. For the conservation of the original structure, temperature must be such that polymer-rich regions are not dissolved (Montanheiro et al., 2022). Different FD parameters, both instrumental and solution conditions, can regulate these properties, resulting in the final biological, mechanical, and physical properties required for different tissues (Fereshteh, 2018). Another advantage of FD technology is the extensive choice of materials which can be applied such as water-soluble polymers, organic-soluble

polymers, and ceramics. The technology produces scaffolds with numerous minute, interlocking pores, through which cell adhesion, cell growth, and cell formation become easy. Porosity should be appropriate so that blood vessels as well as liquid can flow, increasing the biocompatibility between the scaffold and the body. Forming a very ordered porous architecture is especially valuable for tissues that must be aligned with accuracy. It also stabilizes the scaffold without changing its composition (Fereshteh, 2018). FD technology is easy to understand as it only entails physical processes like dissolving, freezing, and sublimation. One of its most significant advantages is that it uses water as a solvent instead of harmful organic solvents, so it is a greener, less expensive, and safer process. FD also allows for the creation of highly biocompatible scaffolds. It can also be combined with other techniques like gas foaming, salt leaching, liquid dispensing, and gel casting to further enhance scaffold properties. Since FD is a non-heat process, it does not interfere with the incorporation of temperature-sensitive additives like proteins, drugs, and growth factors and represents a potential method for drug-delivery 3D scaffold development. A drawback is that equipment is quite costly for initial use in FD (Fereshteh, 2018). Secondly, it is a time-consuming process and hence not very cost-effective in mass production. Another drawback is that the samples so produced can shrink, losing their structural integrity. However, despite these shortcomings, freeze-drying is one of the most common methods to create polymeric scaffolds, particularly in those applications where highly porous scaffolds with bone mesenchymal stem cell compatibility and the ability to support chondrocyte attachment and growth in cartilage tissue engineering are required (Montanheiro et al., 2022). A comprehensive comparison of FD benefits and drawbacks is shown in Table 2.1.

Table 2.1. Advantage and disadvantage of FD

Advantages	Disadvantages
Tunable pore size and structure (Montanheiro et al., 2022)	The process is time-consuming (Montanheiro et al., 2022)
The extensive range of materials that can be used (Fereshteh, 2018)	It less cost-effective for large-scale production (Montanheiro et al., 2022)
Technology is easy to understand because it relies entirely on physical processes (Fereshteh, 2018)	The final samples may experience shrinkage (Montanheiro et al., 2022)
Ideal for incorporating heat-sensitive additives (Fereshteh, 2018)	The initial cost of the equipment needed for FD is quite high (Fereshteh, 2018)

2.8. Cellular Approach

In the scientific field of regenerative medicine, bone tissue regeneration has so far been a significant concern for biological healing. One of the most promising methods to create biological bone substitutes that preserve, restore or enhance bone tissue function is bone tissue engineering, a broad field that includes engineering, biology and medicine, as mentioned in previous sections. Due to their ability to produce a substance that closely resembles natural bone tissue, different stem cells have been viewed as potentially potent tools for the area of bone tissue engineering in light of the ageing population and the global scarcity of donor tissue (Seong et al., 2010). When selecting a cell source for bone tissue engineering, there are a number of fundamental factors to take into account: whether to use the patient's own (autologous) or another person's (allogeneic) cells; whether tissue can be harvested easily and with minimal donor site morbidity; whether to use primary osteogenic cells with limited proliferative potential or self-renewing stem cells; whether to use cells with both osteogenic and vasculogenic potential to support the formation of vascularized bone; whether the obtained cell population is homogeneous and allows for the controlled induction of the osteogenic phenotype; stability and safety of the cell after implantation; and whether automation and quality control measures can be developed for the generation of cells and grafts (Marolt et al., 2010). Finding cell sources that can be implanted to the site of the bone defect and will differentiate into osteoblasts and form neo-vasculature is the main barrier to improving the effectiveness of these cellular therapies (Amini et al., 2012). A number of cell types, including stem cells such as embryonic stem cells (ESCs), bone marrow (BM)-derived or umbilical cord blood (UCB)-derived mesenchymal stem cells (MSCs), adipose tissue-derived stem cells (ADSCs), muscle-derived stem cells (MDSCs) and dental pulp stem cells (DPSCs, Figure 2.5), have been studied thus far for their potential to support bone repair and regeneration. Additionally, by combining these stem cells with an appropriate biomaterial and cultivation technique, three-dimensional (3D) biological bone structures have been produced (Seong et al., 2010).

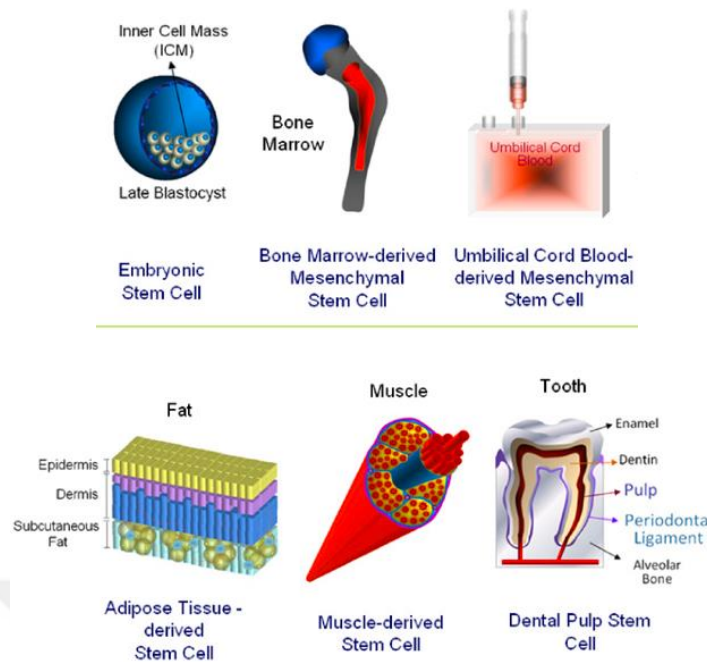


Figure 2.5. Stem cells used in bone tissue engineering applications (Seong et al., 2010).

2.8.1. Embryonic Stem Cells (ESCs)

By definition, embryonic stem cells (ESCs) are derived from "embryos," which are produced during fertilisation and continue to develop until the ninth week of pregnancy. To get around the ethical arguments surrounding their use, ESCs are commonly extracted from additional embryos created using in vitro fertilisation procedures. Due to their high proliferative activity and pluripotency, ESCs may be utilised as a single source to derive several lineages found in adult bone, such as osteogenic cells, vascular cells, osteoclasts, and nerve cells for bone regeneration (Amini et al., 2012). Because of these properties, ESCs are among the best cell sources for tissue engineering and regenerative medicine. It is possible to control the lineage-specific differentiation of ESCs by adjusting the microenvironment and certain culture conditions (Seong et al., 2010). Directing ESC differentiation into osteogenic lineage has received a lot of interest lately. Leukaemia inhibitory factor (LIF) or feeder cell layer removal can both trigger in vitro differentiation of ESCs. In the case of murine ESCs, this also results in the creation of three-dimensional cell aggregates called "embryoid bodies" (EBs). ESCs go through a similar differentiation route during in vitro EB creation as they do during early development in vivo. These cell types exhibit differentiation into mesoderm, ectoderm, and endoderm derivatives that are unique to a given location. EB formation, which produces spontaneous mesoderm formation as a need for terminal differentiation towards osteogenic

lineages, is used in the majority of contemporary methods for in vitro osteogenic differentiation (Figure 2.6). Several chemicals have been used to induce in vitro osteogenic lineage development from spontaneously produced mesoderm within EBs. Following EB creation, EBs or dissociated single cells from EBs are replated onto tissue culture plates (Seong et al., 2010). ESCs can either form embryoid bodies initially or not in order to achieve osteogenic differentiation. The first strategy involves the production of an embryoid body, its dissociation, the re-plating of dissociated single cells, and the introduction of osteogenic supplements, such as 1,25-hydroxy vitamin D3, β -glycerophosphate, ascorbic acid, dexamethasone, and retinoic acid. However, this strategy might only produce a few number of the type of cell needed because of the restricted control over lineage-specific differentiation of ESCs within EBs. Thus, researchers have looked for more effective, straightforward, and practical culture techniques, by directly differentiating ESCs into the osteogenic lineage (Amini et al., 2012).

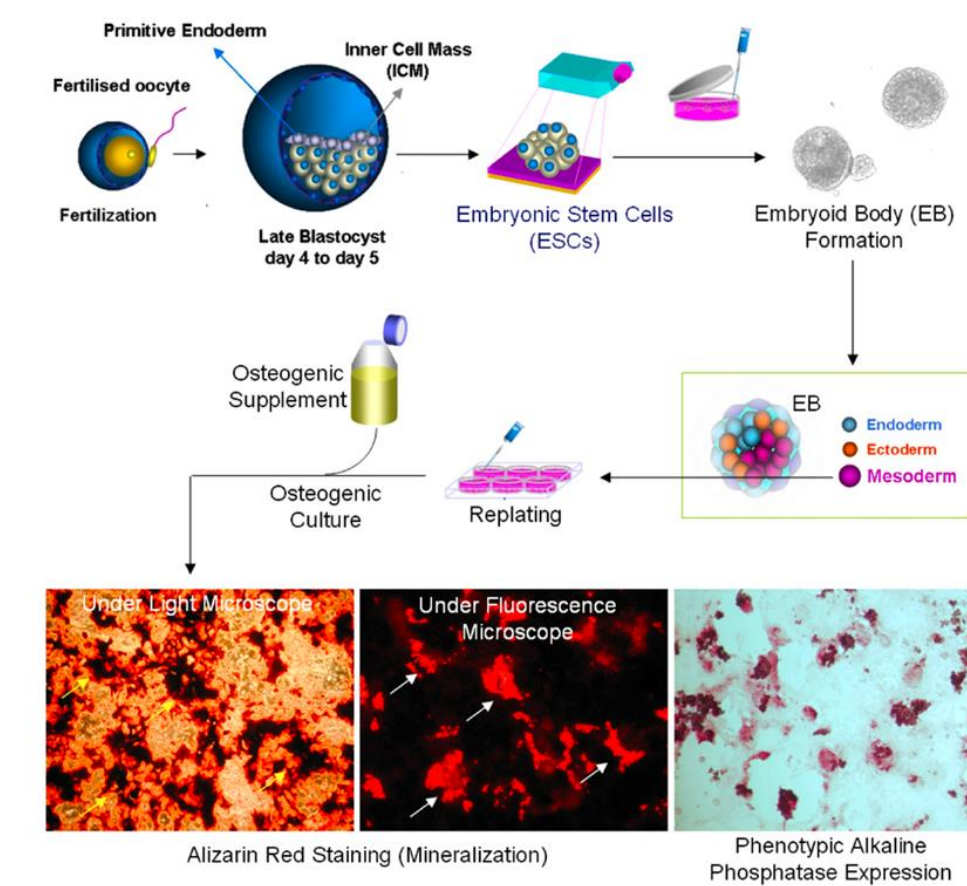


Figure 2.6. Culture procedure for ESC osteogenic differentiation (Seong et al., 2010).

2.8.2. Adult Stem Cells

Mesenchymal Stem Cells (MSCs): Because MSCs differentiate and create bone during the natural bone growth process, they have long been recognised for their potential in bone transplant engineering. Because of the huge opportunity in BTE, they have been characterised and multiple pathways for their isolation have been found. The expression of different CD markers—that is, negative for CD34, CD45, CD14, CD11a, CD19, and HLA-DR and positive for STRO-1, CD29, CD73, CD90, CD105, CD106, CD166, CD146, and CD44—has been used to identify MSCs (Amini et al., 2012).

2.8.3. Bone Marrow Stem Cells

Due to their abundance and ease of harvesting, BMSCs have been utilised most frequently in BTE research. They can be separated from a number of biological sources, including as the placenta, peripheral blood, umbilical cord blood, and BM. They typically make up *0.001%–0.01% of the cells in the BM and are isolated from BM aspirates of volunteer donors, which are often taken from the iliac crests or femoral heads of patients. BM has more osteogenic SCs than peripheral blood, despite the fact that SCs may be separated from peripheral blood (Szpalski et al., 2012). MSCs, which are found in the marrow (Figure 2.7), aid in the regeneration of mesenchymal tissues like bone, cartilage, muscle, ligaments, tendons, adipose, and stroma. Three primary techniques have been used to isolate MSCs from bone marrow: (1) obtaining only adherent cells on tissue culture plates following plating, (2) obtaining populations of STRO-1 or CD105 positive cells, and (3) obtaining populations of CD45 or Gly-A negative cells (Seong et al., 2010). Naturally, a variety of parameters, such as the seeding density, scaffold material, and growth conditions (i.e., medium contents), affect the BMSC osteoblastic differentiation potential (Szpalski et al., 2012). Many findings of BMSC and ASC cultures grown for an extended period of time (≥ 4 months, ≥ 30 doublings) have shown alterations in cell cycle kinetics and the potential for irregular karyotype development, which could result in malignant cell transformation (Marolt et al., 2010).

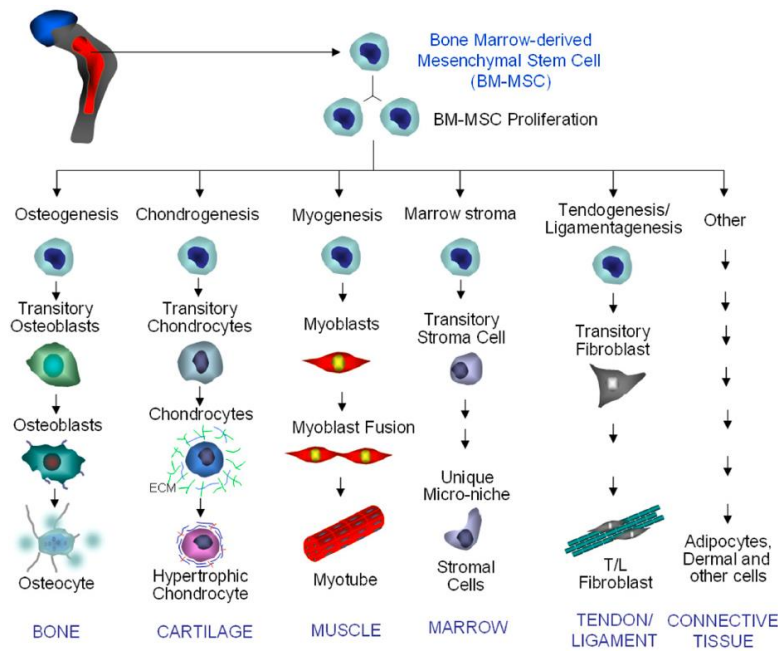


Figure 2.7. Bone marrow MSC differentiation strategy (Seong et al., 2010).

2.9. MC3T3 cell line

Because cell lines may be easily grown in culture indefinitely, they are crucial tools for scientific study (Yan et al., 2014). Osteoblast cell biology has been studied using a variety of cell culture models, such as malignant and immortalised cell lines, generated osteoblasts from pluripotent stem cells, and primary cells from various animals. Because of the phenotypic heterogeneity and restricted accessibility of human osteoblast cells, cells derived from other species can offer replacements for in vitro study model. Mice's calvaria, long bones, and vertebrae are among the skeletal regions from which osteoblast cell populations can be isolated (Czekanska et al., 2012). A popular in vitro osteogenic model system, the MC3T3-E1 cell line was created from newborn mouse calvaria and has been extensively utilised in studies related to bone tissue engineering (Yan et al., 2014). This newborn mouse calvaria clonal cell line has given rise to a number of subclones. When ascorbic acid and inorganic phosphate are added, five of them—subclones 4, 8, 11, 14, 26—show mineralisation. Mineralizing subclones express elevated amounts of BSP, OCN, and PTH/PTHrP receptor mRNA. Only subclones 4 and 14 of these clones have been demonstrated to mineralise the collagenous extracellular matrix. Similar to intramembranous osteogenesis in vivo, subclone 4 was demonstrated to experience temporal shifts from proliferation to nodule formation and mineralisation. On the third day of incubation, cells began to synthesise type I collagen and

proliferated rapidly in vitro. At the same time, mineral deposition was recorded early on day 14, and ALP enzyme activity rose from day 3 to day 21. Supplementing MC3T3-E1 cells with ascorbic acid and β GP is necessary for matrix mineralisation. When these stimulators are not present, ALP activity is minimal and does not lead to mineralisation. The kind of serum utilised in the culture medium, as well as serum-derived growth factors and cytokines, affect how MC3T3E1 cells differentiate. When full foetal bovine serum is added, cells' capacity to proliferate during early differentiation is enhanced (Czekańska, 2014) . Czekańska et al. (2012) compared the proliferation and maturation potential of three osteoblast cell lines (SaOs2, MG-63, and MC3T3-E1) with primary human osteoblast (HOb) cells for establishing their in vitro models suitability in biomaterial testing. They observed that MC3T3-E1 cells exhibited similar proliferation and mineralization patterns with primary osteoblasts and can be taken as an adequate model for biomaterial studies. Yan et al. (2014) analyzed how passage number and osteogenic supplements like ascorbic acid (AsAP) and dexamethasone (Dex) affect the osteogenic capability of MC3T3-E1 cells. Their study considered calcium deposition, alkaline phosphatase activity, and gene expression of osteogenic-related genes such as bone sialoprotein (BSP), osteocalcin (OC), and osteopontin (OPN). Results showed that passaging led to a sharp reduction in mineralization ability, which was totally abolished following passage 34. The combination of AsAP and Dex enhanced mineralization even greater than ATCC-recommended AsAP treatment alone, as demonstrated by augmented depositions of calcium and increased levels of BSP and OPN.

2.10. Effects of boron compounds on bone tissue engineering

Boron is an element that is a member of the metalloid group and has an atomic number of 5. Since 1923, boron has been recognised as a necessary element for plants, and numerous research have examined its impact on plant physiology. The body has between 3–20 milligrammes of boron in total. The quantities of boron compounds in various organs vary. For instance, the highest boron level is found in bones and tissues that contain ancreatine, like hair. It appears that bodily fluids have lower boron concentrations than bodily tissues. Only 0.06 ppm of boron is present in blood, 0.02 ppm in plasma, and 0.75 ppm in urine (Cebeci & Fikretin, 2022) . Studies on bone formation have demonstrated the connection between boron and human health. Mineralisation and bone-related protein expressions in osteoblasts level has been raised using boric acid, a product of boron. Dietary supplements of boron assisted in

lowering the number of natural killer cells in circulation. Antioxidant enzyme activity has been raised by low-dose boron therapy. Growth was stimulated and the plasma's levels of calcium and magnesium were raised by boron administration (Aydın et al., 2019) . It's still unclear exactly how B affects bone health. However, it is well recognised that B plays a significant role in the metabolism of steroid hormones, calcium, magnesium, and vitamin D, all of which have several indirect effects on bone. In vivo histological and histomorphometric analyses revealed that a B shortage increases bone fragility and negatively impacts bone tissue healing. According to Demirer et al., systemic boron injection decreased bone loss and inflammation in rats with periodontal disease (Gumusderelioglu et al., 2015) . According to Gumusderelioglu et al study , By demonstrating its beneficial effects on the proliferation and differentiation of MC3T3-E1 preosteoblastic cells, the cell culture studies' findings demonstrated that the encapsulated boron within the scaffolds can be employed as an osteoinductive agent (Gumusderelioglu et al., 2015) . Pazarçeviren et al study results concluded that 5BHT samples, which contain 5 mol% boron in HT, showed a great deal of promise for utilisation in composite bone tissue constructs (Pazarçeviren et al., 2021) .

2.11. SPP

(SPP), is a Sodium pentaborate pentahydrate (SPP, $\text{NaB}_5\text{O}_{10} \cdot 5\text{H}_2\text{O}$) is a boron compound which is produced using from boric acid and sodium carbonate. SPP compound is used in many fields with its fire retardant, anti-viral and antibacterial properties. In order to reduce bacterial and viral pathogen contamination in shrimp, it can also be added to feed and utilised as a component of enzymatic liquid detergents. Additionally, it can be included in medications that speed up the healing of skin wounds. To reduce bacterial and viral pathogen contamination in shrimp, SPP can be added to feed and used as an ingredient in enzymatic liquid detergents. (Chen & Pei, 2016). Table 2.2 provides a summary of key studies involving sodium pentaborate pentahydrate (SPP).

Table 2.2. Summary of key studies involving sodium pentaborate pentahydrate.

Reference	Aim of the Study	NaB Concentration	Results
(Taşlı et al., 2013a),	Odontogenic and osteogenic differentiation of human tooth germ stem cells (hTGSCs)	5-700 µg/mL	Increased viability of hTGSC at 10 and 20 µg/mL concentrations. Toxicity observed at higher concentrations. Enhances odontogenic and osteogenic

Table 2.2. Summary of key studies involving sodium pentaborate pentahydrate.

Reference	Aim of the Study	NaB Concentration	Results
			differentiation of hTGSCs at 20 µg/mL concentration.
(Doğan et al., 2014)	Using poly-(lactide-co-glycolide-acid) (PLGA) scaffolds with or without rat adipose-derived stem cells (rADSCs), they have assessed the impact of boron inclusion on bone repair both in vitro and in vivo.	0.01% NaB (w/v) and 0.05% NaB (w/v)	The findings showed that scaffolds containing boron enhanced rADSCs' in vitro proliferation, adhesion, and calcium mineralisation.
(Doğan et al., 2017)	Adipogenic differentiation of 3T3-L1 and 3T3-F442A pre-adipocyte cell lines and murine pluripotent C3H10T1/2 cells	50, 100, 250, 500 and 1000 µg/mL	NaB was found to be non-toxic to proliferating cells, including at 1000 µg /mL Reduced expression of adipogenesis related genes, proteins and transcription factors
(E. A. Abdik et al., 2019)	Adipogenic differentiation of human adipose-derived stem cells (hADSCs)	10-150 µg/mL	NaB found to be nontoxic to hADSCs at tested concentrations Inhibited adipocyte differentiation of hADSCs was determined at 10, 20, 50, and 100 µg/mL NaB concentrations
(H. Abdik et al., 2021)	Anti-obesity effects of NaB on BALB/c mice	1.5 mg/g and 0.5 mg/g doses by oral gavage	Decreased weight gain was determined both NaB treated groups when compared to the high fat diet group. Expression levels of adipogenesis related genes decreased in white adipose tissue in NaB treated mice in dose dependent manner.
(Omeroglu Ulu et al., 2023)	Synergistic anti-cancer effect of NaB, curcumin and piperine on HepG2 and Hep3B hepatocellular carcinoma cells	1700 /2500 µM NaB + 30 µM curcumin + 6 µM piperine	Anti-cancer activity of NaB, curcumin and piperine combination on HepG2 and Hep3B cells were demonstrated by cell viability, cell cycle, apoptosis and transcriptome analysis.
(Aydın et al., 2019)	The effects of SPP and BA on pancreatic cells	625 µg/ml, 312.5 µg/ml, 156.25 µg/ml, 78.1 µg/ml, 39.06 µg/ml, 19.5 µg/ml, 9.76 µg/ml, 4.88 µg/ml, 2.44 µg/ml, 1.22 µg/ml and 600 ng/ml	Treatment with 19.5 µg/ml BA or NaB under hypoxia increased β-TC-6 cell viability, insulin secretion, and Gck gene expression at 24 and 48 hours.
(Cebeci & Fikretin, 2022)	In vitro anti-cancer activities of sodium pentaborate pentahydrate (NaB), sodium perborate tetrahydrate (SPT), and boric acid (BA) on the small-cell lung cancer (SCLC)	1000 µg/mL, 1250 µg/mL, and 15 µg/mL for BA, NaB, and SPT	EC50 values for BA, NaB, and SPT were 1000 µg/mL, 1250 µg/mL, and 15 µg/mL, respectively after 72 hours. Cell viability assays showed that all three compounds exerted dose-dependent cytotoxicity on DMS-114 cells. They also upregulated pro-apoptotic and tumor suppressor genes while

Table 2.2. Summary of key studies involving sodium pentaborate pentahydrate.

Reference	Aim of the Study	NaB Concentration	Results
	cell line DMS-114 cells.		downregulating anti-apoptotic genes.
(Demirci et al., 2015)	The current study used an experimental burn model and in vitro cell culture techniques to assess the healing activity of a novel antimicrobial carbopol-based hydrogel made with boron and pluronic block copolymers.	5–20 µg/ml NaB	Gel containing NaB demonstrated exceptional antimicrobial activity (apart from against <i>Proteus mirabilis</i>). On day 1, it was discovered that the NIH-3T3 cells' cell viability was comparatively increased by the 15 µg/ml NaB and 10 µg/ml F127 when compared to other concentrations and control groups..
(Z. Iyigundogdu & Demir, 2017)	Sodium pentaborate pentahydrate and triclosan are applied to cotton fabrics in order to gain antimicrobial and antiviral properties for the first time	SPP (3 %) and 0.03 % triclosan	Remarkable antimicrobial activity against all tested microorganisms was demonstrated by fabrics treated with 3% SPP and 0.03 % T solution, whereas untreated fabrics showed no antimicrobial activity. While <i>S. aureus</i> showed the lowest antimicrobial activity, <i>Staphylococcus epidermidis</i> showed the strongest antibacterial impact when the antimicrobial solution was placed onto cotton materials.
(Z. Iyigundogdu et al., 2022)	To create new, long-lasting, and potent antimicrobial (antiviral, antibacterial, and antifungal) thermoplastic elastomers (TPE) based on styrene-ethylene/butylene-styrene (SEBS)	NaB 15.08% by wt	Adding up to 15% NaB slightly affected tensile strength and elongation, indicating NaB acts as a non-reinforcing filler. Antimicrobial and antiviral additives had little to no effect on the thermal properties (Tm and Tg) of the composites. According to ISO 22196:2011, the tested sample showed strong antimicrobial activity with over 99.9% reduction against <i>E. coli</i> , <i>S. aureus</i> , <i>C. albicans</i> , and <i>A. niger</i> .
(Mokkapat i et al., 2016)	They investigated the effect of a GO + NaB membrane on the growth and proliferation of hASCs.	a 0.1 g /ml	The cell population was positive for mesenchymal stem cell markers (CD29, CD44, CD73, CD90, CD105) and negative for hematopoietic (CD14, CD34, CD45) and endothelial (CD31) markers. MTS assay showed that cells grown on NaB-integrated GO membranes prepared with ethanol had higher viability on days 3, 8, and 13 compared to cells on bare GO membranes.
(Fikrettin, 2024)	In order to address wounds from grade 1 to grade 3 haemorrhoids, the study's goal was to evaluate the therapeutic effects of a topical gel containing sodium pentaborate pentahydrate.	The sodium pentaborate pentahydrate 3% gel	According to a study, topical sodium pentaborate gel helps lessen haemorrhoid symptoms such as anal itching, pain when at rest, pain when defecating, an anus lump sensation, and bleeding when defecating. All of these results together support the effectiveness of topical sodium pentaborate gel in reducing patients' haemorrhoid

Table 2.2. Summary of key studies involving sodium pentaborate pentahydrate.

Reference	Aim of the Study	NaB Concentration	Results
			symptoms.
(Şenkal et al., 2022)	They created an in vivo 3D cell culture method for keratinocyte-based toxicity research using the immortalised human keratinocyte cell line (HaCaT).	NaB High 1000 µg/mL NaB Low 100 µg/L NaB	In 3D HaCaT cell cultures, MMP2, TGFβ1, and VEGF gene expressions were significantly higher than in 2D cultures. Protein levels of IGFBP-2, IGFBP-6, M-CSFR, PDGF-AA, and EGFR were also increased. Cell viability tests showed 3D cultures had more skin-like responses and no toxicity from boron compounds, unlike monolayer cultures which showed significant toxicity.
(Inan et al., 2022)	The objective of this study was to assess the potential effects of sodium pentaborate pentahydrate (NaB) on hair growth and uncover how it affects key molecular mechanisms involved in hair growth, including Wnt-1, β-catenin, vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and transforming growth factor-β1 (TGF-β1) signalling pathways.	NaB 2 mg and 4 mg	In the weekly measurements, it was shown that the NaB 2 mg and 4 mg groups' increased hair density began on day 14. In comparison to both control groups and the NaB 4 mg group, the Wnt-1, β-catenin, PDGF, VEGF, TGF-β1, and collagen I protein levels were significantly greater in the NaB 1 mg and 2 mg groups.

2.12. Effects of small molecules on bone tissue engineering

Optimal tissue healing during the repair process depends on the capacity to regulate and direct cells differentiation towards a specific lineage and to stimulate tissue growth. Many techniques have been studied in this area, including the application of biochemical and physical stimuli. The usage of growth factors (GFs) is one biochemical strategy that has drawn a lot of therapeutic attention. By attaching to cell surface receptors and triggering signalling pathways, GFs can either increase or decrease cellular activity (adhesion, proliferation, and differentiation)(Goonoo & Bhaw-Luximon, 2019). Small molecules can be administered from scaffolds for tissue engineering and are stable, affordable, and simple to make substitutes. Melatonin (N-acetyl5-methoxytryptamine) is a tiny chemical that may be used as a substitute for osteoinductive biological factors such as BMPs. The pineal gland at the base of the brain releases the hormone melatonin, which is physiologically controlled by

light levels in the surrounding environment. Human melatonin levels peak at night, which has an impact on people by reducing body temperature and making them drowsy. Numerous pathways in several organ systems are activated by melatonin, a well-known regulator of circadian (day-night) rhythm. Melatonin's function in bone regeneration has been studied in recent years. According to Aravamudhan et al., preosteoblast (MC3T3-E1) and osteosarcoma 17/2.8 cell lines could induce osteogenic differentiation at A12 days when exposed to melatonin at concentrations as low as 50 nM. Purmorphamine has been shown in the Aravamudhan et al. study to stimulate osteogenesis in mouse mesenchymal progenitor cells (C3H10T1/2). The authors compared the transcriptional regulation caused by purmorphamine and that induced by BMP-4 treatment on C3H10T1/2 cells and discovered similarities in their capacity to up-regulate genes for proliferation (Aravamudhan et al., 2013).

Drugs and organic small molecules. As scaffold additions for bone regeneration, small compounds may penetrate cells and stimulate the signalling pathways linked to osteogenesis or interact with surface receptors. They might modify the expression of the target genes and function as co-activators or inhibitors of the transcription factors, which would affect the creation of bones. Additionally, a lot of them can improve the new tissue's vascularization.

Statins :Statins are among the most well-known classes of osteoinductive chemicals. Originally, this class of chemicals was employed as medications to decrease cholesterol. Many in vitro and in vivo investigations have taken advantage of statins' capacity for osteogenesis since the discovery of their osteogenic activity. According to research on statin mechanisms, they increase the expression of VEGF (the most important growth factor involved in vascularization), osteoblast-related genes, and the BMP and Wnt/ β -catenin signalling pathways (Safari et al., 2021).

In this study, chitosan scaffolds with varying concentrations of sodium pentaborate pentahydrate (SPP) were developed and characterized for bone tissue engineering applications.

3. MATERIALS AND METHODS

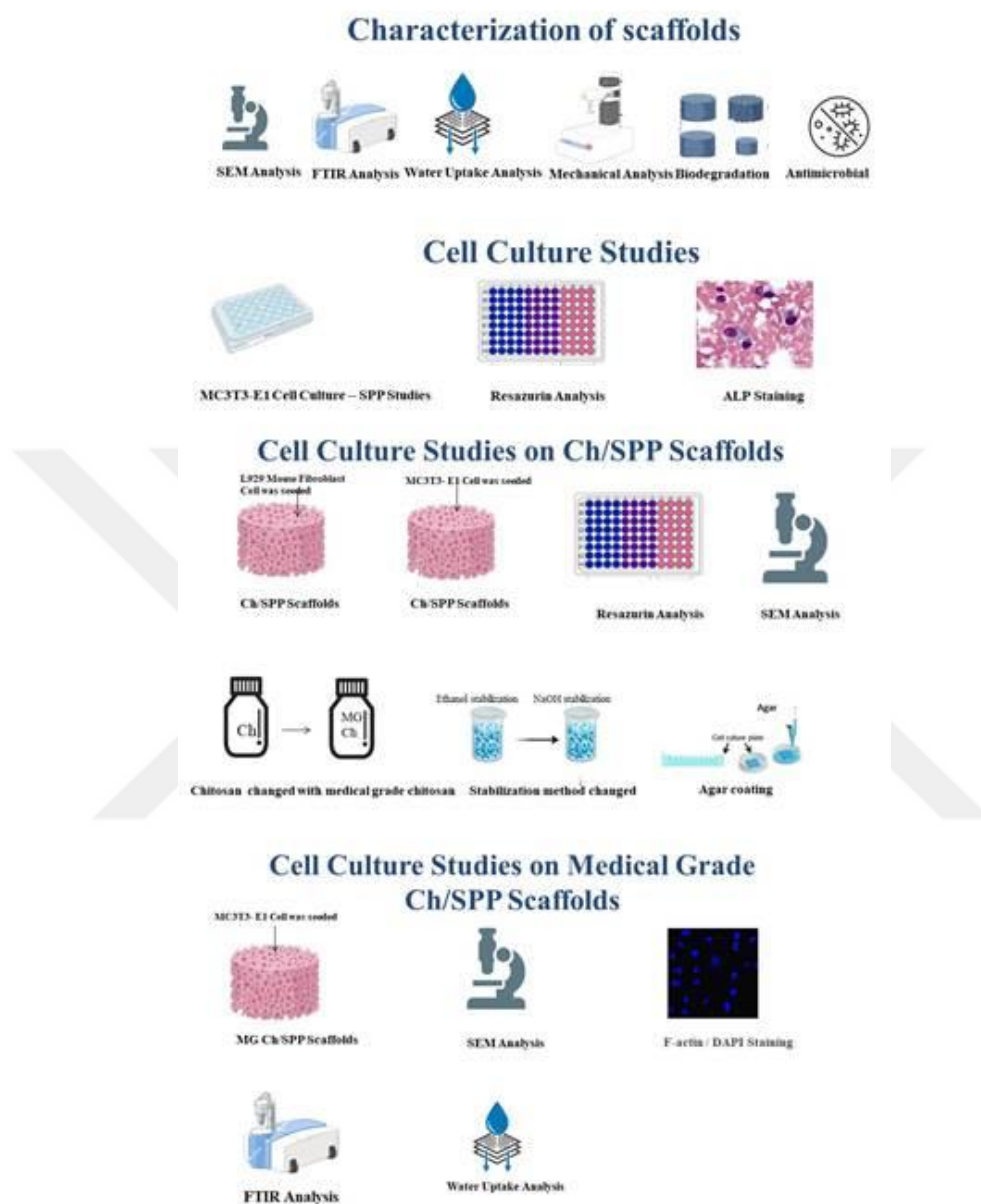


Figure 3.1. Schematic of the main analyses of this thesis

Figure 3.1 provides an overview of the experimental workflow applied in this study after scaffolds fabrication, starting with the characterization of the produced scaffolds and progressing through various stages of cell culture studies. The scaffolds were systematically analyzed using SEM, FTIR, water uptake tests, mechanical analysis, biodegradation assays, and antimicrobial evaluation to determine their physicochemical and biological

properties. Following this, cell culture studies were conducted with MC3T3-E1 cells to assess viability, proliferation, and osteogenic differentiation using resazurin analysis and ALP staining. Additional investigations on Ch/SPP scaffolds started with viability tests with L929 fibroblast cells as preliminary studies and morphological assessments through SEM. To ensure the biocompatibility and reproducibility of the scaffold system, medical-grade chitosan was employed, stabilized through ethanol treatment, sodium pentaborate incorporation, and agar coating. Subsequent studies on medical-grade Ch/SPP scaffolds confirmed cellular interactions through SEM, water uptake analysis, FTIR characterization, and fluorescent staining (F-actin/DAPI). Collectively, Figure 3.1 illustrates the integrated methodological framework designed to validate the suitability of Ch/SPP scaffolds for bone tissue engineering applications.

3.1. Production of Chitosan Scaffolds

Chitosan scaffolds were prepared by dissolving chitosan (4488869-250G, Sigma-Aldrich) in 1% (v/v) acetic acid (27225-2.5L-R, Sigma-Aldrich) at a concentration of 2% (w/v) using ultrapure water. The chitosan solution was stirred magnetically for 24 hours to ensure complete dissolution of the chitosan chains. After this period, the solution was centrifuged at 5000 rpm for 10 minutes to remove any insoluble particles. SPP (sodium pentaborate pentahydrate) was then added to the chitosan solution at concentrations of 0.125%, 0.25%, 0.5%, 0.75%, and 1% (w/w) while stirring continuously on a magnetic stirrer for 45 minutes. The resulting solutions were transferred into 24-well plates and frozen at -20 °C overnight. For clarity, the prepared scaffolds were designated according to their SPP content as shown in Table 3.1.

Table 3.1 Sample names

Chitosan / SPP (w/w)	Medical grade chitosan / SPP (w/w)
Ch - 0 SPP	MG Ch - 0 SPP
Ch - 0.125 SPP	MG Ch - 0.5 SPP
Ch - 0.25 SPP	MG Ch - 0.75 SPP
Ch - 0.5 SPP	
Ch - 0.75 SPP	
Ch - 1 SPP	

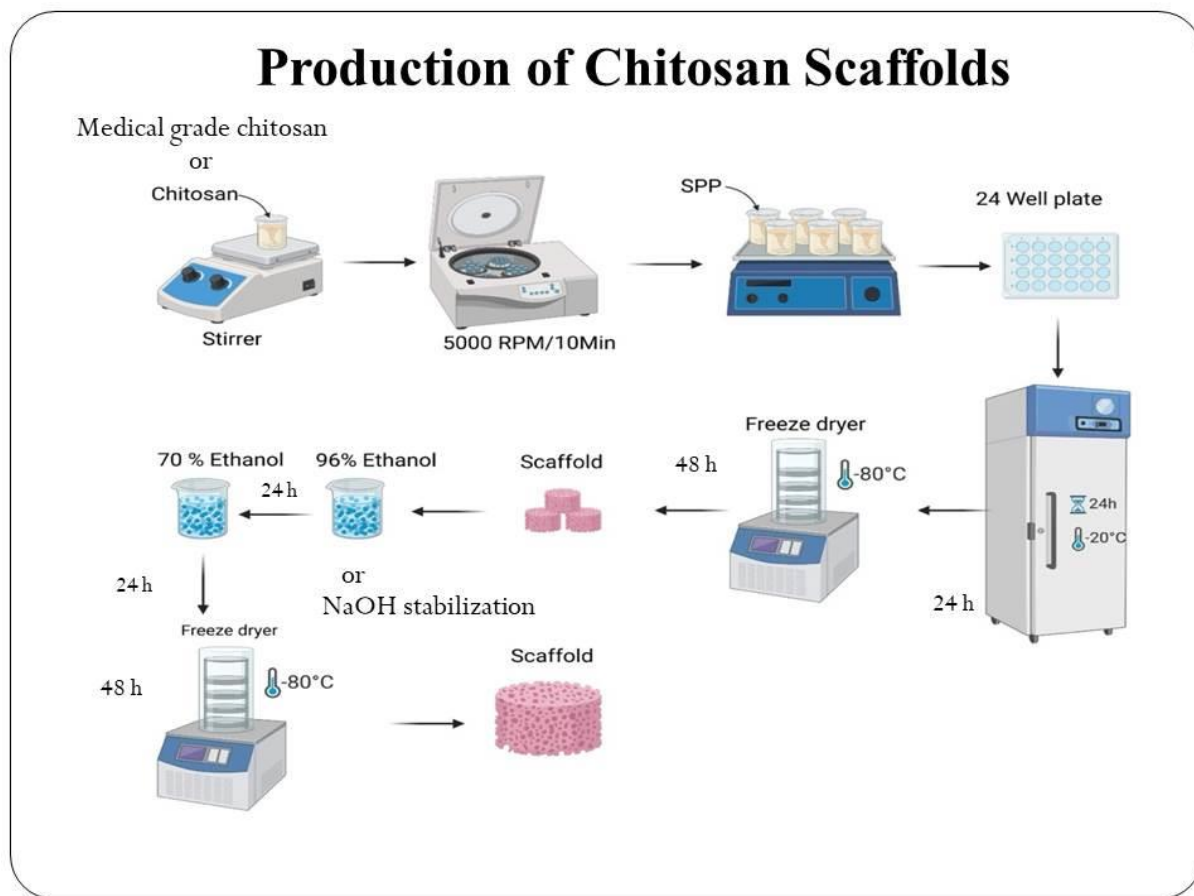


Figure 3.2. Schematic Representation of Scaffold Preparation Process (RW Method)

The scaffolds were subsequently freeze-dried at -80°C under vacuum (0.5) for two nights. To stabilize the scaffolds, they were immersed in 96% ethanol overnight and then transferred into 70 % ethanol (Figure 3.2). After this treatment, the scaffolds were frozen again at -20°C overnight and subjected to a second freeze-drying process using a Labconco freeze-dryer for two nights. Finally, the dried scaffolds were cut into 2 mm x 9 mm dimensions for further analysis. The production of the scaffolds used in this thesis is schematized in Figure 3.1 and macroscopic images are given in Figure 3.3.

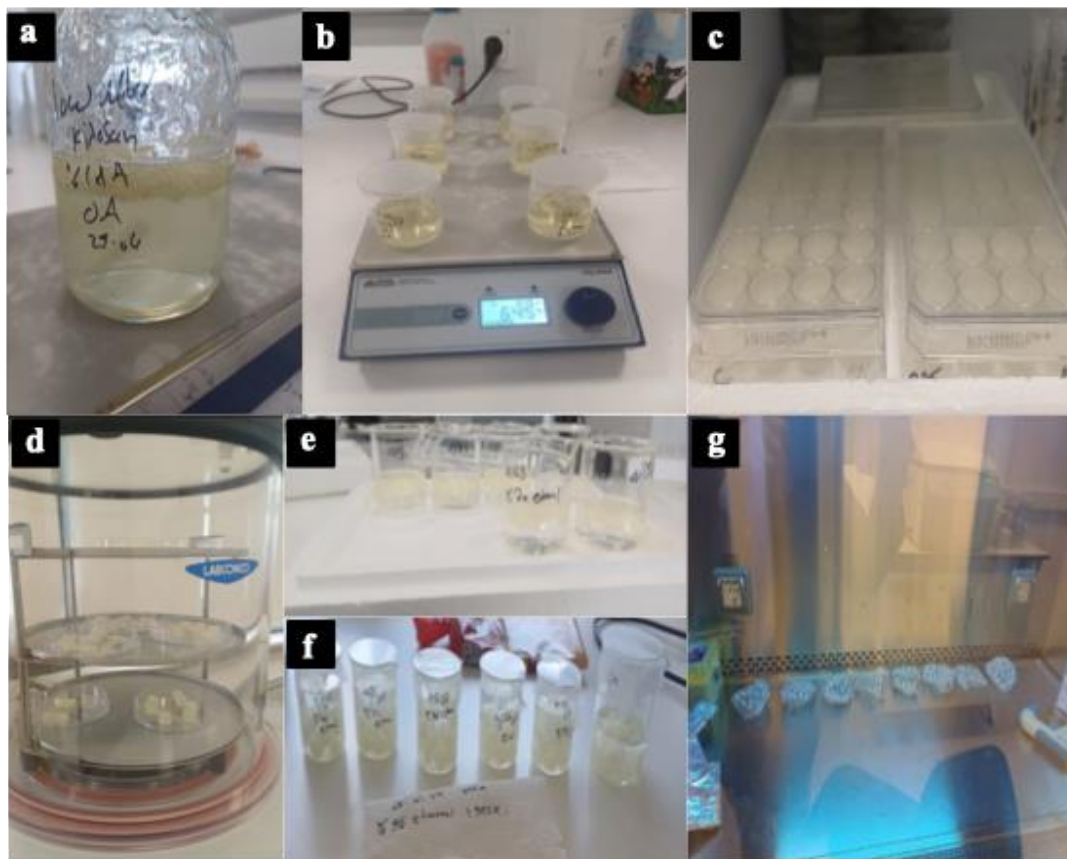


Figure 3.3. Preparation of chitosan scaffolds: **a)** Chitosan solution was magnetically stirred, and **b)** sodium pentaborate pentahydrate (SPP) was added slowly in varying concentrations (0.125%, 0.25%, 0.5%, 0.75%, and 1% w/w) with constant stirring for 45 minutes. **c)** The homogenous solution was cast into 24-well plates and kept at -20°C overnight. Subsequently, **d)** the frozen samples were freeze-dried at -80°C under vacuum (0.5) for two nights. To stabilize the structure, the scaffolds were **e)** soaked in 96% ethanol overnight and transferred to **f)** 70% ethanol. Then, the samples were frozen and freeze-dried. **g)** They were cut into 9 mm diameter and 2 mm thickness for cell culture and characterization studies. They were soaked in 70% (v/v) ethanol for 2 h under vacuum, washed 3 times with PBS in laminar flow cabinet and UV sterilized for cell culture studies.

3.2. Characterization of scaffolds

3.2.1. SEM Analysis

Scanning electron microscopy (SEM, Zeiss Evo 50, Oberkochen, Germany) was used to examine the morphological features of scaffolds. Before visualization the samples are coated

with a thin layer of gold using a sputter coater the devices and images were obtained at 250x, 500x and 1000x magnifications. Pore diameter distributions were analysed by Image J program using SEM images.

At the planned growth times, SEM examination was also used to investigate the adhesion behaviour and morphology of MC3T3–E1 cells on scaffolds. Following the removal of the culture media, the cells- scaffold constructions were rinsed twice with PBS before being fixed for 30 minutes with 2.5% (v/v) glutaraldehyde dissolved in 0.1 M PBS. Following a sequence of increasing ethanol concentrations (30, 50, 70, 90, and 100% v/v) for dehydration, samples were treated for five minutes with hexamethyldisilazane (HMDS). Scaffolds were covered with a gold-palladium layer and examined under a scanning electron microscope once the samples had fully dried.

3.2.2. FTIR Analysis

Fourier transform infrared spectroscopy (FTIR, Jasco, FT/IR-6700, Japan) was performed to examine the chemical structures of the produced samples between 400-4000 cm⁻¹ wavelentghs. FTIR spectre of chitosan polymer and SPP were also recorded for comparision of functional groups with Ch-SPP scaffolds.

3.2.3. Water Uptake Analysis

To determine the water uptake properties (Q) of dried chitosan scaffoolds with approixametly the same weigt, they were immersed into phosphate buffer saline (PBS, pH 7.4) at 37 °C and weighed afer removing excess PBS at defined time intervals (Ws) and at dry state (Wd). Then,

$$Q = (W_s - W_d) / W_d * 100$$

formula was used for calculation water uptake capacities.

3.2.4. Mechanical Analysis

For mechanical analysis the scaffolds were evaluated with (TA.XTPlus Surrey-England). The scaffolds 10 mm in diameter and 10 mm in height were immersed in a PBS solution (0.2 M, pH 7.4) at RT for 2 hours untill they reach equilibrium swelling. Following immersion, the

scaffolds were removed from the PBS and the excess surface liquid was carefully blotted using filter paper. A maximum load of 5 kg was applied. Stress–strain data were obtained from load and displacement measurements, and the compressive modulus was calculated as the initial linear modulus. Toughness (MJ/m^3), Young modulus ($\text{MPa\%}$) and strength (Pa) values for samples were recorded.

3.2.5. Biodegradation Studies

The scaffolds used for biodegradation studies were cut into 9 mm diameter and 2 mm height were placed in 50 mL centrifuge tubes containing 35 mL of PBS at pH 7.4, supplemented with 0.5 mg/mL lysozyme (100,000 U/mg, Sigma Aldrich, 62970-5G-F) and 0.5 mg/mL sodium azide (%1 w/v, NaN_3). The biodegradation properties of the scaffolds were carried out under enzymatic digestion. The vials were then incubated at 37 °C in a BIO.C.33 incubator (GERHARDT/THO00/1) at 100 rpm. Over a period of four weeks, four samples were collected at weekly intervals for each scaffold type. At each sampling point, the scaffolds were carefully removed from the vials, rinsed twice with distilled water to eliminate any residual buffer and lysozyme, and then vacuum-treated to ensure complete removal of any remaining solution. The rinsed scaffolds were subsequently frozen and freeze-dried for 48 hours. After drying, the samples were weighed and analyzed to assess their properties (Ren et al., 2005).

3.2.6. Antimicrobial Activity Evaluation

The antimicrobial activity of the Ch/SPP scaffolds was evaluated using the agar disc diffusion method. Microbial suspensions of bacteria (0.5 McFarland standard), yeast and fungi (1 McFarland standard), were prepared from fresh growth cultures. 100 μL of each microbial suspension were inoculated on nutrient agar (NA) for bacteria, and potato dextrose agar (PDA) for yeast and fungi. Chitosan/SPP scaffolds were cut into 1mm diameter and placed on the inoculated agar. Inoculated plates were incubated for appropriate time and temperature for all microbial species, and antimicrobial activity was determined by measuring the inhibition zone diameters.

3.3. Cell Culture Studies

3.3.1. MC3T3-E1 Cell Culture – SPP Studies (Free SPP in cell culture medium)

MC3T3-E1 cells are pre-osteoblastic cells. They were initially cultured in growth medium, α -Minimum Essential Medium (α -MEM, Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco), 1% (v/v) penicillin-streptomycin (Gibco), and 1% (v/v) L-glutamine (Gibco). Cells were maintained in a humidified incubator at 37°C in 5% CO₂. The culture medium was refreshed every two days. After one week of culture, cells were harvested using trypsin-EDTA solution (Gibco). Briefly, trypsin was added to the flask and incubated for 5 minutes at 37°C to detach the cells. To neutralize the trypsin and inhibit its activity, at least 1:1 of growth medium was added. The cell suspension was collected and centrifuged at 1000 rpm for 5 minutes. The supernatant was discarded, and the cell pellet was resuspended in fresh growth medium. Subsequently, the cells were seeded into multi-well plates or onto scaffolds for further experiments.

3.3.2. Osteogenic Differentiation of MC3T3-E1 Cells in the Presence of SPP

Osteogenic differentiation medium and solution preparations were carried out as follows. A total of 250 mL of normal medium (α -MEM) and 250 mL of osteogenic differentiation medium were prepared. For the β -glycerophosphate (β -G, G9422, Sigma-Aldrich) solution, 0.216 g was dissolved in 10 mL, and 0.5832 g was dissolved in 27 mL of culture medium (excluding serum, L-glutamine, and P/S), then sterilized using a 0.22 μ m filter to achieve a final concentration of 10 mM through a 1/10 dilution in the differentiation medium. For the L-ascorbic acid (AA, A92902, Sigma-Aldrich) solution, 0.035 g was dissolved in 7 mL of UPW and filtered through a 0.22 μ m filter. This was added at a 1/100 dilution to obtain a final concentration of 50 μ g/mL in the differentiation medium. Dexamethasone (D4902, Sigma-Aldrich) was prepared by dissolving 0.0026 g in 6.62 mL of absolute ethanol (99.9%) to obtain a 10⁻³ M stock solution, which was further diluted 1:100 to 10⁻⁵ M in sterile ethanol and then to 1:1000 in the differentiation medium, resulting in a final concentration of 10 nM (10⁻⁸ M). For 250 mL of differentiation medium, 25 mL of β -glycerophosphate, 2.5 mL of L-ascorbic acid, and 250 μ L of dexamethasone solutions were added.

SPP was applied to cells for 13 days at concentrations of 0.125, 0.25 and 0.5 mg/mL in both growth medium and osteogenic differentiation medium. The study groups are as given in Table 3.1. Cells were seeded and cultured as described in 3.3.

Table 3.2 Cell culture experiments, sample names

Control Medium (CM)	Differentiation Medium (DM)
SPP (mg/mL)	SPP (mg/mL)
CM- 0 SPP	DM - 0 SPP
CM- 0.125 SPP	DM - 0.125 SPP
CM- 0.25 SPP	DM - 0.25 SPP
CM- 0.5 SPP	DM - 0.5 SPP

3.3.3. Resazurin Analysis

In this section, resazurin sodium salt was used as an alternative to MTT viability assay to determine cell viability. Resazurin stock solution was prepared as 0.6 mM stock in PBS supplemented with calcium and magnesium, and sterilized using a 0.22 μ m syringe filter. This stock solution was diluted 1:10 in growth medium to prepare the working solution. For the assay, 400 μ L of the 10% resazurin-containing medium was added to the cells in each group. The cells were then incubated at 37 °C in a CO₂ incubator for 2 h. After incubation, 200 μ L of the supernatant from each sample was transferred into a 96-well plate, and the absorbance was measured at wavelengths of 570 nm and 600 nm. Resazurin analysis was performed on days 1, 3, 6, and 13. The culture medium was refreshed every 2 days.

3.3.4. ALP Staining

ALP synthesis is an important marker for bone differentiation. Cultured cells were fixed using 10% neutral buffered formalin for 15 min and washed using UPW for 2 times. Then, they were stored at +4 °C for further ALP staining. The cells were brought to RT and washed with UPW. A fresh substrate solution was prepared by first dissolving 0.3127 g of Tris HCl in 10 mL of UPW and adjusting the pH to 8.0 using 200 μ L of 1 M NaOH. Subsequently, 0.0014 g of naphthol AS was dissolved in 56 μ L of dimethylformamide and mixed with 7 mL of UPW and 7 mL Tris-HCl buffer. Then, 0.0084 g of Fast Red Violet LB salt was added, and the

entire solution was filtered with a 0.22 μm filter. 400 μL of this fresh substrate solution was added to every well, and the samples were incubated for 1 hour. The wells were washed four times with UPW and allowed to stand for 1 hour after incubation. For silver nitrate staining, a 2.5% (w/v) silver nitrate solution was made by dissolving 0.5 g of AgNO_3 in 20 mL of UPW. The samples were incubated in this solution for 30 minutes and then rinsed three times with UPW. The samples were incubated in UPW for 5 minutes and then allowed to dry. The samples were observed under invert phase-contrast microscope.

3.4. Cell Culture Studies on Ch/SPP Scaffolds

3.4.1. Preliminary Studies using L929 Mouse Fibroblast Cell Line

The Ch/SPP scaffolds were cut into 9 mm diameter and 2 mm height for cell culture studies. They were soaked into 70% (v/v) ethanol in UPW for 2 h under vacuum and then washed with PBS three times to remove excess ethanol from the pores in laminar flow cabinet. Then, they were soaked into complete growth medium for preconditioning with the proteins included in culture medium before cell seeding for one night at 37 °C in 5% CO_2 . After conditioning, each scaffold was gently blotted with sterile filter paper to remove excess medium and then transferred individually into a 24-well culture plate.

L929 cells were harvested as follows: the flasks were removed from the incubator, and the existing culture medium was discarded. Each flask was rinsed with 4 mL of PBS to wash the cells, ensuring the entire surface was covered. The PBS was then discarded. Then, 3 mL of trypsin-EDTA was added to each flask. The flasks were incubated at 37 °C in 5% CO_2 for 5 minutes to allow cell detachment. 4 mL of complete medium (DMEM with 10% (v/v) FBS, 1% L-Glutamin and 1% penicillin) was added to each flask to inactivate trypsin enzyme activity. The cell suspension was then transferred to 15 mL centrifuge tubes and centrifuged at 4 °C, 1000 rpm for 5 minutes. After centrifugation, the supernatant was removed, and fresh culture medium was added to the cell pellet. Each scaffold was seeded with 300,000 cells in a volume of 60 μL . The scaffolds were incubated for 3 hours to allow initial cell attachment. After 3 hours, 1 mL of complete medium was added to each well. The plates were then incubated overnight at 37 °C in a humidified incubator with 5% CO_2 . The scaffolds were transferred to a new plate to prevent the inclusion of detached cells in the assay. A 0.8 mM resazurin stock solution was prepared in PBS containing calcium and magnesium, followed

by filtration using a 0.22 μm filter. A medium containing 10% resazurin was then added to the scaffolds, and they were incubated at 37 °C in a 5% CO₂ incubator for 3 hours. After incubation absorbance values were measured using a SPECTROstar spectrophotometer at wavelengths of 570 nm and 600 nm. 600 nm was used as reference wavelength.

3.4.2. Cell Culture Studies Using MC3T3-E1 Cells

MC3T3-E1 cell culture studies were carried out as in L929 cell culture studies with slight differences. The scaffolds were prepared as mentioned in 3.4.1. Agar coating was applied to the surface of wells to prevent cell growth on the well surface. 2% (w/v) agar was prepared in UPW and sterilized by autoclave. Then, 5 drop agar solution was poured onto the well surface and waited in a laminar cabinet at RT for solidification. Scaffolds with excess medium was removed by sterile filter paper were placed on the solidified agar. For each scaffold, 250,000 cells were seeded in a total volume of 60 μL cell suspension and carefully pipetted onto the scaffold surface. The scaffolds were left in the incubator for 2 hours to allow initial cell attachment. After 2 hours, an additional 30 μL of cell culture medium was added to each scaffold to keep them moist and support further attachment. Following a total incubation period of 4 hours, 1 mL of complete cell culture medium was carefully added to each well. Each experimental group was prepared in at least 4 replicates ($n = 4$).

3.4.2.1. Viability MC3T3-E1 Cells on Ch/SPP Scaffolds / Resazurin Analysis

The scaffolds were transferred to a new plate to prevent the inclusion of detached cells in the assay. A 0.6 mM resazurin stock solution was prepared in PBS containing calcium and magnesium, followed by filtration using a 0.22 μm filter. A medium containing 10% resazurin was then added to the scaffolds, and they were incubated at 37 °C in a 5% CO₂ incubator for 3 hours. After incubation Figure 3.3, the fluorescence intensity was measured using a SPECTROstar spectrophotometer at wavelengths of 570 nm and 600 nm. 600 nm was used as reference wavelength.

3.4.2.2. Morphology of MC3T3-E1 Cells on Ch/SPP Scaffolds

Cell morphology of MC3T3 on the scaffolds was investigated by SEM analysis on days 1. In brief, the scaffolds were fixed with 2.5% (v/v) glutaraldehyde (in PBS), washed with PBS twice, and dehydrated immersing into ethanol series (30, 50, 70, 90, and 100%). At the last step, hexamethyldisilazane was applied to the samples for complete drying and the samples

were coated with gold/palladium before SEM analysis (FEI, Quanta 650 Field Emission SEM) at Çukurova University, Central Research Laboratory, at magnifications of 100x, 250x, 500x, and 1000x.

3.4.2.3. DAPI/Factin Fluorescence Staining of MC3T3-E1 Cells on MG Ch/SPP Scaffolds

For cytoskeletal and nuclear staining, cells on the scaffolds were first washed three times with phosphate-buffered saline (PBS) at room temperature (RT) to remove residual culture medium, followed by fixation with 2.5% (v/v) glutaraldehyde in PBS for 30 minutes. After fixation, the samples were washed three additional times with PBS. Samples were stored at 4°C until used for fluorescence staining. On the day of staining, samples were again washed with PBS and then treated with 0.1% (v/v) Triton X-100 in PBS for 20 minutes at RT to permeabilize the cell membranes. Following permeabilization, scaffolds were washed three times with PBS containing 1% (w/v) bovine serum albumin (PBS/A). A staining solution consisting of 1% (v/v) phalloidin for F-actin and 0.1% (v/v) DAPI in PBS/A was prepared, and 400 µL of this solution was added to each sample. Samples were incubated in the dark for at least one hour at RT. At the end of incubation time, staining solution was removed and the samples were washed three times with PBS/A. The stained scaffolds were kept in PBS/A protected from light until fluorescence imaging.

3.5. Cell Culture Studies on Medical Grade Ch/SPP Scaffolds and Using Different Stabilization Techniques for Scaffolds

The chitosan polymer used in the previous sections significantly promoted the proliferation of L929 mouse fibroblast cells but not MC3T3-E1 mouse pre-osteoblast cells. Therefore, different parameters had been tried for scaffold production. In a study (Gültan et al., 2020), it has been reported that chitosan scaffolds stabilized with NaOH supported proliferation of MG-63 human osteosarcoma cells when compared to the ethanol stabilized chitosan scaffolds. Therefore, the chitosan used was stabilized with both ethanol and NaOH and MC3T3-E1 study was performed. The same processes were tried with Heppe Medical Grade chitosan, which has a high degree of deacetylation (>92.6%) and low viscosity (1% in 1% acetic acid, 20 °C, <7 mPa) (Çapkin Yurtsever et al., 2022). The viability analysis of MC3T3-E1 cell culture studies on these samples showed that chitosan scaffolds produced from Heppe

Medical grade chitosan by freeze-drying method supported MC3T3-E1 cell growth when stabilized with NaOH. Therefore, in the last part of the thesis, scaffold characterization and cell culture studies for the scaffolds produced with Heppe Medical Grade chitosan and stabilized with NaOH were performed again as described in the previous sections. Ch - 0.5 SPP and Ch - 0.75 SPP groups were selected as samples due to their mechanical and biocompatible properties (the efficiency of obtaining uniform 3D scaffolds was low due to fragile properties of Ch - 1 SPP scaffolds so, they were not selected for further studies). Ch scaffold was used as the control group.

Table 3.3 Comparison of chitosan scaffold types in the study

Chitosan Type			Stabilization	Attachment and Proliferation of MC3T3-E1 Cells
Sigma-Aldrich, 4488869-250G			Ethanol	X
Sigma-Aldrich, 4488869-250G			NaOH	X
Heppe Medical GmbH, Chitosan 95/5, 24700	Chitosan,	Ethanol		X
Heppe Medical GmbH, Chitosan 95/5, 24700	Chitosan,	NaOH		√

3.6. Statistical Analysis

“Graph-Pad Prism” (version 10.2.3, USA) program was used for statistical analysis. In addition, the Real Statistics Resource Pack software (Release 8.9.1). Copyright (2013 – 2023) from Charles Zaiontz (www.real-statistics.com) was also used for the statistical analysis. One-way ANOVA test was applied to determine the significant differences between the groups and “Tukey-Kramer multiple comparisons test” was applied as a “post Hoc test”.

4. RESULTS AND DISCUSSION

4.1. Characteristics of Scaffolds

4.1.1. Morphology of Scaffold

The porous architecture of the chitosan–SPP scaffolds varied with SPP concentration. Scanning electron microscopy (SEM) images (Figure 4.1) showed an overall trend of

decreasing pore size as SPP content increased, albeit with some irregularities. Pure chitosan scaffolds (0% SPP) had the largest pores ($p < 0.01$) with average pore size $155 \pm 62 \mu\text{m}$, providing a highly open structure. Introducing a low level of SPP (0.125%) led to a notable reduction in average pore size. Interestingly, intermediate SPP concentrations (0.25% and 0.5%) produced slightly larger pores compared to the 0.125% group – a deviation possibly caused by minor inconsistencies in the freeze-drying process during fabrication. At 0.75% SPP, the pore size again became smaller, and the scaffolds with 1% SPP (the highest concentration) exhibited the smallest ($p < 0.001$) pore size ($65 \pm 24 \mu\text{m}$), most compact pores of all. These observations suggest that SPP incorporation tends to tighten the scaffold's pore network, with higher SPP levels generally yielding finer porosity. Figure 4.1 displays representative SEM micrographs highlighting these pore structure changes, while Figure 4.2 quantifies the pore size distributions for each formulation.

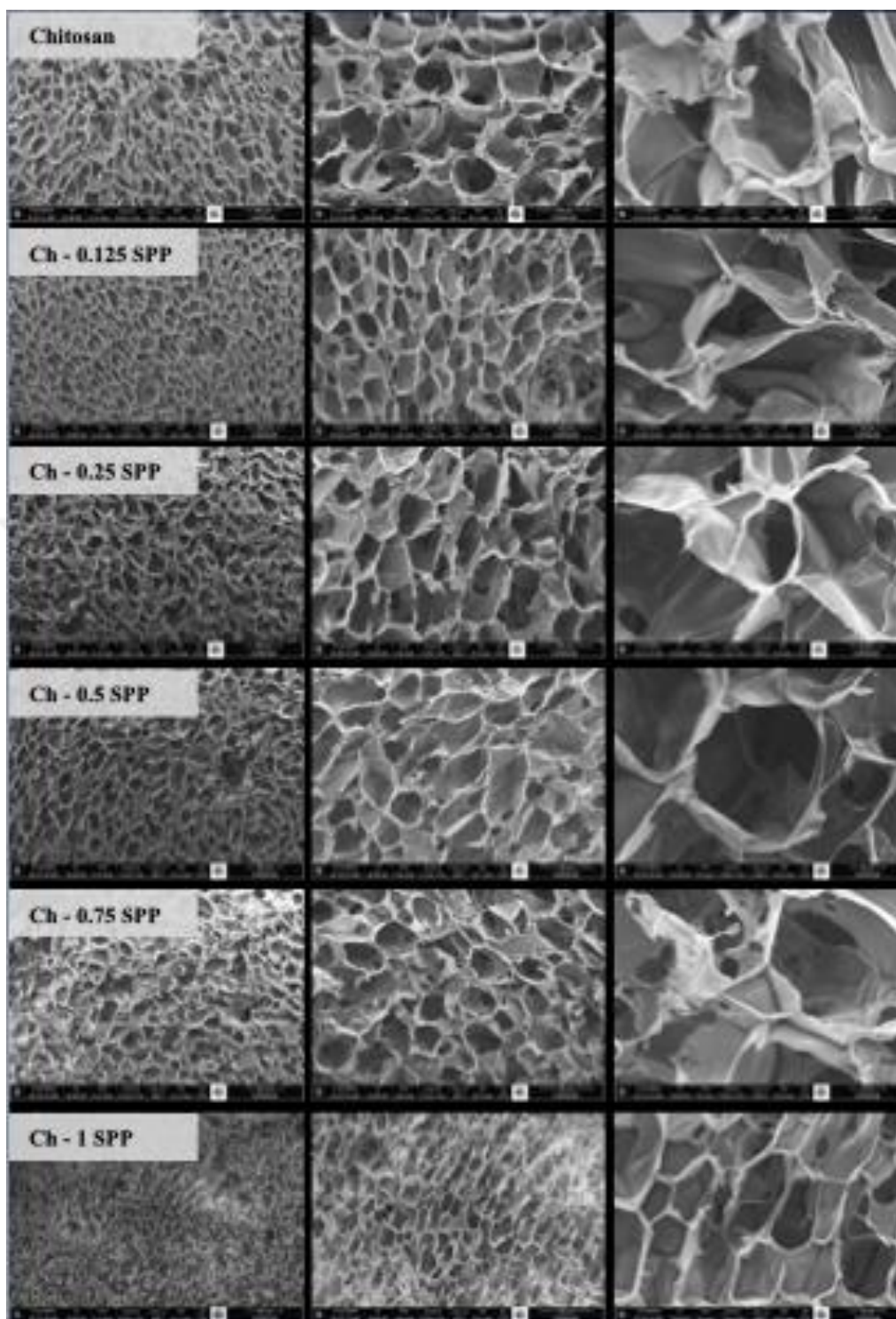


Figure 4.1. SEM images of chitosan and Ch-SPP scaffolds at various SPP concentrations (0%, 0.125%, 0.25%, 0.5%, 0.75%, and 1% w/w). Images at different magnifications illustrate changes in scaffold morphology and pore structure with increasing SPP content. Image magnifications are 100x, 250x, and 1000x, respectively.

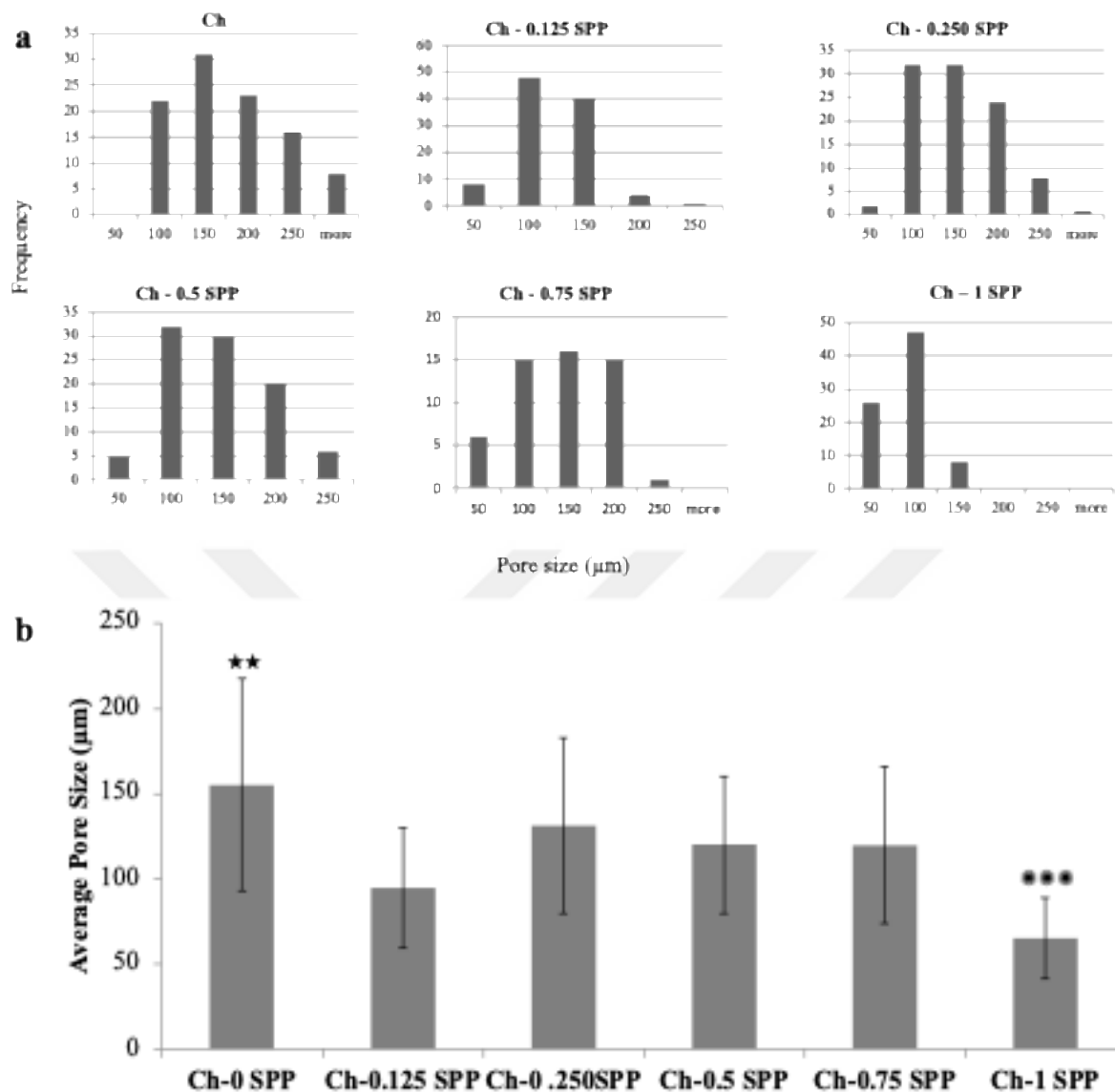


Figure 4.2. a) Pore size distribution histograms for chitosan and chitosan–SPP scaffolds, calculated from SEM images using ImageJ. Each histogram corresponds to a scaffold formulation (0% through 1% SPP), demonstrating the shift toward smaller pore sizes with higher SPP concentrations. b) Average pore sizes of the scaffolds. $\star\star$ means (p<0.01) significant increase compared to other groups. $\star\star\star$ means (p<0.001) significant decrease compared to other groups.

4.1.2. Biodegradation

Macroscopic Evaluation of Biodegradation. Scaffold degradation was monitored over four weeks of incubation in phosphate-buffered saline (PBS) containing lysozyme to mimic

physiological enzymatic conditions. All scaffolds showed visible signs of degradation after 4 weeks, manifesting as a progressive reduction in size and mass as seen in Figure 4.3. The extent of macroscopic breakdown correlated with SPP content: higher SPP-loading led to more pronounced degradation. Pure chitosan scaffolds retained much of their original structure (exhibiting the least visible change), whereas scaffolds with the highest boron content (1% SPP) underwent the greatest apparent degradation, often fragmenting and noticeably shrinking in size by week 4. These qualitative observations indicate that SPP accelerates the breakdown of chitosan scaffolds under enzyme-rich conditions.

Quantitative Analysis of Biodegradation. Weight loss measurements over the 4-week period reinforced the visual findings (Figure 4.4) and (4.5) All formulations showed a steady increase in percent mass loss over time, and scaffolds with higher SPP levels degraded significantly faster. By the end of week 4, pure chitosan scaffolds lost only about 20% of their initial mass. In contrast, scaffolds containing moderate-to-high SPP concentrations ($\geq 0.75\%$ SPP) experienced approximately 30–35% mass loss over the same period. Scaffolds with lower SPP (0.125–0.5%) exhibited intermediate degradation levels between these extremes. Additionally, the borate components of SPP may create more enzymatically susceptible linkages, thereby facilitating more rapid enzymatic cleavage of glycosidic bonds. In summary, adding SPP yields scaffolds that break down more readily in physiological-mimicking conditions, which could be advantageous for applications requiring faster scaffold resorption. There was a small increase in the weight of degraded scaffolds at week 4. This may be due to crystallization of salts in PBS. The scaffolds were washed with UPW before drying for weight measurement, but there may still be some salt crystals on the scaffold surfaces.



Figure 4.3. Photographs of chitosan and chitosan–SPP scaffolds (0%, 0.125%, 0.25%, 0.5%, 0.75%, 1% SPP) taken before and after 4 weeks of incubation in PBS with lysozyme.

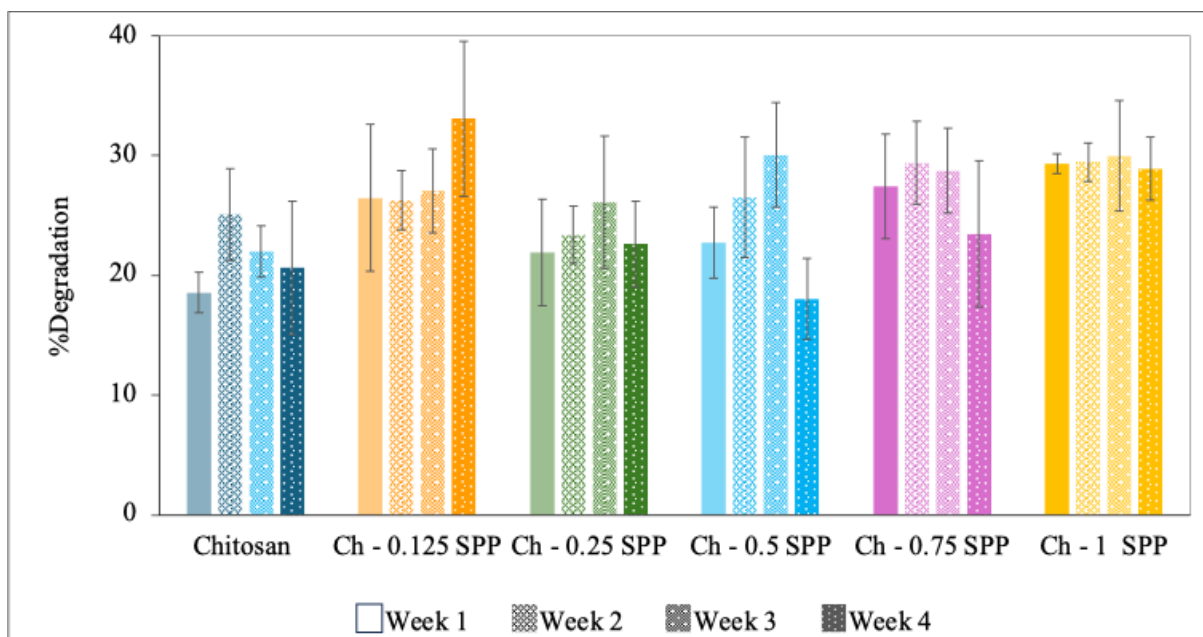


Figure 4.4. Percentage mass loss (degradation) of chitosan and Ch-SPP scaffolds over a 4-week incubation in PBS with lysozyme. Data are presented as mean $\pm$ standard deviation (SD) for each time point.

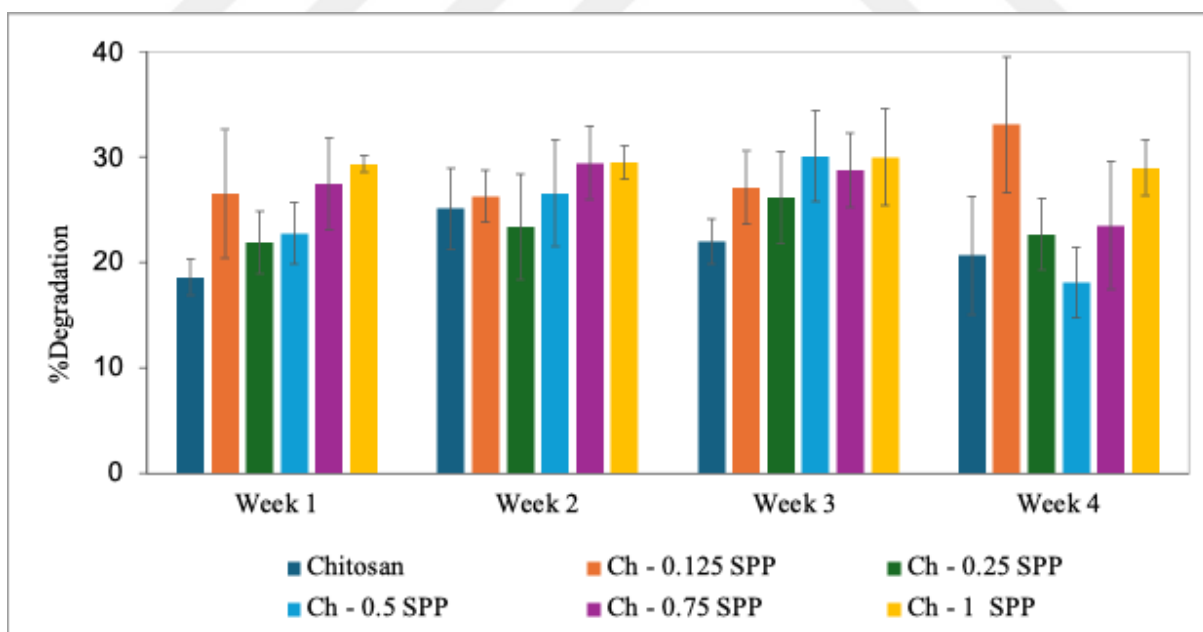


Figure 4.5. Representation of percentage mass loss (degradation) of chitosan and Ch-SPP scaffolds compared to each other in the same week.

4.1.3. Water uptake

All scaffold formulations were evaluated for their swelling behavior in PBS. The scaffolds absorbed water rapidly within the first 5–10 minutes of immersion, then gradually approached a saturation point. Equilibrium swelling was reached at roughly 30 minutes for most samples, after which the water content remained relatively constant. This two-phase swelling profile – an initial steep uptake followed by a plateau – was observed in both pure chitosan and SPP-containing scaffolds, indicating that the presence of SPP did not fundamentally alter the overall kinetic pattern of water absorption. However, the capacity for water uptake (i.e. the equilibrium degree of swelling) was markedly affected by SPP content. Macroscopic images of dry and swollen Ch-SPP scaffolds are given in Figure 4.6. It provides a visual comparison of the scaffolds before and after immersion in PBS for 1 hour, confirming that the pure chitosan scaffolds expand the most, while those with higher SPP remain more dimensionally stable in the wet state.

Figure 4.7 and 4.8 summarize the equilibrium water uptake (Q) for each scaffold composition, and a clear inverse relationship is evident between SPP concentration and water uptake. As the SPP percentage increased, the scaffold's ability to sorb water decreased. In practical terms, higher SPP loadings yielded scaffolds that were less swollen and more structurally stable in PBS, whereas the pure chitosan scaffolds swelled the most. Specifically:

Chitosan (0% SPP): Exhibited the highest swelling capacity ($p < 0.001$), reflecting a very porous and hydrophilic network that allows extensive water absorption. The scaffold became soft and fully hydrated quickly due to the ample free space within its structure.

0.125% SPP: Showed a slight reduction in swelling compared to pure chitosan. Even a small amount of SPP likely introduced additional cross-linking or filler effect within the chitosan matrix, increasing polymer–polymer interactions and reducing the free volume available for water.

0.25% SPP: Demonstrated a further decrease in water uptake. The scaffold network at this concentration is denser, which limits its flexibility and the extent to which water can infiltrate and expand the matrix.

0.5% SPP: Continued the trend of diminishing swelling capacity. The presence of SPP at 0.5% likely reinforces the scaffold structure through physical or ionic interactions, thereby restricting water diffusion and holding the material in a less swollen state.

0.75% SPP: Displayed even lower swelling, indicating that the scaffold has become considerably more compact. The polymer structure is notably rigidified by this SPP content, and water molecules find it difficult to penetrate deeply.

1% SPP: Produced the lowest swelling capacity of all groups. The scaffold remained relatively compact and only absorbed a limited amount of water, consistent with it having the tightest pore structure and greatest rigidity due to the high SPP incorporation.

This systematic decrease in equilibrium swelling with higher SPP validates that SPP serves to control and moderate the hydrophilicity of the scaffold. By occupying space in the polymer network and possibly introducing ionic cross-links (Figure 4.9), SPP reduces the tendency of chitosan to over- swell. From a tissue engineering perspective, this is beneficial: excessive swelling of scaffolds can compromise mechanical integrity and alter pore architecture, whereas a controlled swelling behavior helps maintain scaffold shape and strength upon implantation.

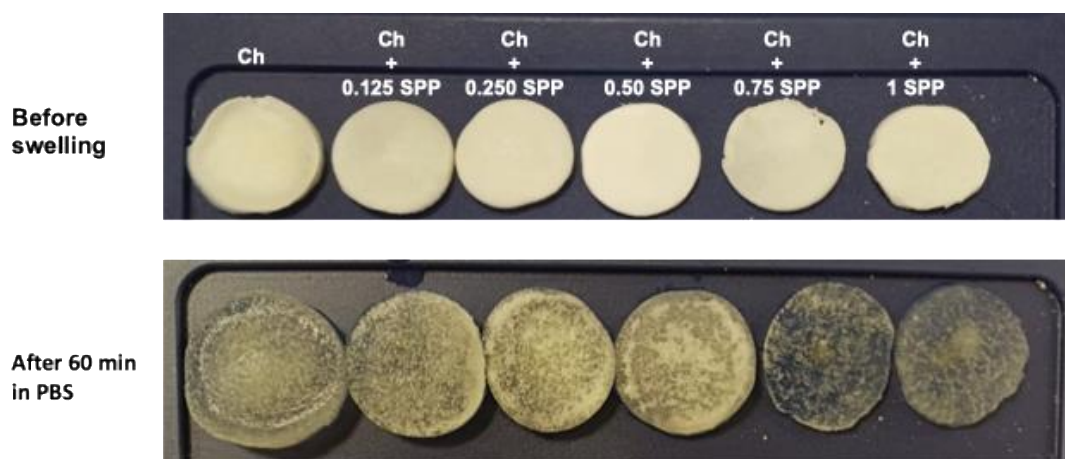


Figure 4.6. Macroscopic appearance of chitosan and chitosan–SPP scaffolds (0%, 0.125%, 0.25%, 0.5%, 0.75%, 1% SPP) before and after 60 minutes of immersion in PBS.

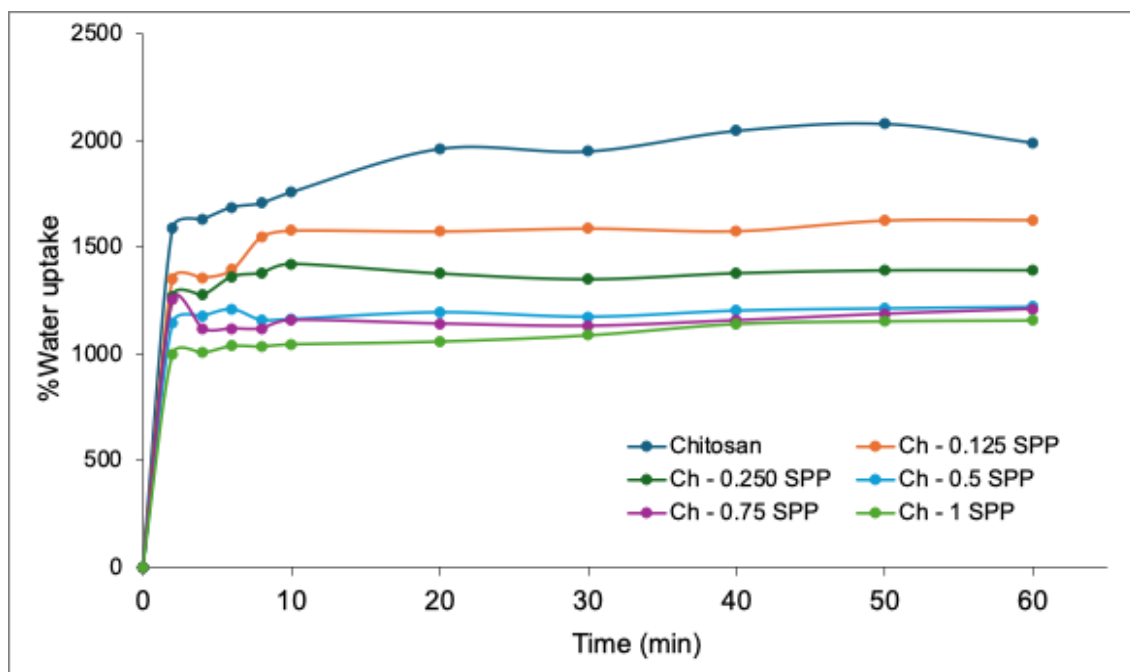


Figure 4.7. Water uptake profiles of chitosan and chitosan–SPP scaffolds during a 60-minute immersion in PBS. Each curve represents a different scaffold formulation (0% up to 1% SPP). All scaffolds show rapid initial water absorption in the first 10 minutes followed by a plateau as they approach swelling equilibrium (typically by ~30 minutes). This graph highlights the kinetic similarity of swelling among formulations, as well as differences in the final degree of swelling.

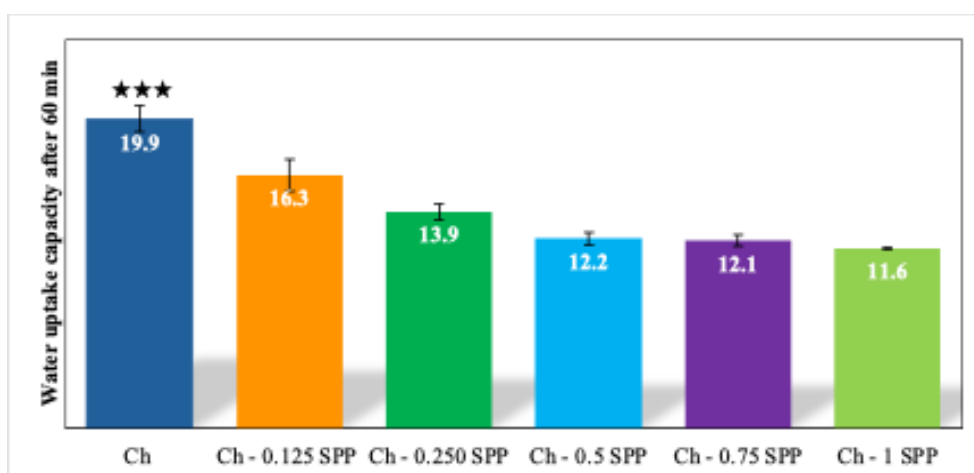


Figure 4.8. Equilibrium water sorption capacity (Q) of chitosan and chitosan–SPP scaffolds with varying SPP concentrations, presented as a bar chart. Q is defined as $(W_s - W_d) / W_d$, where W_s is the swollen weight at 1 hour and W_d is the dry weight. ★★★ means ($p < 0.001$).

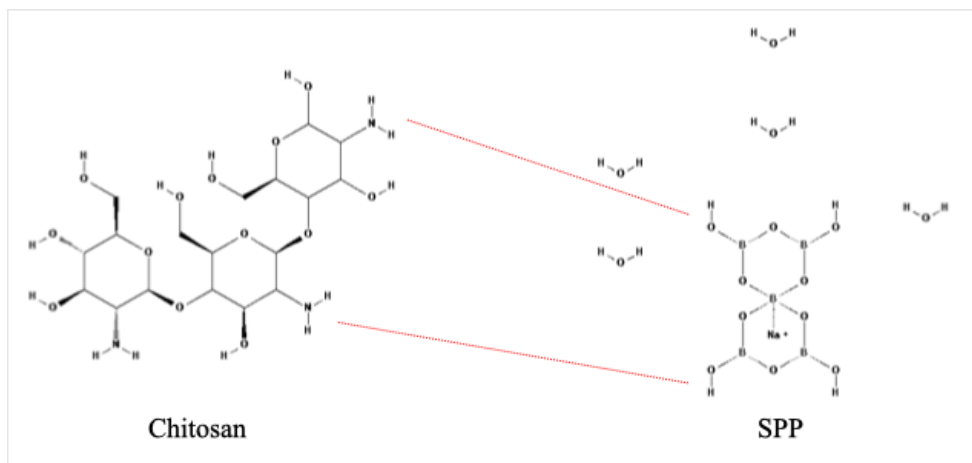


Figure 4.9. Possible interaction between SPP and chitosan for the conditions stated in this study. Chemical Structure Depiction images were taken from PubChem, July, 2025.

4.1.4. FTIR Analysis

FTIR analysis gives information about the functional groups of the materials. FTIR spectra of Ch-SPP scaffolds, chitosan polymer and SPP were given in Figure 4.10. Since SPP particles may be embedded in the chitosan polymer and FTIR analysis results show surface scanning results, peaks belonging to SPP were not significantly observed in Ch-SPP scaffolds (Figure 4.10.a). There was a slight increase at the peaks at 469 cm^{-1} , 600 cm^{-1} and 710 cm^{-1} with increasing SPP concentration, especially at Ch-0.75 SPP and Ch-1 SPP scaffolds, indicate the presence of SPP in the structure of chitosan scaffolds (Figure 4.10.b).

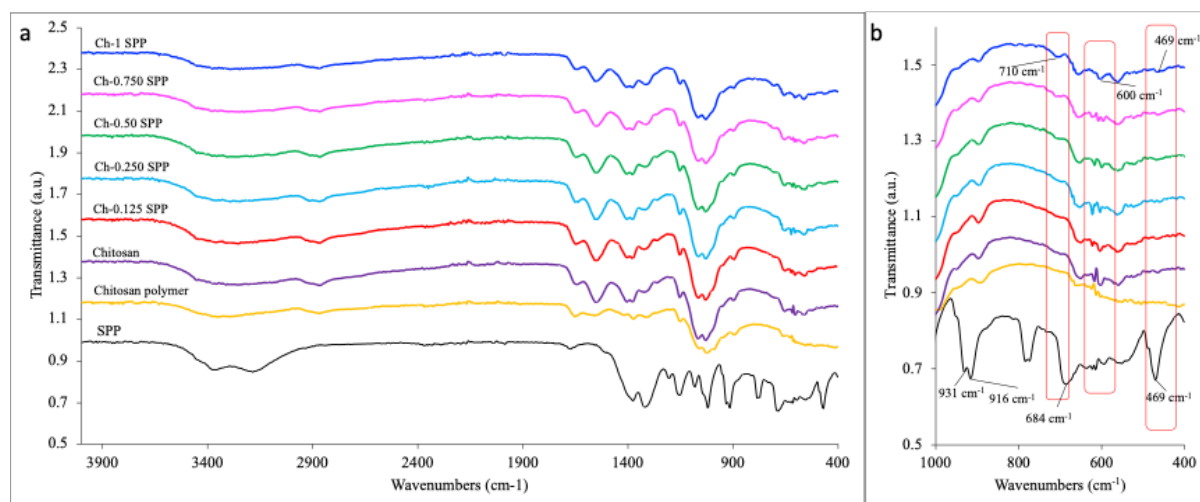


Figure 4.10. a) FTIR analysis spectra of chitosan polymer, SPP, Ch and Ch-SPP scaffolds. b) Magnified spectra from 400 to 1000 cm^{-1} for detailed visualization of peaks varying with SPP concentration.

4.1.5. Mechanical Analysis

Mechanical analysis (compression test) results of Ch-SPP scaffolds are given in Figure 4.11. The results showed that the strength and toughness values of the scaffolds increased in scaffolds containing SPP including Ch-0.75 SPP compared to the control group. The characteristics of the brittle structure observed in Ch-SPP scaffolds were also confirmed by the mechanical analysis results. While the strength and toughness values of Ch-1 SPP scaffolds decreased compared to the Ch-0.75 SPP group, it was determined that they were still higher than the control group. Strength of scaffolds were calculated as 33.46 ± 5.02 kPa and 15.68 ± 0.92 for Ch – 0.5 SPP and Ch control group. The results showed that the addition of 0.5 (w/w) SPP to the Ch structure increased the strength up to 2 times compared to the control samples. This increase was concentration dependent upto 0.5 SPP (w/w), then strength values started to decrease with increasing SPP content.

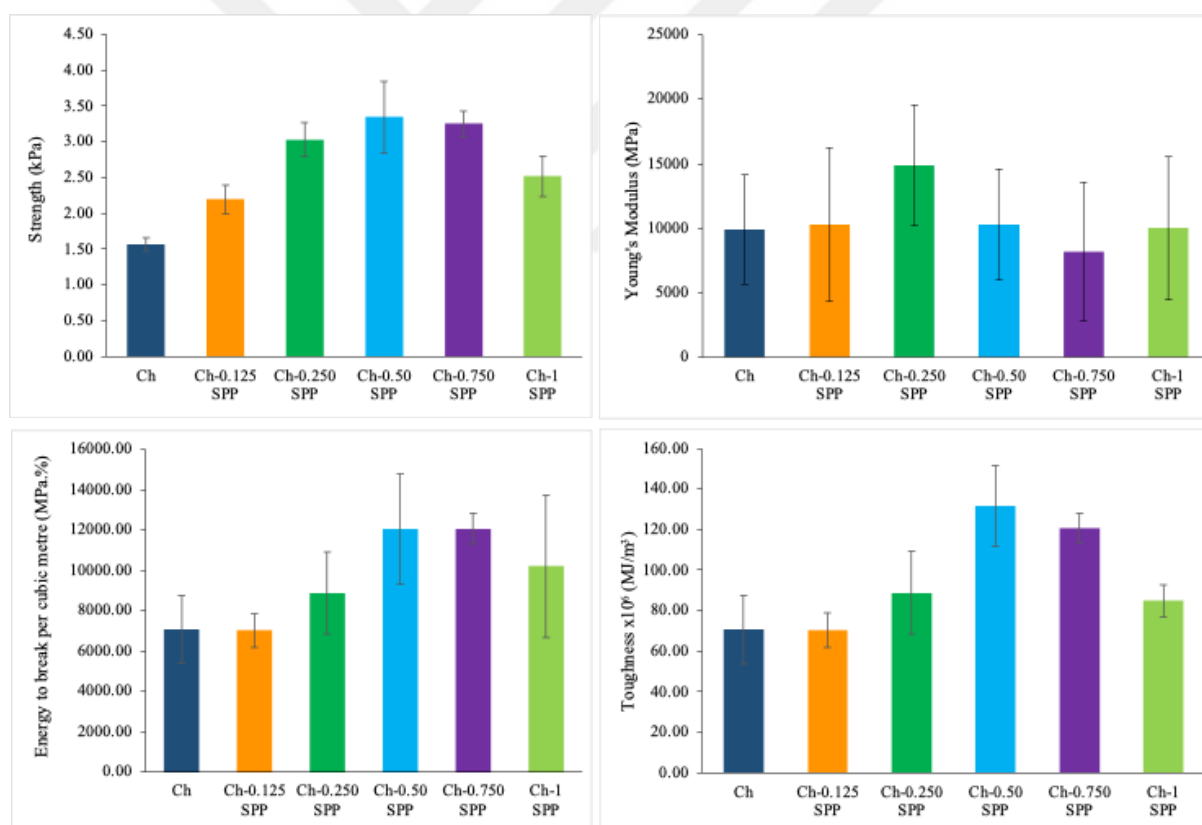


Figure 4.11. Compression test results of Ch-SPP scaffolds.

4.1.6. Antimicrobial Activity

The antifungal efficacy of the chitosan–SPP composite scaffolds was examined using an agar disk-diffusion assay. Disks of each scaffold formulation were placed on agar plates inoculated with two representative opportunistic fungal pathogens: *Candida albicans* (a yeast) and *Aspergillus niger* (a filamentous fungus). After incubation, clear zones of inhibited microbial growth around the disks indicated antifungal activity. Scaffolds containing SPP produced measurable inhibition halos on the fungal lawns, whereas control scaffolds without SPP (pure chitosan) showed little to no inhibition zone (consistent with the relatively weak antifungal effect of chitosan alone at neutral pH). The diameters of the inhibition zones were recorded for scaffolds with 0.5%, 0.75%, and 1% SPP w/w, and the results are summarized as follows. For *C. albicans*, all SPP-loaded scaffolds created distinct inhibition zones of roughly 17–18 mm in diameter. Specifically, the Ch - 0.5 SPP scaffold disk (approximately 11.7 mm in its own diameter) produced an inhibition halo of 18.2 mm. Similarly, 0.75% SPP w/w disks (~8.9 mm disk diameter) and 1% SPP disks (~9.0 mm disk) yielded inhibition zones of 17.1 mm and 17.6 mm, respectively. These values indicate that even the lower SPP concentrations tested were sufficient to suppress *C. albicans* growth beyond the edge of the scaffold (with the zone extending a few millimeters out from the disk). The antifungal effect was even more pronounced against *A. niger*. Scaffolds with 0.5% SPP w/w (disk ~10.4 mm) produced an inhibition zone of about 20.2 mm. At 0.75% SPP w/w, the zone grew to 22.5 mm, and the 1% SPP scaffold achieved a 30.2 mm zone of inhibition – more than triple the diameter of the scaffold disk itself. This dose-dependent increase in zone size with SPP concentration for *A. niger* suggests that higher boron content in the scaffold corresponds to greater diffusion of the antifungal agent and/or a stronger growth-suppressing effect on this fungus. The markedly larger zones observed for *A. niger* (compared to *Candida*) also imply that *A. niger* is particularly sensitive to the presence of SPP, or that the agar diffusion of the active species (likely borate ions) was more effective in the agar medium used for *A. niger* (potato dextrose agar, PDA). In all cases, the inhibition zones around SPP-containing scaffolds were well-defined and no fungal colonization was visible immediately surrounding the disks, confirming the antifungal activity imparted by SPP. Figure 4.12 shows representative plates from these experiments, where clear halos around the scaffold disks can be seen. Higher SPP-loaded scaffolds (especially 1% SPP w/w) resulted in larger clear zones, visually reinforcing the quantitative measurements. There was no evidence of microbial inhibition on plates with

control chitosan scaffolds (no clear zone beyond the disk edge), underscoring that the antifungal effect is due to the SPP component. These findings demonstrate that incorporating SPP into chitosan scaffolds endows them with potent antimicrobial (particularly antifungal) properties in a concentration-dependent manner. Antibacterial activity was tested against *E. coli*, *C. albicans*, *P. aeruginosa*, *E. faecalis*, *Z. rouxii*, *S. aureus*, and MRSA, but no clear inhibition zones were observed in any group. The inhibition zone diameters (mm) for different microorganisms at various SPP concentrations are as follows:

Table 4.1. Antifungal activity results

Microorganism	SPP Concentration (w/w)	Scaffold Diameter (mm)	Inhibition Zone (mm)
<i>Candida albicans</i>	0.5%	11.7	18.18
<i>Candida albicans</i>	0.75%	8.9	17.15
<i>Candida albicans</i>	1%	9	17.62
<i>Aspergillus niger</i>	0.5%	10.4	20.2
<i>Aspergillus niger</i>	0.75%	10.17	22.45
<i>Aspergillus niger</i>	1%	9.74	30.2

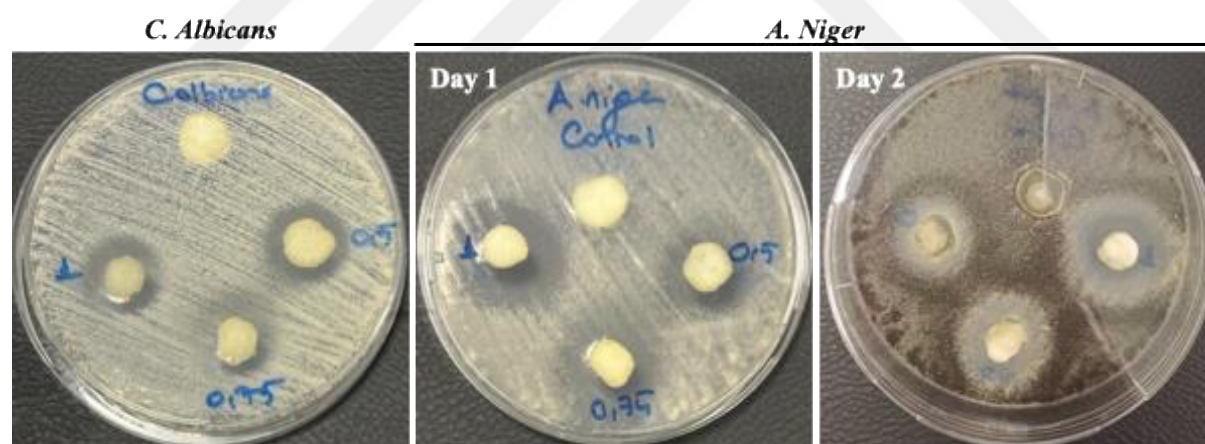


Figure 4.12. Antifungal activity of Ch-SPP composite scaffolds demonstrated by an agar diffusion test.

4.2. MC3T3-E1 Cell Line – SPP Studies

In this part of the thesis, the effects of SPP on viability of MC3T3-E1 cells and their osteogenic differentiation properties were investigated. When different concentrations of SPP

were applied to the cells for 24 hours, it was determined that cell viability was not adversely affected up to a concentration as high as 2 mg/mL Figure 4.13. It was determined that cells cultured in 0.25 mg/mL SPP concentration showed significantly higher viability compared to the control group without SPP. The cell viability decreased 92.85% and 49.1% at 4 mg/mL and 8 mg/mL SPP concentrations ($p < 0.001$ compared to all groups), respectively.

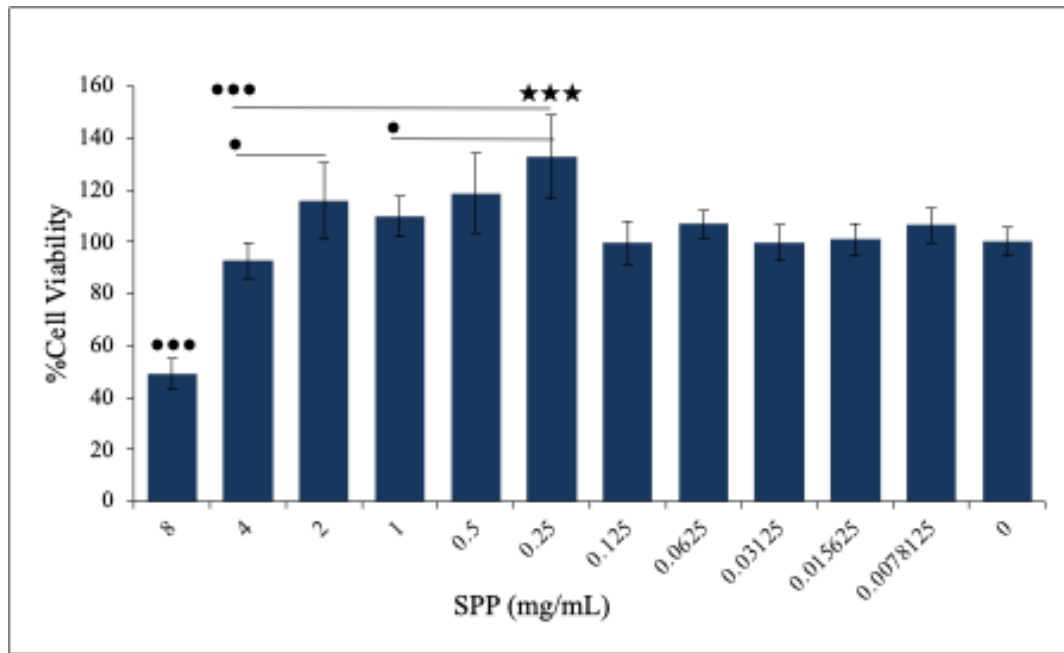


Figure 4.13. The effect of SPP on viability of MC3T3-E1 cells at 24 h culture. “★” is significantly higher and “●” is significantly lower than all other groups or compared group.

The osteogenic differentiation promoting properties of different boron compounds have been demonstrated in the literature with various cell lines and stem cells (Taşlı et al., 2013b). SPP has been used less in the literature than other boron compounds. One reason for this may be that SPP is not a natural boron compound but is synthesized using boric acid and sodium carbonate (Şahin et al., 2023). In this study, the effect of SPP on the osteogenic differentiation potential of MC3T3-E1 cells was analyzed by both resazurin assay and ALP staining. SPP was applied to the cells at selected concentrations both in growth medium and in osteogenic differentiation medium. 0.125, 0.25 and 0.5 mg/mL concentrations of SPP were selected according to the results of viability analysis. 0.25 mg/mL SPP was the group that supported cell viability the most compared to the control group. 0.125 and 0.5 mg/mL SPP groups were

selected as groups that did not affect cell viability positively or negatively. Inverted phase contrast images of the cells both for growth medium and osteogenic differentiation medium with or without SPP are given in Figure 4.14. The morphology of MC3T3-E1 cells is generally spindle like at the beginning of culture. When the cells become dense and compacted in culture and with osteogenic differentiation, they gain a more cubic shape (Yan et al., 2014). In our study, since the cells completely covered the surface and reproduced intensively on the 7th and 14th days, it is not possible to comment on the single cell morphology. However, the difference between control groups and SPP including groups were significant in invert phase contrast images. The density of the cells decreased with increasing SPP concentration resulting less cell viability as also given in resazurin analysis results in Figure 4.15 Unlike what is expected in osteogenic differentiation, cell extensions were also more prominent in SPP-treated groups compared to the control group.

Viability of MC3T3-E1 cells with or without SPP and with or without osteogenic differentiation medium was determined by resazurin analysis. The results are given in Figure 4.15 and Figure 4.16 All groups are shown comparatively in Figure 4.15 while the results of the 13th day are shown in detail in Figure 4.16 by statistical analysis. Increased cell viabilities were recorded for all group from day 1 to day 13.

When the effect of differentiation medium on cell proliferation was examined, it was observed that the viability of the cells cultured in the differentiation medium was significantly lower than those cultured in the control medium ($p < .05$), while in the presence of 0.125 mg/mL SPP in the medium, the cell viability in the differentiation group was significantly ($p < 0.01$) higher than in the control group (Figure 4.16a). The significant differences in viability between the control and SPP groups at day 13 are better illustrated in Figure 4.16b. Cells in growth medium without SPP showed highest cell viability when compared to the SPP including groups. However, in differentiation medium, cells cultured with 0.125 mg/mL SPP showed higher viability than those cultured in differentiation medium alone ($p < 0.05$). The control group showed significantly higher viability than the groups with 0.25 mg/mL SPP and 0.5 mg/mL SPP in both media. In line with these results, the suppressive effect of SPP on cell viability was shown at values of 0.25 mg/mL and above as the culture time was prolonged. When the first day results were analyzed, it was observed that SPP concentrations, which did

not show a negative effect on cell viability, suppressed cell proliferation as the culture time was prolonged.

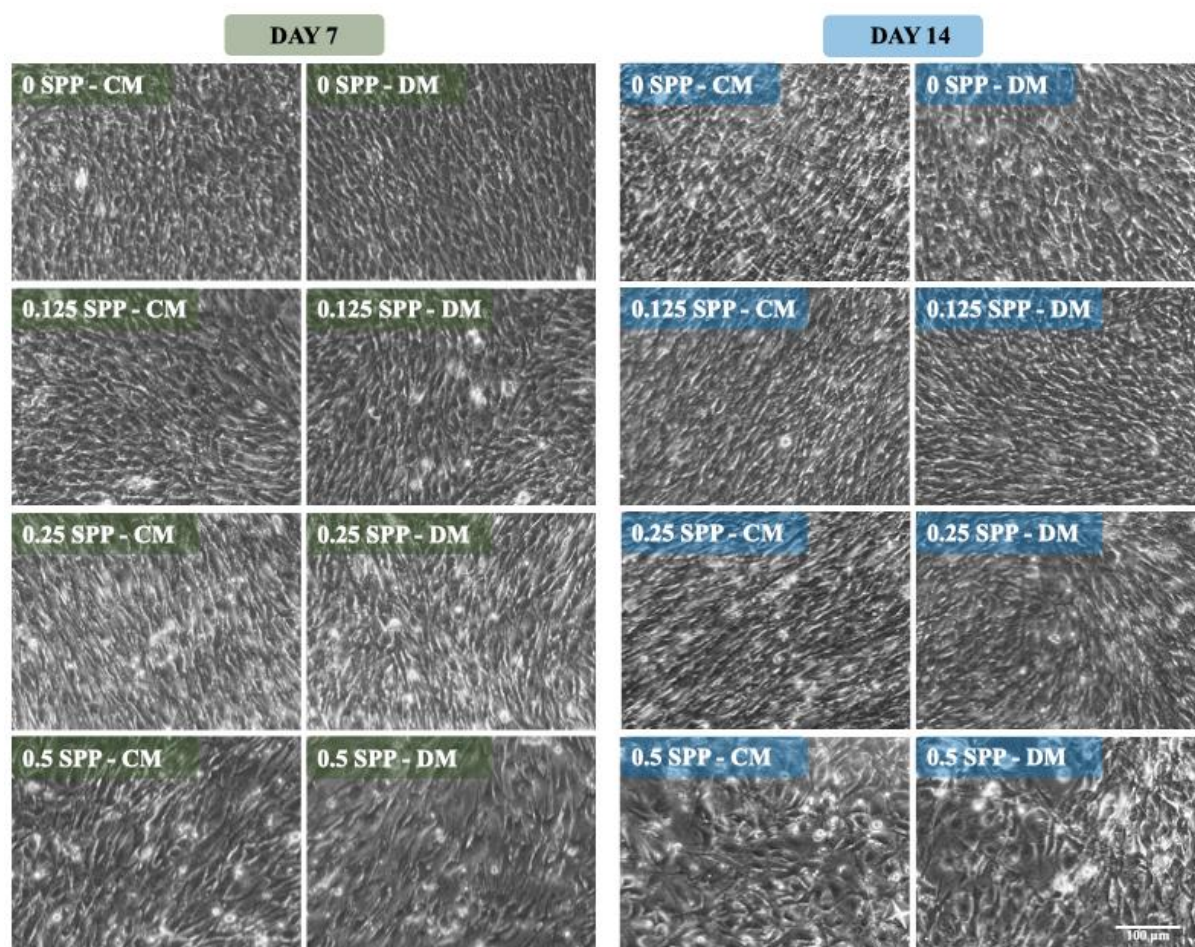


Figure 4.14. Invert phase contrast images of MC3T3-E1 cells with or without SPP at 0.125, 0.25 and 0.5 mg/mL concentrations both in culture medium and osteogenic differentiation medium (20X). Nomenclature is described in Table 1.

Alkaline phosphatase (ALP) activity is one of the most important and widely used parameters to demonstrate osteogenic differentiation of osteoblast cells and stem cells (Ansari et al., 2022). In this study, the effect of SPP on the osteogenic differentiation potential of MC3T3-E1 cells was analyzed by ALP staining. Inverted phase contrast images of ALP stained osteogenically induced MC3T3-E1 cells in control medium without SPP after 14 days of *in vitro* culture are given in Figure 4.17 ALP activity was observed only in osteogenic-induced MC3T3-E1 cells without SPP application. No ALP activity was observed in other groups

(images not shown). The pink areas in the images show high ALP activity, while the darker areas show mineralization. Although there are studies in the literature investigating the effects of SPP on different cell lines and stem cells, no study with MC3T3-E1 cell line was found within the scope of our research. In a study, osteogenic and odontogenic differentiation of human tooth germ stem cells was investigated in the presence of 20 μ g/mL SPP. Increased mineralization, ALP activity and expression of osteogenic and odontogenic markers were found for human tooth germ stem cells after 14-day of culture in differentiation media (Taşlı et al., 2013b). This study differs from ours in that it uses different cells and has a lower SPP concentration (6.25 times lower than the lowest concentration utilized in our study, 0.125 mg/mL). In another study, the effects of SPP on primary human dermal fibroblasts and cutaneous wound healing was investigated. 0.15 μ g/mL SPP concentration significantly increased migration capacity and superoxide dismutase activity of the cells. In addition, combination of SPP in Pluronic gel supported cutaneous wound healing with increased collagen synthesis and contraction in wound area (Doğan et al., 2014).

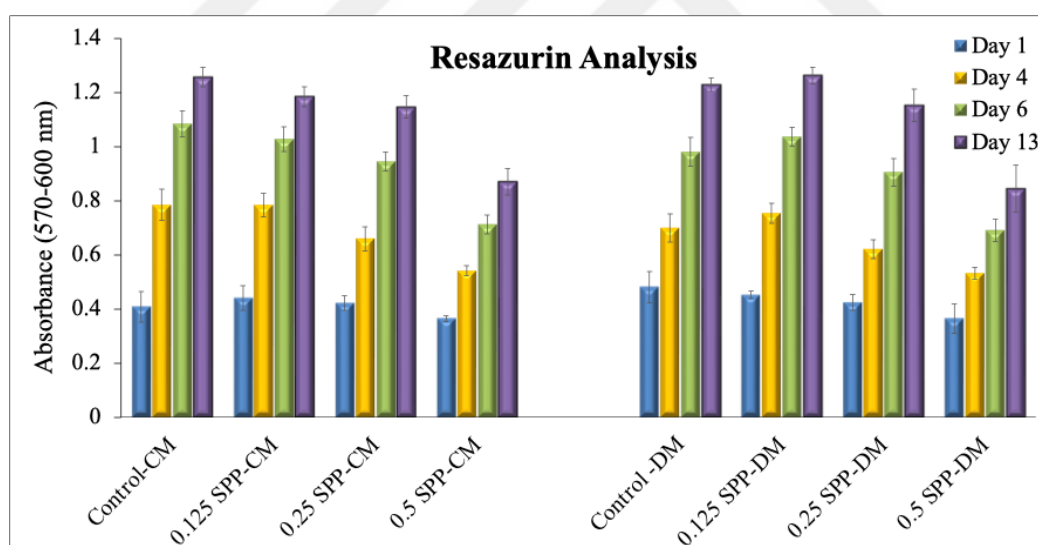


Figure 4.15. Resazurin analysis results of osteogenically induced and control MC3T3-E1 cells with / without SPP in culture medium at days 1, 4, 6, and 13.

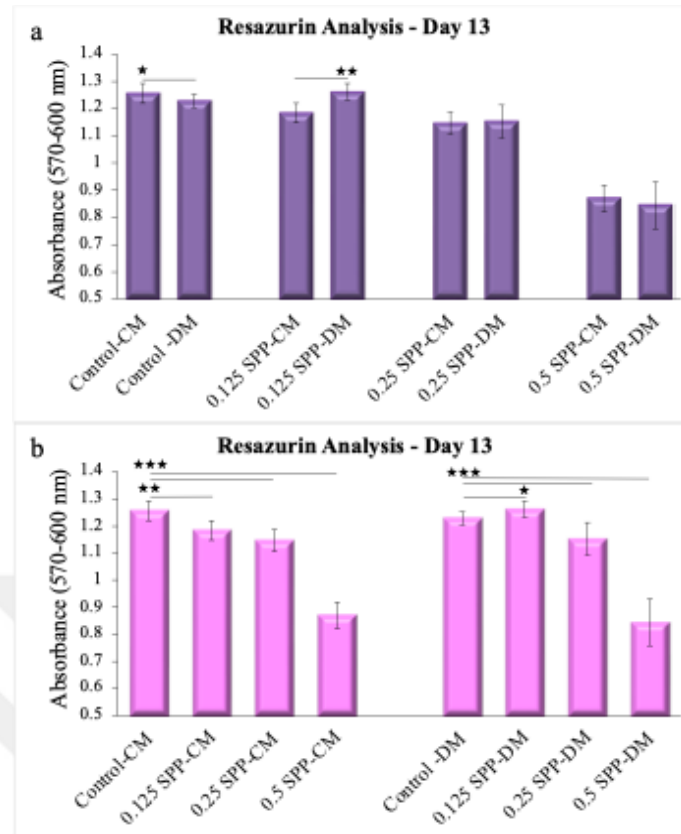


Figure 4.16. Resazurin analysis results of osteogenically induced and control MC3T3-E1 cells with / without SPP in culture medium at day 13. ★ ($p<0.05$), ★★ ($p<0.01$), and ★★★ ($p<0.001$) mean significant difference between compared groups. a) Comparison of groups containing the same amount of SPP b) Comparison of cells cultured in control medium or differentiation medium containing different amounts of SPP. CM: control medium, DM: differentiation medium.

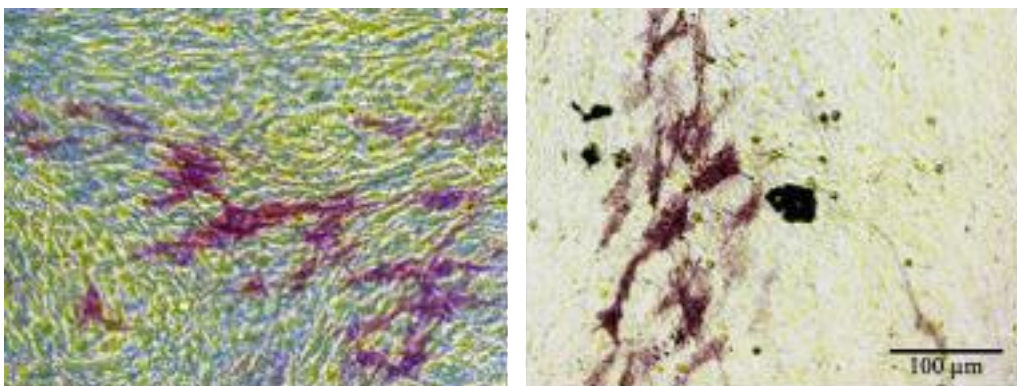


Figure 4.17. ALP staining results of osteogenically induced MC3T3-E1 cells in control medium without SPP after 14 days of *in vitro* culture (20x).

4.3. L929 Cell Line / Ch-SPP Scaffold Studies (Preliminary Studies)

In this part of the study, whether the scaffolds support cell adhesion and proliferation was performed with mouse fibroblast cells, L929 cell line, which are also recommended for use in ISO standards for biomaterials. Resazurin analysis results are given in Figure 4.18. All Ch-SPP scaffolds supported L929 cell proliferation for 7 day culture. There was a slight decrease in Ch – 0.25 SPP group when compared to other groups for all days. Ch – 0.75 SPP scaffolds showed the highest cell viability when compared to the other groups ($p < 0.001$) on day 1.

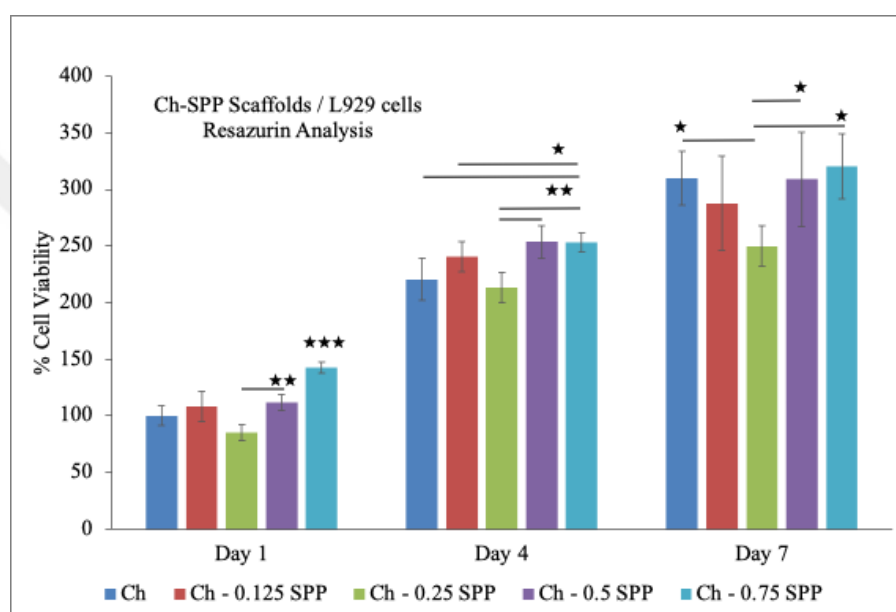


Figure 4.18. Resazurin analysis results of L929 cells on Ch-SPP scaffolds. ★ ($p < 0.05$), ★★ ($p < 0.01$), and ★★★ ($p < 0.001$) mean significant difference between compared groups.

4.4. MC3T3-E1 Cell Line / Ch-SPP Scaffold Studies

4.4.1. Resazurin Analysis

Resazurin assay was conducted to assess the metabolic activity of MC3T3-E1 cells cultured on Ch scaffolds containing various concentrations of sodium pentaborate pentahydrate (SPP): 0% (control), 0.125%, 0.250%, 0.5%, 0.75%, and 1% (w/w). As shown in Figure 4.19, cell viability on days 7 and 10 was generally low across all groups. Resazurin (blue-purple) turns into resarufin after metabolic activation which is pinkish when compared to resazurin. The macroscopic images of resazurin applied scaffolds after 3 h incubation are given in Figure

4.19 b. It was clearly seen that, pink discoloration was less in day 4 and day 7 analyses indicating lower cell viability when compared to the pinkish color on day 1.

However, among the tested concentrations, the Ch – 0.5 SPP group exhibited good viability on day 4, suggesting that this concentration may provide a more favorable environment for maintaining cell metabolic activity over time. To further assess cell attachment and morphology, scanning electron microscopy (SEM) analysis was performed for the samples collected at day1 and images are given in Figure 4.20. SEM images showed that cells successfully adhered to the surface of all scaffolds, exhibiting typical spread morphology, which indicates that SPP-containing scaffolds supported cell attachment and were not cytotoxic. Even at higher concentrations, no signs of structural damage were observed. However, cell number on the scaffolds were very low when compared to the seeded cell number (250000 cells/scaffold) and they were not able to proliferate on the scaffolds as seen in the decreased viabilities on day 4. So further observations were carried out to understand whether the cells were not able to attach on the scaffolds or they were died.

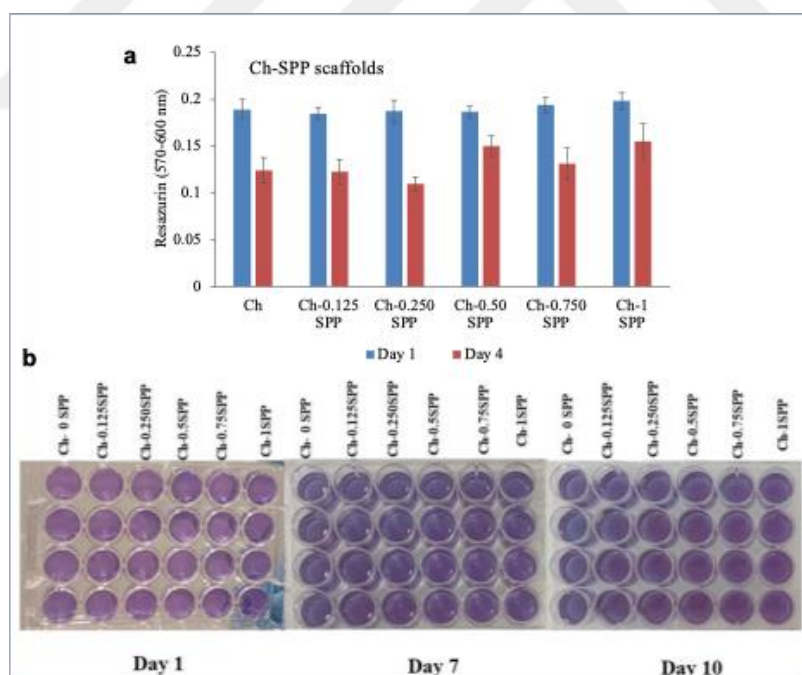


Figure 4.19. Resazurin analysis results of MC3T3-E1 cells on Ch-SPP scaffolds. a) Graphical representation of absorbance values. b) Macroscopic images of the scaffolds after resazurin application for 3 h incubation.

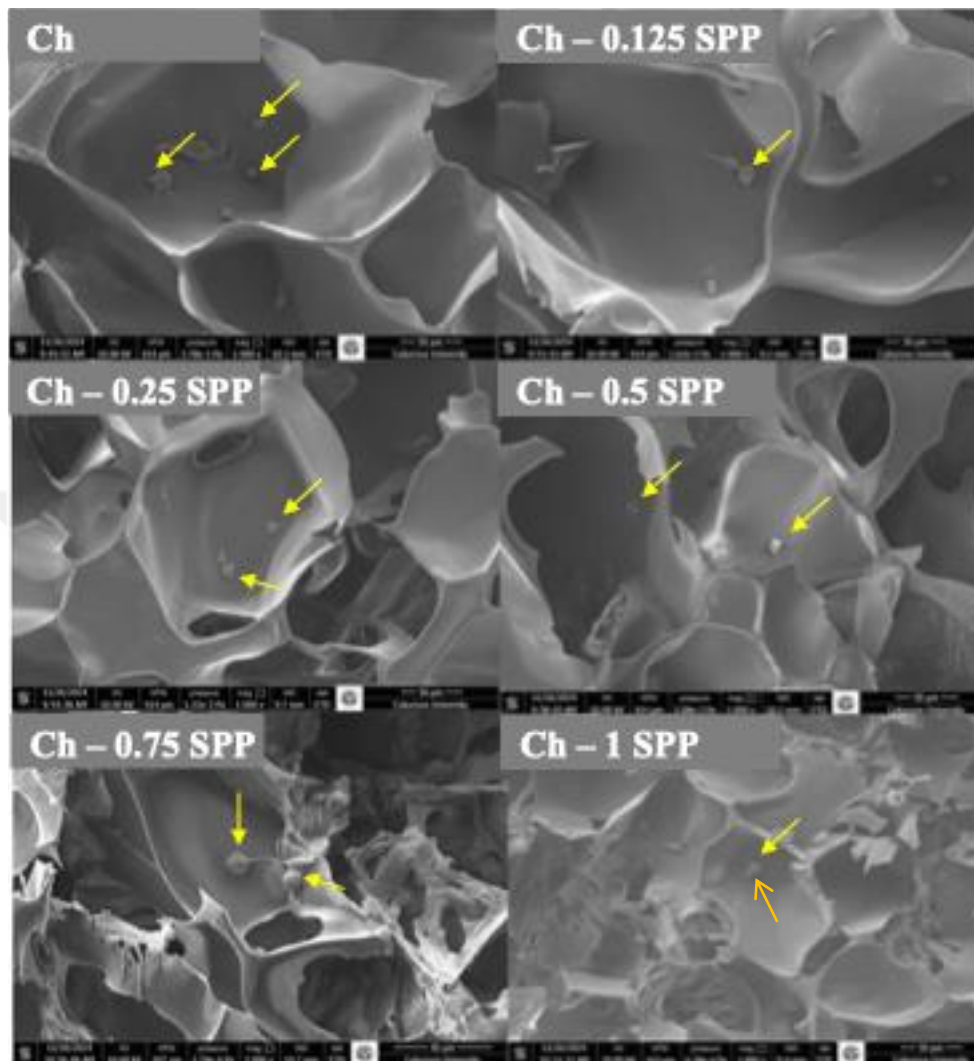


Figure 4.20. (SEM) images of MC3T3 cells cultured on Chitosan/SPP scaffolds were taken on Day 1 to evaluate cell attachment and morphology.

Inverted phase contrast microscopy was used to visualize the bottom of the wells after scaffold removal on day 7 and the images are given in Figure 4.21. These images revealed that many cells remained attached onto the surface of the wells, rather than to the scaffolds. In addition there were no dead cells on the surface indicating that the scaffolds were not cytotoxic for the cells however the cells were not able attach on the surface of the scaffolds and they migrated to the well surface. Taken together, these findings suggest that 0.5 and 0.75 SPP are the most promising concentrations, offering both high cell viability and favorable morphology, with no signs of cytotoxicity.

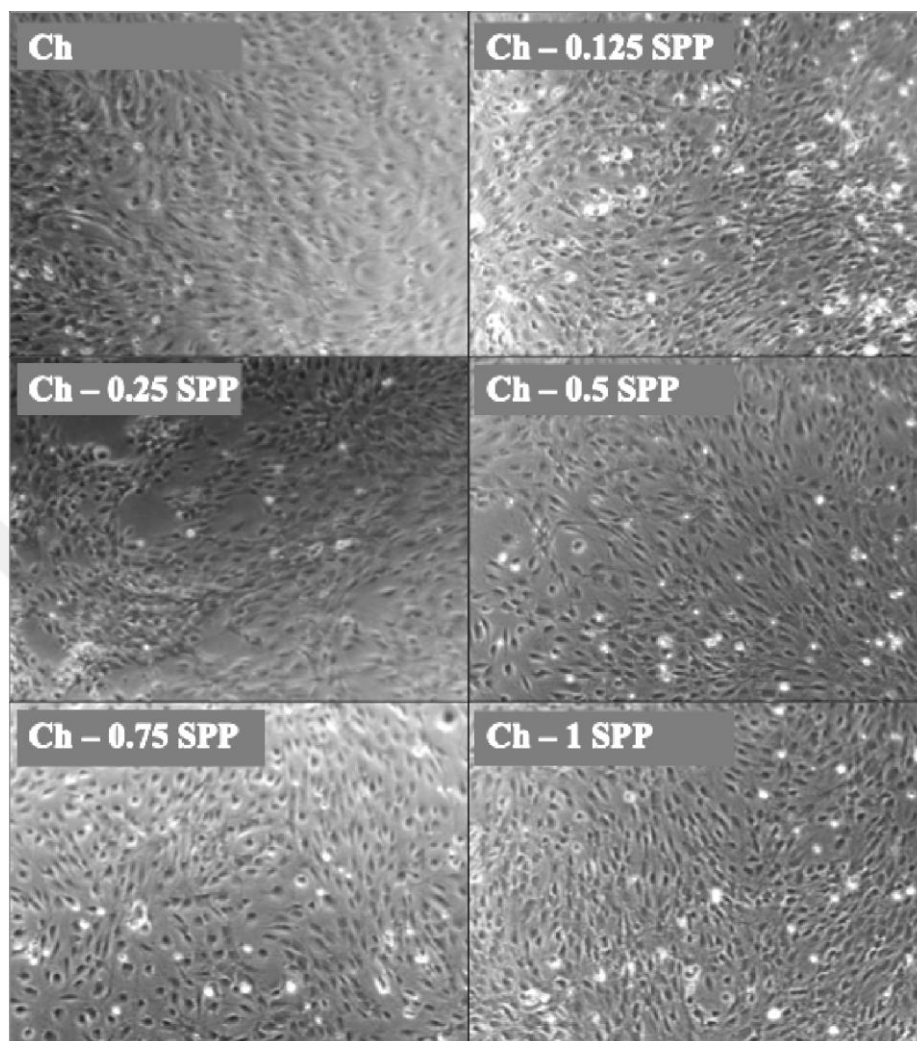


Figure 4.21. Inverted phase contrast images (10x) of MC3T3-E1 cells under the scaffolds after they were taken out from the wells for cell viability analysis (Day 7).

For further analysis, some parameters given below were checked to enhance the adhesion and proliferation of MC3T3-E1 cells to Ch and Ch-SPP scaffolds:

1. Type of chitosan used for scaffold production
2. Stabilization method for chitosan scaffolds
3. Different cell seeding strategies

4.5. Comparison of Stabilization Methods for Ch and Medical Grade Ch for MC3T3-E1 Cell Attachment and Proliferation

Resazurin analysis results for chitosan-based scaffolds prepared with different stabilization methods are presented in Figure 4.22. Four groups were tested:

- **Ch-Et** (chitosan stabilized with ethanol),
- **Ch-NaOH** (chitosan stabilized with NaOH),
- **MG Ch-Et** (medical-grade chitosan stabilized with ethanol), and
- **MG Ch-NaOH** (medical-grade chitosan stabilized with NaOH).

On Day 1, all groups exhibited relatively high cell viability, with absorbance values above 0.3, indicating good initial cell attachment and proliferation. However, by Day 4, a decline in metabolic activity was observed in all groups, most notably in Ch-Et and MG Ch-Et. Interestingly, on Day 7, the MG Ch-Et group showed a recovery in viability, suggesting possible cell adaptation or delayed proliferation. In contrast, Ch-Et continued to show reduced viability, indicating that ethanol stabilization in chitosan may not support long-term cell growth. Among all tested groups, MG Ch-NaOH consistently exhibited the highest cell viability at day 7 ($p < 0.01$, when compared to Ch-Et and Ch-N), suggesting that NaOH-stabilized medical-grade chitosan scaffolds provide the most favorable environment for cell survival and proliferation.

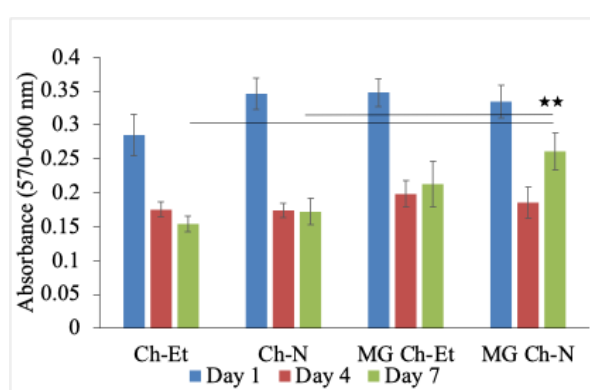


Figure 4.22. Resazurin analysis results of MC3T3-E1 cells on chitosan and medical grade chitosan scaffolds stabilized by ethanol or NaOH. Ch: chitosan, MG Ch: medical grade chitosan, Et:ethanol, N:NaOH. ★★ ($p < 0.01$) means significant difference between compared groups.

4.6. Effect of Sodium Pentaborate Pentahydrate Concentration in Medical-Grade Chitosan Scaffolds on MC3T3-E1 Cell Viability

4.6.1. Resazurin Analysis

To evaluate the effect of SPP incorporation on cell viability, MC3T3-E1 cells were cultured on NaOH-stabilized medical-grade chitosan (MG-Ch) scaffolds containing 0 (Ch), 0.5, and 0.75 SPP. To prevent cell attachment to the well surface, a thin agar layer was first applied to the plates prior to cell seeding. As shown in Figure 4.23 and 4.25, all groups exhibited high cell viability on Day 1, indicating good initial cell adhesion and metabolic activity. However, a sharp decline in viability was observed on Day 4 across all groups, which may be attributed to changes in cell-scaffold interaction or early nutrient limitations. Interestingly, cell viability increased again on Day 7 and remained relatively stable on Day 10, suggesting recovery and adaptation of the cells over time. Among the groups tested, the MG Ch - 0.5 SPP scaffold consistently showed the highest viability at Days 7 and 10, indicating its favorable effect on promoting sustained cell growth compared to pure chitosan and the MG Ch - 0.75 SPP group. These findings confirm that Ch - 0.5 SPP in NaOH-stabilized MG-chitosan scaffolds offers the best support for long-term cell viability ($p < 0.05$), and that boron incorporation at optimal concentration enhances cell compatibility without cytotoxicity.

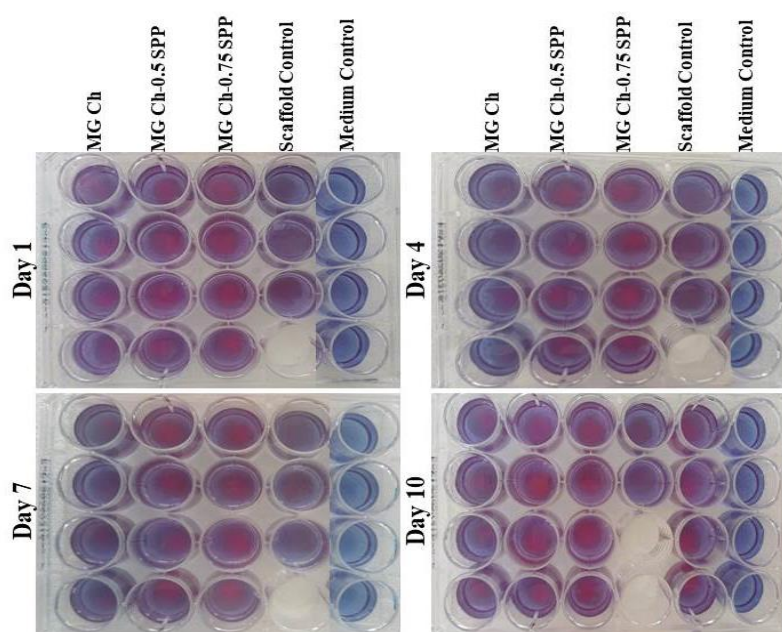


Figure 4.23. Macroscopic images of MG Ch-SPP scaffolds after resazurin analysis.

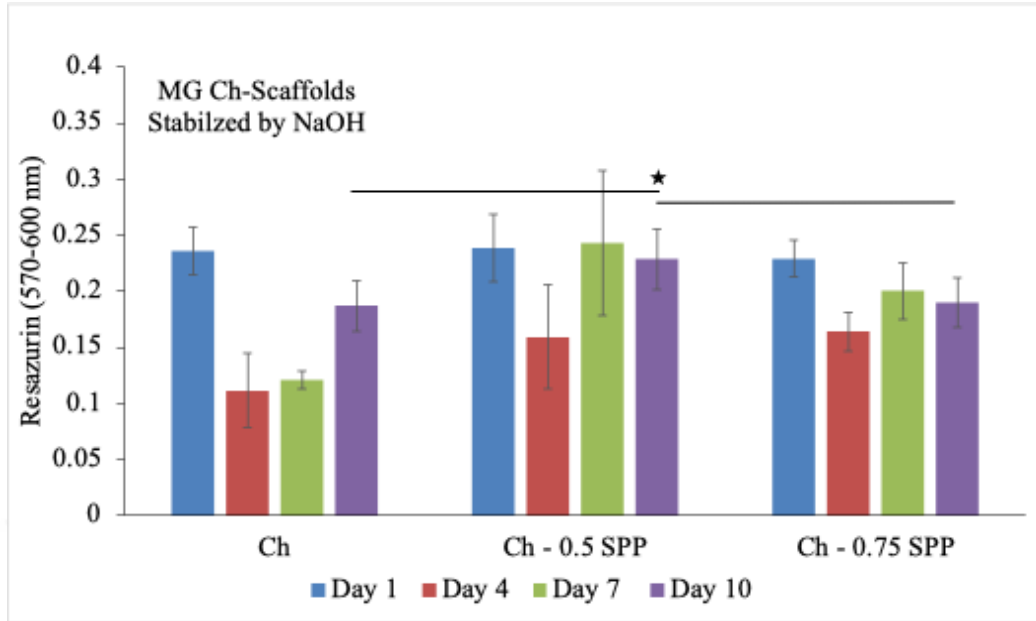


Figure 4.24. Resazurin analysis results of MC3T3-E1 cells on medical grade (MG) chitosan scaffolds stabilized with NaOH and containing different SPP concentrations. ★ ($p < 0.05$) represents the significant difference between the groups.

4.6.2. SEM Analysis

SEM images presented in Figure 4.25 show the surface morphology and cell attachment behavior on MG Ch, MG Ch-0.5 SPP, and MG Ch-0.75 SPP scaffolds. It was difficult to see cell cluster on the scaffolds at day 10 however, there were some individual cells on all scaffolds. Particularly in the MG Ch-0.5 SPP group, cells appeared more spread on the scaffold structure, with extensions interacting with the pore walls—suggesting enhanced cell-scaffold interaction. Regarding scaffold structure, MG Ch scaffolds displayed relatively larger and more irregular pores, while SPP-containing scaffolds showed smaller and more compact pores. The incorporation of SPP reduced pore size and resulted in a more organized, interconnected network—especially at 0.5% concentration, which appears optimal for both structure and cellular attachment.

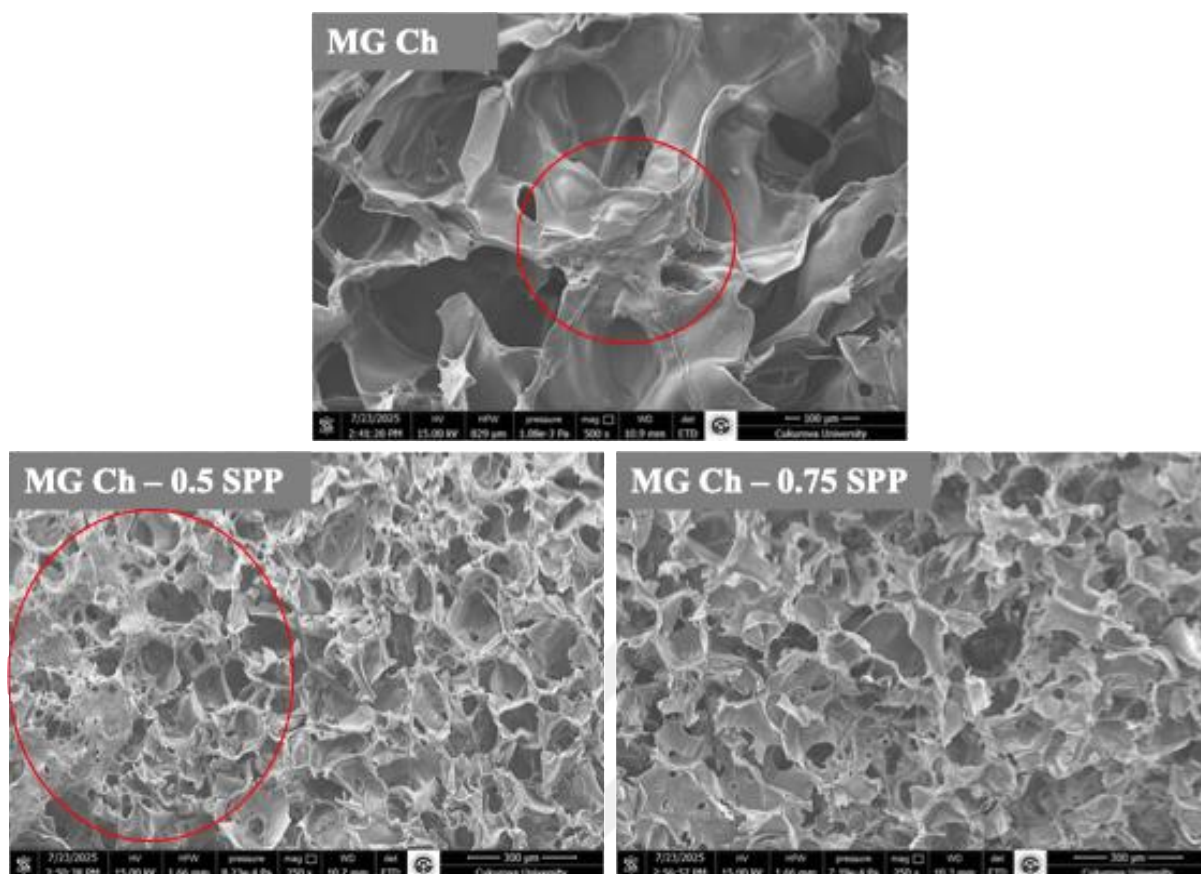


Figure 4.25. SEM) images of MC3T3-E1 cells cultured on MG Ch scaffolds at various SPP amounts (0%, 0.5% and 0.75% w/w) were taken on Day 10 to evaluate cell proliferation and morphology. Red circles show cell dense areas on the scaffolds.

4.6.3. DAPI/F-actin Fluorescence Staining of MC3T3-E1 Cells on MG Ch-SPP Scaffolds

DAPI staining was used to observe the number and distribution of cell nuclei on chitosan scaffolds with different SPP concentrations over time. Images were taken at 4×, 10×, and 20× magnifications for three groups: MG Ch, MG Ch - 0.5 SPP, and MG Ch - 0.75 SPP on day 1 and 10. Scaffolds are 3D materials and it is difficult to visualize fluorescence stained cells on 3D structure. In this study, DAPI staining could be clearly imaged due to the clear boundaries of the cell nucleus, but F-actin staining could not be imaged due to scaffold staining and difficulties in clarifying the imaging for scattered cells.

As seen in figures 4.26 and 4.27, the number of cells increased by Day 10 in all groups compared to Day 1, showing that the cells continued to proliferate on the scaffolds. The increase was most noticeable in the MG Ch - 0.5 SPP group, followed by the MG Ch - 0.75 SPP group, which suggests that SPP supports cell proliferation. The increase in the cell number and distribution of the cells on the scaffolds were more clear when compared to the resazurin and SEM analysis. The samples preparation protocol may affect the image quality for SEM analysis. The apparent increase in cell number is clearly visible in the DAPI staining images but not in the viability analysis, which may be due to decreased metabolic activity in the cells, but this is not an expected result when the cell number increases so much.

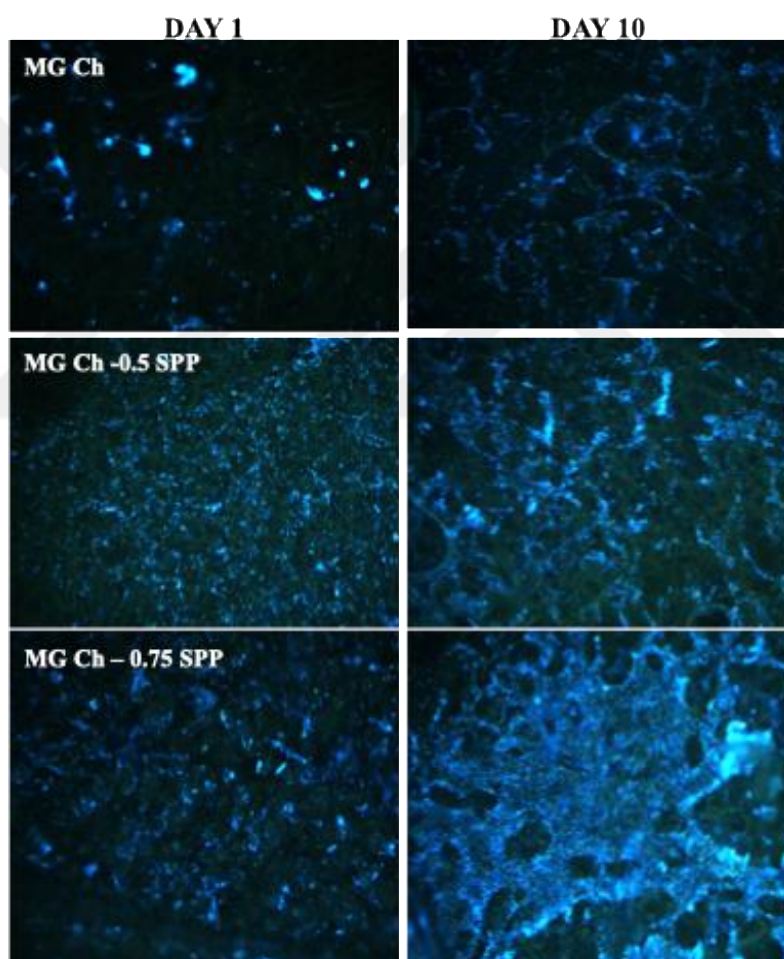


Figure 4.26. Fluorescence microscopy images (10× magnifications) of DAPI stained MC3T3-E1 cells on the MG Ch – SPP scaffolds at day 1 and day 10. MG: Medical grade, Ch: chitosan, SPP: sodium pentaborate pentahydrate.

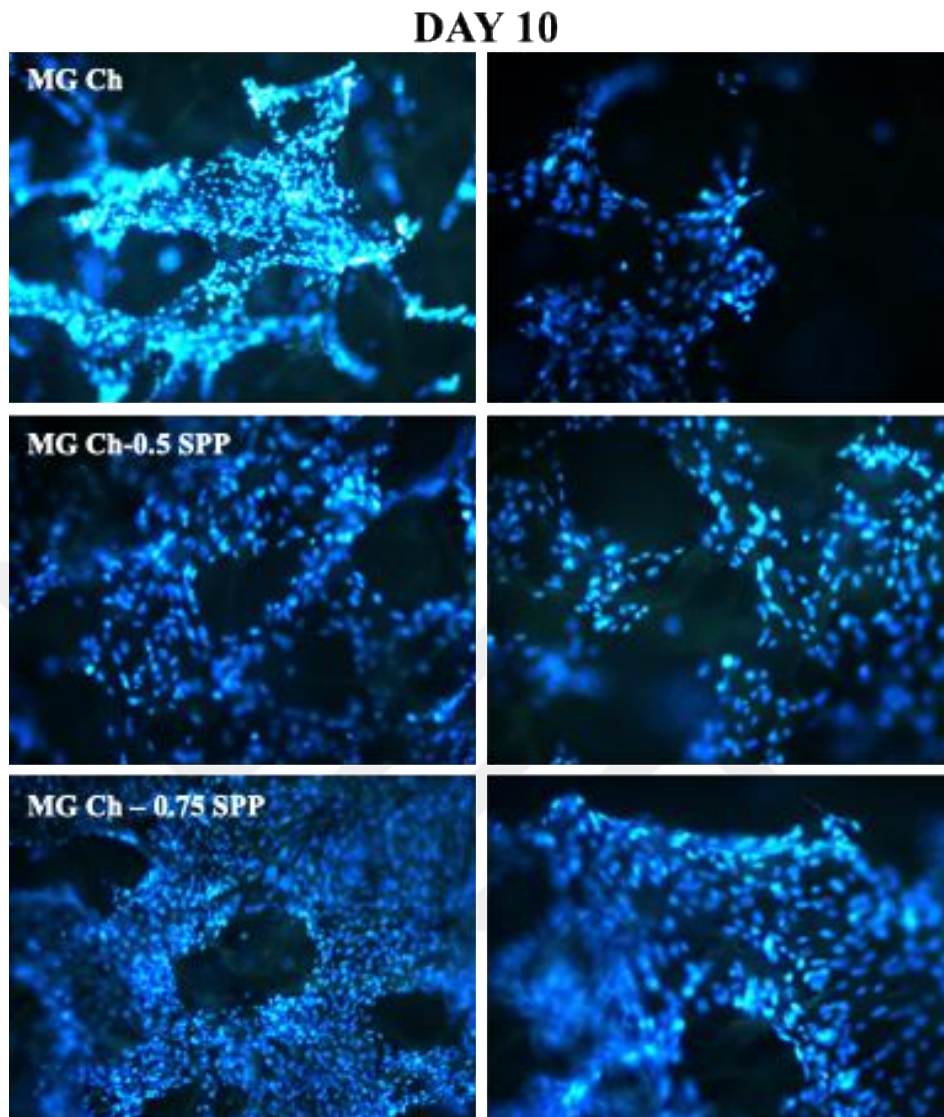


Figure 4.27. Fluorescence microscopy images (20x) of DAPI stained MC3T3-E1 cells cultured on MG Ch – SPP scaffolds on day 10. MG: Medical grade, Ch: chitosan, SPP: sodium pentaborate pentahydrate.

MC3T3-E1 cells were able to proliferate both on the surface of the scaffolds and in the pores. The cells which were spread and proliferated in the pore are shown in Figure 4.28.

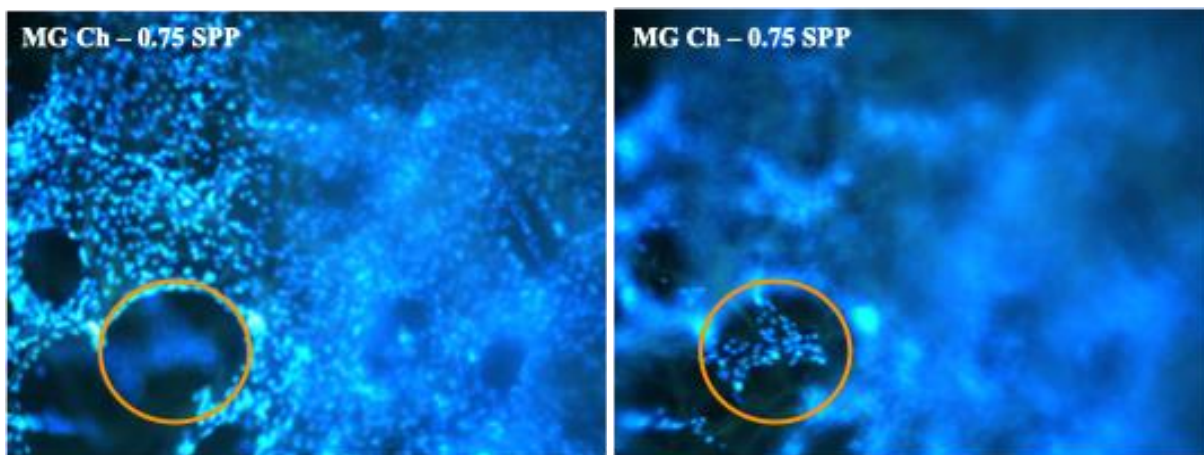


Figure 4.28. Fluorescence microscopy images (10x) of DAPI stained MC3T3-E1 cells cultured on MG Ch – 0.75 SPP scaffolds on day 10. Images of the same area taken from different focusing points to show cells in the pores. MG: Medical grade, Ch: chitosan, SPP: sodium pentaborate pentahydrate.

4.6.4. Water Uptake Analysis

All scaffold formulations were evaluated for their swelling behavior in PBS. Macroscopic images of MG Ch scaffolds before and after swelling in PBS for 60 min at room temperature are given in Figure 4.29. As with previous formulations, the scaffolds exhibited a two-phase swelling profile characterized by an initial rapid water uptake within the first 5–10 minutes, followed by a gradual approach to equilibrium. Equilibrium swelling was reached at approximately 30 minutes for all samples. However, the extent of equilibrium swelling was clearly influenced by the SPP concentration. Figure 4.30 and 4.31 summarize the equilibrium water uptake (Q) for MG Ch - 0SPP, MG Ch - 0.5 SPP, and MG Ch - 0.75 SPP scaffolds after 1 hour of PBS immersion. A marked decrease in swelling capacity was observed with SPP incorporation, confirming the role of SPP in modulating scaffold hydrophilicity and structural integrity. Specifically:

MG Ch- 0 SPP: Exhibited the highest swelling capacity ($Q \approx 1500\%$), consistent with its highly porous and hydrophilic nature.

MG Ch - 0.5 SPP: Showed a substantial reduction in swelling ($Q \approx 780\%$). The incorporation of 0.5% SPP (w/w) likely may enhanced polymer–polymer interactions through ionic or

physical cross-linking, reducing the free volume available for water and increasing structural rigidity. As a result, water diffusion was more restricted compared to the pure MG - Ch scaffold ($p < 0.01$).

MG Ch - 0.75 SPP: Demonstrated an intermediate swelling capacity ($Q \approx 981\%$), greater than MG Ch - 0.5 SPP but still significantly lower than MG Ch ($p < 0.05$). This suggests that while 0.75% SPP (w/w) continues to restrict water absorption, the specific distribution or interaction of SPP at this concentration may result in a slightly less compact network than at 0.5%.

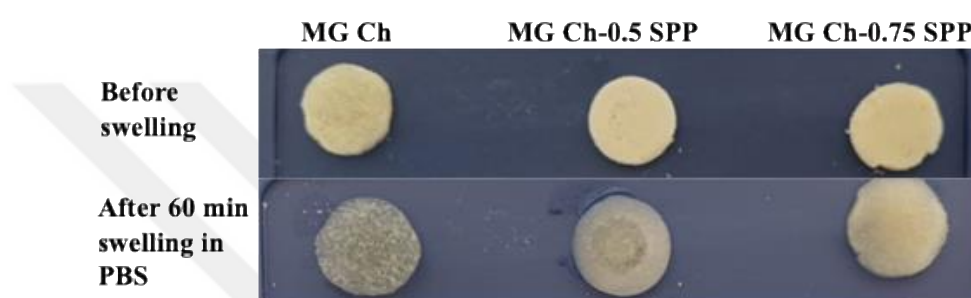


Figure 4.29. Macroscopic images of MG Ch scaffolds before and after swelling in PBS for 60 min at room temperature.

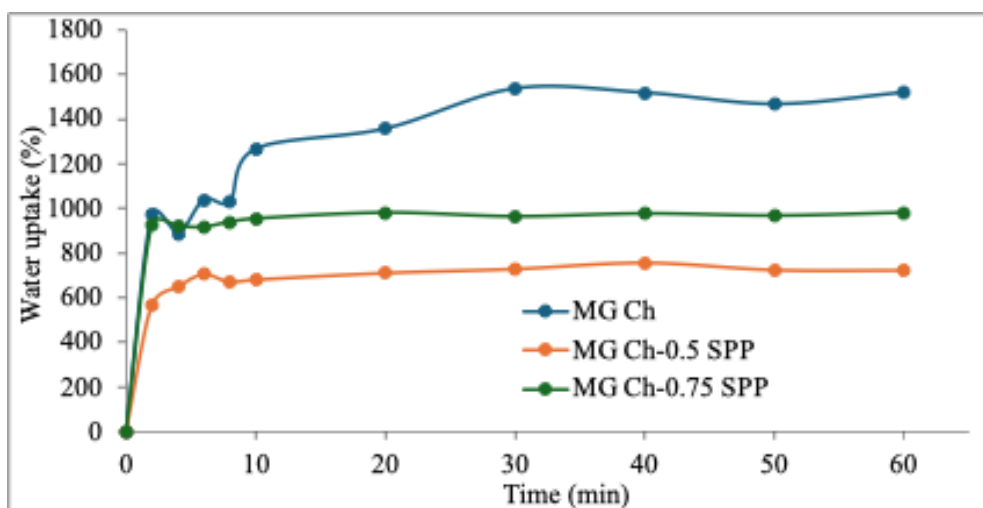


Figure 4.30. Water uptake profiles of MG Ch - SPP scaffolds during a 60-minute immersion in PBS at RT.

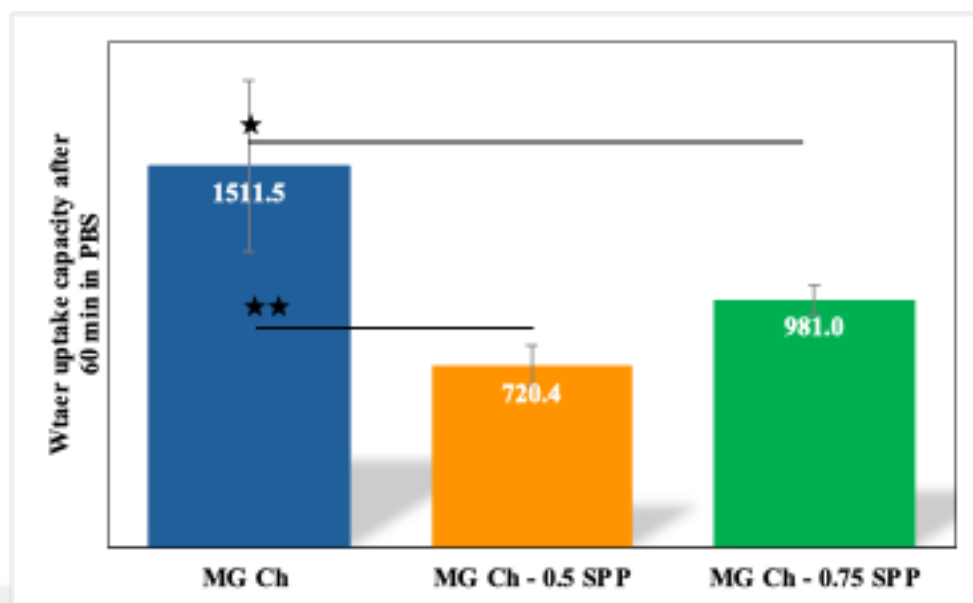


Figure 4.31. Comparison of water uptake capacity of MG Ch-SPP scaffolds after immersion in PBS for 60 min. ★ ($p < 0.05$) and ★★ ($p < 0.01$) represents the significant difference between the groups.

4.6.5. FTIR Analysis

FTIR analysis was conducted to show successful integration of SPP molecules into chitosan polymer structure. Peaks observed below 1000 cm^{-1} wave number range are particularly common in inorganic compounds and metal-oxygen bonds. In our study, peaks at 931 cm^{-1} , 780 cm^{-1} , 550 cm^{-1} and 469 cm^{-1} are observed in the FTIR spectrum of SPP. These peaks were not observed in the chitosan structure. As mentioned in the FTIR analysis of the previous chitosan samples, the functional groups seen in the SPP structure in FTIR due to the embedding of SPP in the chitosan polymer are difficult to be seen in Ch - SPP scaffolds. The most prominent peaks were observed with slight shifts in the Ch - 0.75 SPP group, where SPP was added at the highest rate. While the peaks at 706 cm^{-1} and 458 cm^{-1} seen in Ch 0.75 SPP indicate the presence of SPP, it was thought that these peaks could not be seen clearly due to the lower SPP content in the other groups.

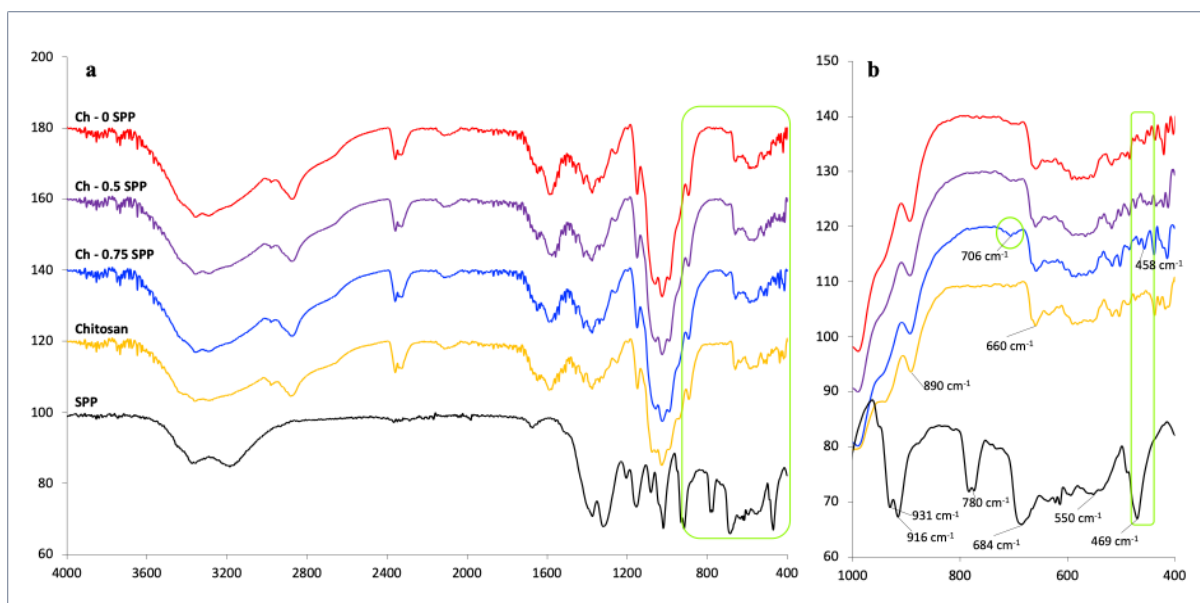


Figure 4.32. a) FTIR analysis spectra of medical grade chitosan polymer, SPP, MG Ch and MG Ch-SPP scaffolds. b) Magnified spectra from 400 to 1000 cm^{-1} for detailed visualization of peaks varying with SPP concentration.

5. DISCUSSION

Bone defects, especially large or critical-sized defects resulting from trauma, infection, or degenerative disease, remain a significant clinical challenge. Tissue engineering strategies focus on developing biocompatible scaffolds that can support bone regeneration by serving as temporary matrices for cell attachment, proliferation, and differentiation. Chitosan-based scaffolds have attracted great interest in this context due to chitosan's favorable biological properties such as good biocompatibility, natural antibacterial activity, biodegradability and ease of chemical modification (Hutmacher, 2001a; Huang et al., 2024). Chitosan is a natural polysaccharide whose cationic nature and structural similarity to glycosaminoglycans make it attractive for cell scaffold application (Sedighi-Pirsaraei et al., 2024). However, pure chitosan scaffolds also have well-documented limitations. They degrade rather slowly in vivo and lack strong osteoconductivity, which can lead to suboptimal bone healing outcomes. For instance, undegraded chitosan fragments may persist at the implant site and invoke chronic inflammation or encapsulation by fibrous tissue instead of being replaced by new bone. Moreover, the minimal osteoinductive cues provided by pure chitosan often result in

incomplete ossification, with defects healing by fibrous connective tissue rather than mineralized bone. To overcome these drawbacks, composite and functionalized scaffold approaches have been widely explored. By combining chitosan with other materials or bioactive factors, one can enhance its mechanical, biological, and degradation properties. For example, Oryan and Sahviah (2017) demonstrated that a chitosan-based scaffold supplemented with gelatin and platelet-derived growth factor significantly improved bone repair in a rat radial defect model, achieving more robust bone regeneration than chitosan alone. This highlights how biomaterial functionalization can critically influence therapeutic outcomes. Similarly, Yang et al. (2010) fabricated porous chitosan scaffolds via freeze-drying of chitosan hydrogels pre-gelled with dibasic sodium phosphate. They reported that using a lower chitosan concentration yielded scaffolds with higher porosity and water uptake but lower compressive strength. These findings mirror part of our results: in the present study, the scaffold with no SPP had the largest pore size and the highest swelling capacity, in line with a more open, hydrated network as noted by Yang et al. Furthermore, Yang et al. observed near-complete in vitro degradation of their chitosan scaffolds in a lysozyme-containing medium by 28 days. In our study, pure chitosan scaffolds were more resistant to degradation ($\approx 20\%$ mass loss at 28 days), possibly due to differences in scaffold preparation or lower enzyme concentration. Notably, however, the incorporation of SPP in our scaffolds substantially accelerated degradation to $\sim 30\text{--}35\%$ mass loss over the same period, underscoring that additives can modulate chitosan's biodegradation dynamics. Indeed, enhanced biodegradability in SPP-containing scaffolds could be beneficial for ensuring timely scaffold resorption and space formation for new tissue, addressing the issue of persistent scaffold remnants observed with pure chitosan. Beyond combining chitosan with polymers or growth factors, incorporating inorganic bioactive components is another strategy to improve scaffold performance.

Bioactive ceramics (like hydroxyapatite or bioactive glass) and trace elements such as boron have been studied for their osteogenic and antimicrobial contributions. Recent research has shown that boron, in particular, can play an osteoinductive role in bone repair (Wu et al., 2011) and other studies indicated that boron-doped bioactive glass scaffolds significantly upregulated osteoblast differentiation markers (Gümüřderelioğlu et al., 2015). In a study it was reported that boron-containing nanoparticles embedded in chitosan scaffolds stimulated

the proliferation and differentiation of MC3T3-E1 preosteoblasts, effectively acting as an osteogenic enhancer within the scaffold (Gümüşderelioglu et al., 2015). Boron is thought to influence bone formation by activating the Wnt/ β -catenin signaling pathway in osteoblasts and by promoting angiogenesis in the healing tissue (Hadidi et al., 2021). This ion also has anti-inflammatory and antioxidant effects that can create a more regenerative environment (Sedighi-Pirsaraei et al., 2024). These attributes make boron compounds particularly appealing as multifunctional additives for tissue engineering scaffolds, as they can concurrently encourage bone regeneration and combat infection. In light of this, we incorporated sodium pentaborate pentahydrate (SPP), a boron-rich compound, into chitosan scaffolds to capitalize on these potential benefits. SPP has gained attention in various biomedical contexts for its bioactivity. Iyigündoğdu and Demir (2017) showed that cotton fabric treated with 3% SPP (along with a small amount of triclosan) acquired broad-spectrum antimicrobial properties, whereas untreated fabric had no such effect. In their study, SPP-treated textiles exhibited pronounced activity against a range of microorganisms, with particularly strong inhibition of *Staphylococcus epidermidis* biofilms. This demonstrates SPP's capacity to confer lasting antimicrobial functionality to a material. Taşlı et al. (2013) provided evidence of SPP's osteogenic potential by treating human tooth germ stem cells with SPP in vitro. At a concentration of 20 $\mu\text{g/mL}$, SPP significantly elevated alkaline phosphatase activity and upregulated the expression of genes associated with osteogenesis and odontogenesis (Taşlı et al., 2013). The treated stem cells showed enhanced differentiation toward bone- and dentin-forming lineages compared to controls, indicating that boron (in the form of SPP) can directly stimulate cellular pathways involved in hard tissue formation. Together, these prior studies suggested that adding SPP to chitosan scaffolds might simultaneously impart antimicrobial and osteoinductive features – a hypothesis that our findings support. In the current work, SPP was integrated into chitosan scaffolds to evaluate its dual role in enhancing scaffold degradation and providing antifungal activity, alongside any effects on swelling behavior and cytocompatibility. Our results confirmed several advantageous effects of SPP incorporation. Degradation of chitosan scaffolds in the presence of SPP increased. This controlled increase in degradability is desirable for bone tissue engineering scaffolds, which should persist long enough to support healing but not so long as to impede new tissue formation. Additionally, the presence of SPP moderated the scaffold's swelling in aqueous conditions, preventing excessive expansion. Excessive swelling can be

problematic in vivo by reducing mechanical stability or altering pore geometry; thus, the reduced swelling of SPP-laden scaffolds can be seen as an improvement in maintaining structural integrity upon implantation. The compression test results in our study revealed that the incorporation of sodium pentaborate pentahydrate (SPP) into chitosan scaffolds significantly influenced their mechanical performance. The addition of 0.5 SPP (w/w) resulted in the highest strength and toughness among all tested groups, indicating an optimal concentration for enhancing the mechanical integrity of the scaffolds. The scaffolds' computed strengths for the Ch – 0.5 SPP and Ch control groups were 33.46 ± 5.02 kPa and 15.68 ± 0.92 , respectively. In comparison to the control samples, the results demonstrated that the strength increased by up to two times when 0.5 (w/w) SPP was added to the Ch structure. In a study, based on the ionic strength and pH-dependent solubility of chitosan, a novel compression technique was created in this study to create chitosan scaffolds with high mechanical strength and tuneable topography. The scaffold's compressive elastic modulus rises from 5.2 kPa to 520 kPa and its porosity falls from 94.1% to 82.5% when the compressive ratio rises from 1 to 8 (Xu et al., 2017).

The antimicrobial tests further demonstrated the value of SPP. Ch scaffolds with SPP were able to inhibit the growth of both *C. albicans* and *A. niger* in vitro, and the effect was clearly dose-dependent. Higher SPP content yielded larger inhibition zones, a result that is consistent with the known concentration-dependent antimicrobial action of boron compounds (Öztaş et al., 2024). The mechanism of SPP's antifungal action in the scaffold context is likely linked to the slow release of borate ions $[\text{BH}_4\text{O}_4]^-$ or related species into the surrounding medium. Boric acid, which forms in equilibrium with borate, is well-documented to have fungistatic and fungicidal effects. It inhibits oxidative metabolism in fungal cells and interferes with critical virulence factors such as biofilm formation and hyphal development in *Candida* (De Seta et al., 2009). In our study's agar diffusion setting, it is probable that borate ions diffusing out of the SPP–chitosan scaffold create a localized microenvironment that is inhospitable to fungal growth, thereby producing the observed clear zones. Notably, *A. niger* appeared more sensitive than *C. albicans*, which could be due to differences in cell structure or metabolism between filamentous fungi and yeasts; alternatively, the PDA medium used for *A. niger* might facilitate better diffusion or stability of the active boron species. Regardless, the ability of the SPP-enhanced scaffolds to inhibit fungi is a significant benefit, as implant-related fungal

infections (though less common than bacterial infections) are serious complications in orthopedic surgery. Moreover, SPP's antimicrobial spectrum is not limited to fungi – prior studies have shown efficacy against bacteria as well (including *E. coli* and *S. aureus*) when SPP is embedded in polymeric matrices (Öztaş et al., 2024). Although we focused on antifungal activity here, it is reasonable to expect broader antimicrobial protection from these composite scaffolds, which could be crucial for preventing post-surgical infections. Neither Ch without SPP nor Ch SPP scaffolds show antibacterial activity in our study. The antibacterial activity of chitosan can vary depending on its production parameters (such as molecular weight and degree of deacetylation) and the type or source of chitosan used (Kong et al., 2010). Additionally, some bacteria—especially gram-negative species like *Escherichia coli*—may exhibit greater resistance to boron compounds compared to gram-positive strains (Nielsen, 2014).

The cytocompatibility outcomes in this study further highlight the benefits of SPP and appropriate scaffold processing. We observed that using NaOH to neutralize and stabilize the chitosan scaffolds (after freeze-drying) resulted in significantly higher osteoblast viability compared to scaffolds that were ethanol stabilized. This supports the findings of Gültan et al. (2021), who reported improved cellular adhesion and proliferation on NaOH-stabilized chitosan scaffolds prepared via gas foaming. The NaOH treatment likely removes residual acids and increases the surface roughness or exposes favorable chemical groups (–OH and –NH₂) on chitosan, thus creating a more conducive surface for cell attachment. In our case, all scaffolds (with or without SPP) showed good cytocompatibility with MC3T3-E1 cells, but those prepared with the NaOH post-treatment had a clear edge in supporting cell viability. Importantly, SPP did not cause any cytotoxic effect at the concentrations used; on the contrary, the scaffolds containing SPP maintained cell viabilities on par with or better than pure chitosan scaffolds. This is an encouraging result, suggesting that the release of boron from the scaffold is at a biologically tolerable level and may even positively influence cell metabolic activity (as low-dose boron can activate certain cell signaling pathways related to growth and differentiation (Hadidi et al., 2021)). Huang et al. (2024) recently showed that functionalizing chitosan scaffolds via glucose-mediated cross-linking enhanced osteogenic differentiation of MC3T3 cells, emphasizing that smart modifications to chitosan can boost its bioactivity. Our approach of integrating SPP aligns with this principle, using a bioactive

inorganic additive to elevate the scaffold's performance. Taken together, the combination of chitosan and SPP presents a promising strategy for engineering next-generation bone scaffolds that are multifunctional. The SPP-enriched chitosan scaffolds in this study demonstrated several key improvements over unmodified chitosan scaffolds: more predictable and accelerated degradation, a more controlled (reduced) swelling behavior, increased mechanical properties and significant antifungal (and presumably broader antimicrobial) capabilities, all while maintaining biocompatibility and even supporting better cell viability when properly processed. Despite these promising results, there are several limitations in our study that should be considered. One key limitation is the observed decline in cell viability over time in the early scaffold formulations. This prompted us to transition to using medical-grade chitosan, which is chitosan that meets strict quality, purity, and safety standards for biomedical use. In a comparative study of medical grade chitosan and chitosan, the results clearly showed that both genipin crosslinked and non-crosslinked medical-grade chitosan scaffolds significantly enhanced stem cell viability, attachment, and proliferation, as confirmed by MTT, SEM, and live/dead assays (Çapkin Yurtsever et al., 2022).

In this study, the effects of sodium pentaborate pentahydrate (SPP) on the viability and osteogenic differentiation of MC3T3-E1 pre-osteoblast cells culture on tissue culture polystyrene were also investigated. Interestingly, 0.125 mg/mL SPP improved viability under differentiation conditions compared to the control. Despite this, ALP staining revealed that osteogenic activity was observed only in the SPP-free group, suggesting that higher concentrations may suppress differentiation over time. These results highlight the dose- and time-dependent nature of SPP's biological effects. Supporting this, (Taşlı et al., 2013b) showed that 20 µg/mL SPP enhanced osteogenic and odontogenic differentiation in human tooth germ stem cells, while (Demirci et al., 2014) found that as low as 0.15 µg/mL SPP promoted fibroblast migration and wound healing. Compared to these studies, the concentrations used in our experiments were considerably higher, which may explain the suppressed differentiation responses observed at later time points in MC3T3-E1 cells.

Despite these promising results, there are several limitations to our study that warrant discussion. First, all experiments were conducted in vitro. While in vitro tests are invaluable

for screening scaffold properties and biological interactions, they cannot fully replicate the complexity of a living organism. Factors such as immune response, blood flow, and mechanical loading in a real bone defect could influence the scaffold's performance. Therefore, the true efficacy and safety of SPP–chitosan scaffolds need to be validated in vivo in an appropriate bone injury or defect model. Second, our antimicrobial evaluation focused on fungal species; however, bacterial infections are also a major concern in orthopedic implants. While SPP has shown antibacterial effects in other studies (Iyigünoğlu and Demir, 2017; Öztaş et al., 2024), testing the scaffold against common bone infection bacteria (e.g., *S.aureus* or *S. epidermidis*) would strengthen the evidence for its clinical utility in preventing osteomyelitis. Additionally, the molecular mechanisms by which boron influences bone cells were not explored here. The literature suggests boron can modulate signaling pathways (like Wnt/ β -catenin and perhaps growth factor signaling) that promote osteoblast activity and angiogenesis (Hadidi et al., 2021). Investigating these pathways in cells exposed to Ch - SPP scaffolds could yield insight into how the scaffolds might actively stimulate bone regeneration on a molecular level. Finally, the long-term biocompatibility and potential systemic effects of SPP release need consideration.

The resazurin assay conducted on L929 fibroblast cells demonstrated that all Ch-SPP scaffolds supported cell proliferation over 7 days, with the Ch–0.75% w/w SPP group showing the highest viability ($p < 0.001$), indicating good compatibility of the scaffolds with this cell line. In contrast, when various concentrations of SPP (0% to 1% w/w) were tested on MC3T3-E1 pre-osteoblasts, cell viability declined significantly by days 7 and 10, suggesting potential cytotoxic effects or limited nutrient diffusion over time. However, when the same MC3T3-E1 cells were cultured on medical-grade chitosan (MG-Ch) scaffolds stabilized with NaOH and containing 0%, 0.5%, and 0.75% SPP w/w, a more favorable pattern was observed: although viability dropped on day 4, it rebounded on day 7 and remained stable on day 10, especially in the 0.5% SPP w/w group. These results indicate that scaffold formulation—including chitosan grade and stabilization method—strongly influences cellular response. Specifically, NaOH-stabilized MG-Ch with 0.5% SPP w/w provided the best long-term environment for MC3T3-E1 viability, while standard chitosan with higher SPP (0.75% w/w) was more effective for L929 fibroblasts. This highlights the importance of scaffold customization based on cell type and application.

The mechanical performance of the Ch–SPP scaffolds showed a notable improvement over pure chitosan controls, with the Ch-0.5 SPP formulation achieving a compressive strength of approximately 33.46 ± 5.02 kPa, nearly double that of the untreated chitosan (15.68 ± 0.92 kPa). However, these values remain significantly lower than physiological benchmarks. Human cancellous (trabecular) bone from femoral heads has been shown to exhibit ultimate compressive strengths ranging between 145 and 1,355 kN/m² (~0.145–1.355 MPa) (Schoenfeld et al., 1974). Importantly, mimicking the comprehensive biomechanical behavior of natural bone—including stiffness, elastic response, and resistance to stress shielding—is essential in scaffold design. Failure to replicate these properties may lead to impaired osteointegration or bone resorption, ultimately compromising implant longevity. Additionally, the degradation profile of scaffolds must be carefully balanced; premature loss of mechanical integrity can result in scaffold collapse before new bone formation occurs, especially when initial strength is already limited. Although the Ch–SPP scaffolds do not meet the rigorous mechanical requirements for load-bearing bone tissue engineering, their enhanced performance compared to pure chitosan is encouraging they might be appropriate candidates for non-load-bearing applications.

In summary, our discussion underscores that integrating a boron compound (SPP) into a chitosan scaffold yields a composite biomaterial with enhanced performance across several metrics relevant to bone tissue engineering. The scaffolds supported MC3T3-E1 pre-osteoblast viability, especially in the NaOH-stabilized and 0.5% SPP group in medical grade chitosan, as shown by resazurin assays and DAPI staining. Since MC3T3-E1 cells are bone-forming precursor cells, their survival and proliferation on the scaffolds indicate a potential to promote osteogenic activity. This suggests that the scaffold environment may not only supports cell presence but also encourages bone tissue regeneration. Additionally, existing literature supports boron's role in enhancing osteogenic differentiation and bone matrix formation. Such multifunctional biomaterials represent an important evolution in scaffold design, moving beyond passive frameworks to therapeutically active constructs that can improve healing outcomes.

6. CONCLUSION

In this study chitosan–sodium pentaborate pentahydrate (SPP) composite scaffolds with enhanced functionality for bone tissue engineering were developed, characterized and investigated for their cell supportive properties. SPP incorporation improved scaffold degradation under enzymatic conditions, reduced swelling in a concentration-dependent manner, and imparted dose-dependent antifungal activity against *Aspergillus niger* and *Candida albicans*. NaOH-stabilized scaffolds also promoted higher MC3T3-E1 osteoblast viability, confirming good cytocompatibility. These findings highlight SPP's dual role as a degradation enhancer and antifungal agent without compromising cell compatibility.

The biocompatibility and performance of medical-grade chitosan (MG Ch) scaffolds incorporated with %0.5 and %0.75 (w/w) SPP were evaluated through multiple assays, including resazurin analysis, SEM imaging, swelling behavior, and DAPI staining. Resazurin assay results demonstrated a general decrease in metabolic activity by Day 10 across all scaffold groups however MG Ch – 0.5 SPP group showed the highest cell viability when compared to other groups ($p < 0.05$). Interestingly, DAPI staining images showed an increased number of nuclei on Day 10 compared to Day 1 in all scaffold groups. This indicates cell proliferation and attachment, which contrasts with the reduced metabolic activity reported by the resazurin assay. This may be attributed to the decreased metabolic activity of the cells due to environmental stress, contact inhibition, or nutrient limitation. In conclusion, freeze dried and NaOH stabilized medical grade chitosan scaffolds containing SPP are cytocompatible and may be an alternative biomaterial for bone regeneration studies with their good mechanical and antifungal properties.

7. FUTURE WORKS

The findings of this study have shown that SPP-incorporated chitosan scaffolds offer promising characteristics for bone tissue engineering, especially in terms of biocompatibility, porosity, and cell support. However, to build on these results and better understand the full potential of these scaffolds, several areas of future research are recommended. One important next step is to carry out long-term in vitro studies to provide a clearer picture of how cells

interact with the scaffolds over extended periods—beyond the 10 days explored in this study. This is especially important for understanding how stable and supportive the material remains over time. It would also be valuable to explore the differentiation potential of the scaffolds in more detail. Specifically, future work could focus on how well the scaffolds promote osteogenic and / or chondrogenic differentiation by looking at relevant gene and protein expression markers such as osteocalcin, collagen types I and II, and FABP4 and using stem cells. SPP incorporation added antifungal properties to the chitosan scaffolds. Although SPP is an antibacterial agent it did not show antibacterial properties in chitosan scaffolds. The maximum cell supporting supportive SPP dosage in chitosan scaffolds can be determined in such a way that it can show antibacterial properties or this effect could be enhanced using different antibacterial agents in composite scaffolds. In addition to bone tissue engineering, it can be suggested that these scaffolds can be used in different tissue engineering fields where antifungal properties are also effective. In vivo animal experiments can be conducted to evaluate how well these scaffolds perform in a living system. This would allow evaluation of their ability to support bone healing, how they integrate with surrounding tissues, how they degrade over time, and whether they trigger any unwanted immune responses. Lastly, further improvements to scaffold design—such as refining SPP concentration or applying advanced fabrication methods like 3D bioprinting—could help enhance cell distribution, mechanical strength, and biological performance of the scaffolds.

References

- Abdik, E. A., Abdik, H., Taşlı, P. N., Deniz, A. A. H., & Şahin, F. (2019). Suppressive Role of Boron on Adipogenic Differentiation and Fat Deposition in Human Mesenchymal Stem Cells. *Biological Trace Element Research*, 188(2), 384–392.
<https://doi.org/10.1007/s12011-018-1428-5>
- Abdik, H., Cumbul, A., Hayal, T. B., Avşar Abdik, E., Taşlı, P. N., Kırbaş, O. K., Baban, D., & Şahin, F. (2021). Sodium Pentaborate Pentahydrate ameliorates lipid accumulation and pathological damage caused by high fat diet induced obesity in BALB/c mice. *Journal of Trace Elements in Medicine and Biology*, 66, 126736.
<https://doi.org/https://doi.org/10.1016/j.jtemb.2021.126736>

- Amini, A. R., Laurencin, C. T., & Nukavarapu, S. P. (2012). Bone tissue engineering: Recent advances and challenges. *Critical Reviews in Biomedical Engineering*, 40(5), 363–408. <https://doi.org/10.1615/CritRevBiomedEng.v40.i5.10>
- Ansari, S., Ito, K., & Hofmann, S. (2022). Alkaline Phosphatase Activity of Serum Affects Osteogenic Differentiation Cultures. *ACS Omega*, 7(15), 12724–12733. <https://doi.org/10.1021/acsomega.1c07225>
- Aravamudhan, A., M. Ramos, D., Nip, J., Subramanian, A., James, R., D. Harmon, M., Yu, X., & G. Kumbar, S. (2013). Osteoinductive Small Molecules: Growth Factor Alternatives for Bone Tissue Engineering. *Current Pharmaceutical Design*, 19(19), 3420–3428. <https://doi.org/10.2174/1381612811319190008>
- Aydın, S., Demirci, S., Sa, D., Ka, E., & Fikretin, Ş. (2019). Boron containing compounds promote the survival and the maintenance of pancreatic β -cells. 5465–5478.
- Brougham, C. M., Levingstone, T. J., Shen, N., Cooney, G. M., Jockenhoevel, S., Flanagan, T. C., & O'Brien, F. J. (2017). Freeze-Drying as a Novel Biofabrication Method for Achieving a Controlled Microarchitecture within Large, Complex Natural Biomaterial Scaffolds. *Advanced Healthcare Materials*, 6(21). <https://doi.org/10.1002/adhm.201700598>
- Çapkin Yurtsever, M., Akdere, Ö. E., & Gümüşderelioğlu, M. (2022). Scaffolds from medical grade chitosan: A good choice for 3D cultivation of mesenchymal stem cells. *Turkish Journal of Biology = Turk Biyoloji Dergisi*, 46(6), 475–487. <https://doi.org/10.55730/1300-0152.2633>
- Cebeci, E., & Fikretin, S. (2022). *Journal of Trace Elements in Medicine and Biology Anti-cancer effect of boron derivatives on small-cell lung cancer*. 70(January).
- Chen, X., & Pei, Y. (2016). Effects of sodium pentaborate pentahydrate exposure on *Chlorella vulgaris* growth, chlorophyll content, and enzyme activities. *Ecotoxicology and Environmental Safety*, 132, 353–359. <https://doi.org/10.1016/j.ecoenv.2016.06.024>
- Collins, M. N., Ren, G., Young, K., Pina, S., Reis, R. L., & Oliveira, J. M. (2021). Scaffold Fabrication Technologies and Structure/Function Properties in Bone Tissue Engineering.

Advanced Functional Materials, 31(21). <https://doi.org/10.1002/adfm.202010609>

Czekańska, E. M. (2014). *In vitro cell and culture models for osteoblasts and their progenitors* By Supervised by. April.

Czekanska, E. M., Stoddart, M. J., Richards, R. G., & Hayes, J. S. (2012). In search of an osteoblast cell model for in vitro research. *European Cells and Materials*, 24, 1–17. <https://doi.org/10.22203/eCM.v024a01>

Deb, P., Deoghare, A. B., Borah, A., Barua, E., & Das Lala, S. (2018). Scaffold Development Using Biomaterials: A Review. *Materials Today: Proceedings*, 5(5), 12909–12919. <https://doi.org/10.1016/j.matpr.2018.02.276>

Demirci, S. (2016). *Boron promotes streptozotocin-induced diabetic wound healing : roles in cell proliferation and migration , growth factor expression , and inflammation*. 119–133. <https://doi.org/10.1007/s11010-016-2719-9>

Demirci, S., Doğan, A., Şişli, B., & Şahin, F. (2014). Boron increases the cell viability of mesenchymal stem cells after long-term cryopreservation. *Cryobiology*, 68(1), 139–146. <https://doi.org/10.1016/j.cryobiol.2014.01.010>

Demirci, S., Karaku, E., Hal, Z., & Topçu, A. (2015). *Boron and Poloxamer (F68 and F127) Containing Hydrogel Formulation for Burn Wound Healing*. 169–180. <https://doi.org/10.1007/s12011-015-0338-z>

Doğan, A., Demirci, S., Apdik, H., Bayrak, O. F., Gulluoglu, S., Tuysuz, E. C., Gusev, O., Rizvanov, A. A., Nikerel, E., & Şahin, F. (2017). A new hope for obesity management: Boron inhibits adipogenesis in progenitor cells through the Wnt/ β -catenin pathway. *Metabolism: Clinical and Experimental*, 69, 130–142. <https://doi.org/10.1016/j.metabol.2017.01.021>

Doğan, A., Demirci, S., Bayir, Y., Halici, Z., Karakus, E., Aydin, A., Cadirci, E., Albayrak, A., Demirci, E., Karaman, A., Ayan, A. K., Gundogdu, C., & Şahin, F. (2014). Boron containing poly-(lactide-co-glycolide) (PLGA) scaffolds for bone tissue engineering. *Materials Science and Engineering C*, 44, 246–253. <https://doi.org/10.1016/j.msec.2014.08.035>

- Echeverria Molina, M. I., Malollari, K. G., & Komvopoulos, K. (2021). Design Challenges in Polymeric Scaffolds for Tissue Engineering. *Frontiers in Bioengineering and Biotechnology*, 9(June), 1–29. <https://doi.org/10.3389/fbioe.2021.617141>
- Ek, Z. G. E., & Prof, A. Y. (2013). *ĞSKELELERĞNĞN GELĞğTĞRĞLMESĞ VE KEMĞK DOKU MÜHENDĞSLĞĞĞNDEKĞ ETKĞNLĞKLERĞNĞN ARAğTIRILMASI DEVELOPMENT OF BORON CONTAINING TISSUE SCAFFOLDS BY BIOMIMETIC METHOD AND INVESTIGATION OF THEIR EFFECTIVENESS IN BONE TISSUE ENGINEERING.*
- Eltom, A., Zhong, G., & Muhammad, A. (2019). Scaffold Techniques and Designs in Tissue Engineering Functions and Purposes: A Review. *Advances in Materials Science and Engineering*, 2019. <https://doi.org/10.1155/2019/3429527>
- Fereshteh, Z. (2018). Freeze-drying technologies for 3D scaffold engineering. In *Functional 3D Tissue Engineering Scaffolds: Materials, Technologies, and Applications*. Elsevier Ltd. <https://doi.org/10.1016/B978-0-08-100979-6.00007-0>
- Fikretin, S. (2024). *Heliyon The efficacy of topical sodium pentaborate formulation on hemorrhoid disease : A randomized , double-blind ,. 10*(February).
- Goonoo, N., & Bhaw-Luximon, A. (2019). Mimicking growth factors: Role of small molecule scaffold additives in promoting tissue regeneration and repair. *RSC Advances*, 9(32), 18124–18146. <https://doi.org/10.1039/c9ra02765c>
- Gültan, T., Yurtsever, M. Ç., & Gümüşderelioğlu, M. (2020). NaOH-etched/boron-doped nanohydroxyapatite-coated PEEK implants enhance the proliferation and differentiation of osteogenic cells. *Biomedical Materials (Bristol, England)*, 15(3), 35019. <https://doi.org/10.1088/1748-605X/ab7198>
- Gumusderelioglu, M., Tunçay, E. T., Kaynak, G., Demirtaş, T. T., Aydın, S. T., & Hakki, S. S. (2015). Encapsulated boron as an osteoinductive agent for bone scaffolds. *Journal of Trace Elements in Medicine and Biology*, 31, 120–128. <https://doi.org/10.1016/j.jtemb.2015.03.008>
- Henkel, J., Woodruff, M. A., Epari, D. R., Steck, R., Glatt, V., Dickinson, I. C., Choong, P. F.

- M., Schuetz, M. A., & Hutmacher, D. W. (2013). Bone Regeneration Based on Tissue Engineering Conceptions-A 21st Century Perspective. *Bone Research*, 1, 216–248. <https://doi.org/10.4248/BR201303002>
- Hutmacher, D. W. (2001). Scaffold design and fabrication technologies for engineering tissues - State of the art and future perspectives. *Journal of Biomaterials Science, Polymer Edition*, 12(1), 107–124. <https://doi.org/10.1163/156856201744489>
- Inan, E., Demir, B., Cicek, D., Sahin, K., Tuzcu, M., Orhan, C., Calik, I., & Sahin, F. (2022). *Journal of Trace Elements in Medicine and Biology Sodium pentaborate pentahydrate promotes hair growth through the Wnt / β -catenin pathway and growth factors.* 73(March), 0–2.
- Iyigundogdu, Z., Basar, B., Couvreur, R., Tamrakar, S., Yoon, J., Ersoy, O. G., Sahin, F., Mielewski, D., & Kiziltas, A. (2022). *Thermoplastic elastomers containing antimicrobial and antiviral additives for mobility applications.* 242(April), 1–8.
- Iyigundogdu, Z. U., & Demir, O. (2017). *Developing Novel Antimicrobial and Antiviral Textile Products.* 1155–1166. <https://doi.org/10.1007/s12010-016-2275-5>
- Kennedy, S. W., Roy Choudhury, N., & Parthasarathy, R. (2023). 3D printing soft tissue scaffolds using Poly(caprolactone). *Bioprinting*, 30, e00259. <https://doi.org/https://doi.org/10.1016/j.bprint.2023.e00259>
- Kong, M., Chen, X. G., Xing, K., & Park, H. J. (2010). Antimicrobial properties of chitosan and mode of action: A state of the art review. *International Journal of Food Microbiology*, 144(1), 51–63. <https://doi.org/10.1016/j.ijfoodmicro.2010.09.012>
- Maia, F. R., Bastos, A. R., Oliveira, J. M., Correlo, V. M., & Reis, R. L. (2022). Recent approaches towards bone tissue engineering. *Bone*, 154(June 2021), 116256. <https://doi.org/10.1016/j.bone.2021.116256>
- Manzini, B. M., Machado, L. M. R., Noritomi, P. Y., & da Silva, J. V. L. (2021). Advances in Bone tissue engineering: A fundamental review. *Journal of Biosciences*, 46(1). <https://doi.org/10.1007/s12038-020-00122-6>
- Marolt, D., Knezevic, M., & Novakovic, G. V. (2010). Bone tissue engineering with human

- stem cells. *Stem Cell Research and Therapy*, 1(2). <https://doi.org/10.1186/scrt10>
- Mokkapati, V. R. S. S., Tasli, N. P., Khan, Z., Tufani, A., & Pandit, S. (2016). *RSC Advances enhanced cell viability and stem cell properties of*. 56159–56165. <https://doi.org/10.1039/c6ra07414f>
- Montanheiro, T. L. D. A., Schatkoski, V. M., de Menezes, B. R. C., Pereira, R. M., Ribas, R. G., de Freitas, A. de S. M., Lemes, A. P., Fernandes, M. H. F. V., & Thim, G. P. (2022). Recent progress on polymer scaffolds production: Methods, main results, advantages and disadvantages. *Express Polymer Letters*, 16(2), 197–219. <https://doi.org/10.3144/EXPRESSPOLYMLET.2022.16>
- Nielsen, F. H. (2014). Update on human health effects of boron. *Journal of Trace Elements in Medicine and Biology*, 28(4), 383–387. <https://doi.org/https://doi.org/10.1016/j.jtemb.2014.06.023>
- Omeroglu Ulu, Z., Degirmenci, N. S., Bolat, Z. B., & Sahin, F. (2023). Synergistic anti-cancer effect of sodium pentaborate pentahydrate, curcumin and piperine on hepatocellular carcinoma cells. *Scientific Reports*, 13(1), 14404. <https://doi.org/10.1038/s41598-023-40809-y>
- Oryan, A., Alidadi, S., & Moshiri, A. (2013). Current concerns regarding healing of bone defects. *Hard Tissue*, 2(2), 1–12. <https://doi.org/10.13172/2050-2303-2-2-374>
- Oryan, A., & Sahvieh, S. (2017). Effectiveness of chitosan scaffold in skin, bone and cartilage healing. *International Journal of Biological Macromolecules*, 104, 1003–1011. <https://doi.org/10.1016/j.ijbiomac.2017.06.124>
- Pazarçeviren, A. E., Tezcaner, A., Keskin, D., Kolukisa, S. T., Sürdem, S., & Evis, Z. (2021). Boron-doped Biphasic Hydroxyapatite/ β -Tricalcium Phosphate for Bone Tissue Engineering. *Biological Trace Element Research*, 199(3), 968–980. <https://doi.org/10.1007/s12011-020-02230-8>
- Safari, B., Aghanejad, A., Roshangar, L., & Davaran, S. (2021). Osteogenic effects of the bioactive small molecules and minerals in the scaffold-based bone tissue engineering. *Colloids and Surfaces B: Biointerfaces*, 198(October), 111462.

<https://doi.org/10.1016/j.colsurfb.2020.111462>

Şahin, E., Orhan, C., Erten, F., Şahin, F., Şahin, N., & Şahin, K. (2023). The effect of different boron compounds on nutrient digestibility, intestinal nutrient transporters, and liver lipid metabolism. *Turkish Journal of Medical Sciences*, 53(3), 619–629.

<https://doi.org/10.55730/1300-0144.5624>

Schoenfeld, C. M., Lautenschlager, E. P., & Meyer, P. R. (1974). Mechanical properties of human cancellous bone in the femoral head. *Medical & Biological Engineering*, 12(3), 313–317. <https://doi.org/10.1007/BF02477797>

Şenkal, S., Burukçu, D., Hayal, T. B., Kiratli, B., Burcu, H., Sağraç, D., Asutay, A. B., Sümer, E., & Şahin, F. (2022). 3D CULTURE OF HaCaT KERATINOCYTE CELL LINE AS AN *in vitro* TOXICITY MODEL. 23(2), 211–220.

<https://doi.org/10.23902/trkjnat.1158811>

Seong, J. M., Kim, B. C., Park, J. H., Kwon, I. K., Mantalaris, A., & Hwang, Y. S. (2010). Stem cells in bone tissue engineering. *Biomedical Materials*, 5(6).

<https://doi.org/10.1088/1748-6041/5/6/062001>

Szpalski, C., Barbaro, M., Sagebin, F., & Warren, S. M. (2012). Bone tissue engineering: Current strategies and techniques-Part II: Cell types. *Tissue Engineering - Part B: Reviews*, 18(4), 258–269. <https://doi.org/10.1089/ten.teb.2011.0440>

Taşlı, P. N., Doğan, A., Demirci, S., & Şahin, F. (2013a). Boron enhances odontogenic and osteogenic differentiation of human tooth germ stem cells (hTGSCs) *in vitro*. *Biological Trace Element Research*, 153(1–3), 419–427. <https://doi.org/10.1007/s12011-013-9657-0>

Taşlı, P. N., Doğan, A., Demirci, S., & Şahin, F. (2013b). Boron enhances odontogenic and osteogenic differentiation of human tooth germ stem cells (hTGSCs) *in vitro*. *Biological Trace Element Research*, 153(1–3), 419–427. <https://doi.org/10.1007/s12011-013-9657-0>

Vacanti, C. (2006). The history of tissue engineering. *Journal of Cellular and Molecular Medicine*, 1(3), 569–576. <https://doi.org/10.2755/jcmm010.003.20>

- Verrier, S., Alini, M., Alsberg, E., Buchman, S. R., Kelly, D., Laschke, M. W., Menger, M. D., Murphy, W. L., Stegemann, J. P., Schütz, M., Miclau, T., Stoddart, M. J., & Evans, C. (2016). Tissue engineering and regenerative approaches to improving the healing of large bone defects. *European Cells and Materials*, 32(July), 87–110.
<https://doi.org/10.22203/eCM.v032a06>
- Xu, Y., Xia, D., Han, J., Yuan, S., Lin, H., & Zhao, C. (2017). Design and fabrication of porous chitosan scaffolds with tunable structures and mechanical properties. *Carbohydrate Polymers*, 177(May), 210–216.
<https://doi.org/10.1016/j.carbpol.2017.08.069>
- Xue, N., Ding, X., Huang, R., Jiang, R., Huang, H., Pan, X., Min, W., Chen, J., Duan, J. A., Liu, P., & Wang, Y. (2022). Bone Tissue Engineering in the Treatment of Bone Defects. *Pharmaceuticals*, 15(7), 1–24. <https://doi.org/10.3390/ph15070879>
- Yan, X. Z., Yang, W., Yang, F., Kersten-Niessen, M., Jansen, J. A., & Both, S. K. (2014). Effects of continuous passaging on mineralization of MC3T3-E1 cells with improved osteogenic culture protocol. *Tissue Engineering - Part C: Methods*, 20(3), 198–204.
<https://doi.org/10.1089/ten.tec.2012.0412>