T.C.
BAHCESEHIR UNIVERSITY
GRADUATE SCHOOL
DEPARTMENT OF TISSUE ENGINEERING AND REGENERATIVE MEDICINE (INTERDISCIPLINARY)

CREATING TWO DIFFERENT ARTIFICIAL BONE MODELS BY USING A SYNTHETIC POLYMER (POLY (LACTIC-CO-GLYCOLIC ACID) (PLGA)) AND NATURAL POLYMER (COLLAGEN) AS A SCAFFOLD FOR BETTER BONE CELL GROWTH EVALUATION

MASTER'S THESIS

GHAITH SULTAN AZEEZ

ISTANBUL 2024

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Institute Director

This thesis was read by us, quality and content as a Master's thesis has been seen and accepted as sufficient.

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Ghaith Sultan Azeez

Signature:…….

ABSTRACT

Creating two different artificial bone models by using a synthetic polymer (Poly (lactic-co-glycolic acid) (PLGA)) and natural polymer (collagen) as scaffold for better bone cell growth evaluation

Ghaith Sultan Azeez

Master's Program in Tissue Engineering and Regenerative Medicine

Supervisor: Dr. Öğr. Üyesi BIRCAN DINÇ

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These limits can further hinder the development of functional artificial bone models in scaffold-based bone tissue engineering. This paper attempts to address these issues by designing and evaluating two models of such bones: one is based on synthetic polymer, PLGA, while the other uses a natural polymer, collagen, as a scaffold for enhancing the development of bone cells. Among the primary goals are intricate scaffold fabrication and its characterization including biocompatibility studies, and evaluation of the scaffold's utility in regenerating bone tissue.

In order to achieve the above mentioned goals, PLGA-collagen nano-hydroxyapatite-chitosan scaffolds were fashioned applying a solvent casting/particulate leaching approach, for optimization 75% PLGA, 20% nHA and 5% CS were combined in several ratios. Differentiated Thermal Analysis and in particular the Dsc and Fourier Transform Infrared Spectroscopy were employed for Characterization. The biocompatibility of the scaffolds and interactions of the cell with scaffold materials were performed by growing L929 cells.

The hypothesis suggests that biodegradable polymer, PLGA, sustained drug delivery and enhanced biocompatibility of the synthetic polymer based scafolds compared to collagen based scaffolds towards cell growth. The effective incorporation of the components into both scaffold types was reported. Cell cultures showed better attachment and proliferation of cells on PLGA scaffolds compared to collagen scaffolds or even combinations of the two beams were less toxic to cells without the cross linker.

In general, the results also show that PLGA scaffolds can be considered as having favorable prospects for use in bone tissue engineering, suggesting that synthetic polymer scaffolds would be better than natural ones towards achieving bone regeneration. Other studies, particularly those using certain bone cell lines or in vivo techniques, will need to be carried out in order to further this knowledge and its clinical potential.

Key Words: Scaffolds, PLGA, Collagen, Biocompatibility, Bone regeneration

ÖZET

Daha iyi kemik hücresi büyümesi için sentetik polimer (Poli(laktik-ko-glikolik asit) (PLGA)) ve doğal polimer (kolajen) iskelesi kullanılarak iki farklı yapay kemik modelinin oluşturulması.

Ghaith Sultan Azeez

DOKU MÜHENDİSLİĞİ VE REJENERATİF TIP Yüksek Lisans Programı

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Bu sınırlamalar, iskele tabanlı kemik doku mühendisliğinde işlevsel yapay kemik modellerinin geliştirilmesini engelleyebilir. Bu tez, iki farklı kemik modeli tasarlayıp değerlendirerek bu sorunları ele almaktadır: biri sentetik polimer PLGA'ya dayanırken, diğeri doğal bir polimer olan kolajeni kullanmaktadır. Amaçlar arasında uygun iskele sentezi, biyouyumluluk çalışmaları ve iskelenin kemik dokusunu yenilemedeki etkilerinin değerlendirilmesi yer almaktadır.

Bu hedeflere ulaşmak için, PLGA-kolajen nano-hidroksiapatit-kitosan iskeleleri, %75 PLGA, %20 nHA ve %5 CS oranlarının birleştirildiği bir çözücü döküm/partikül süzme yöntemiyle şekillendirilmiştir. Karakterizasyon için Diferansiyel Taramalı Kalorimetre ve Fourier Dönüşümlü Kızılötesi Spektroskopisi kullanıldı. İskelelerin biyouyumluluğu, L929 hücrelerinde değerlendirildi.

Hipotez, biyolojik olarak parçalanabilir PLGA'nın, kollajen bazlı iskelelere kıyasla sürdürülebilir ilaç iletimi ve geliştirilmiş biyouyumluluk sunduğunu önermektedir. Araştırma, PLGA iskelelerinde hücrelerin daha iyi tutunduğunu ve çoğaldığını göstermiştir.

Sonuçlar, PLGA iskelelerinin kemik doku mühendisliğinde olumlu beklentilere sahip olduğunu ve sentetik polimer iskelelerinin kemik rejenerasyonunu sağlamada doğal olanlardan daha etkili olabileceğini göstermektedir. Bu bilgiyi daha ileri götürmek

için, belirli kemik hücre hatları veya in vivo teknikleri kullanan ek çalışmalar gerekmektedir.

Anahtar Kelimeler: İskeleler, PLGA, Kolajen, Biyouyumluluk, Kemik rejenerasyonu

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LIST OF ABBREVIATION

BMPs	Bone Morphogenetic Proteins
BMSSCs	Bone Marrow Stromal Stem Cells
BSA	Bovine Serum Albumin
Co-BS	Collagen-Based Scaffold
CS	Chitosan
DCM	Dichloromethane
DMEM	Dulbecco's Modified Eagle Medium
DMF	Dimethylformamide
DSC	Differential Scanning Calorimetry
ECM	Extracellular Matrix
FDA	Food and Drug Administration
FTIR	Fourier Transform Infrared Spectroscopy
GPa	Gigapascal
GF	Growth factors
IPN	interpenetrating network
nHA	nano-Hydroxyapatite
OB	Osteoblast Cells
PEM	Polyelectrolyte Multilayer
PBS	Phosphate Buffered Saline
PCL	Polycaprolactone
PEG	poly(ethylene glycol)
PVA	poly(vinyl liquor)
PLGA-BS	PLGA Based Scaffold
PLGA	Poly (lactic-co-glycolic acid)
PGA	Polyglycolic acid
PLA	Polylactic acid
PLGA/nHA/CS	Poly (lactic-co-glycolic acid)/nano-Hydroxyapatite/Chitosan
3D printing	3 dimensional printing
TE	Tissue Engineering
TEM	Transmission Electron Microscopy

Tg	Temperature of glass transition
TNF	Tumor Necrosis Factor
Tm	Temperature of melting
Tris-HCL	(hydroxymethyl) aminomethane (THAM) hydrochloride
OH	Hydroxyl group.
UV	Ultraviolet light
VEGF	Vascular Endothelial Growth Factors
WNTs	Wingless- related integration site
Xc	Crystallinity

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FIGURES

Chapter 1

Introduction

1.1 Theoretical Framework

The skeletal system is the main foundation of the body, providing protection and structural support for the organs and the body. The skeletal system consists of bones, cartilage, tendons, and ligaments. It also plays a major role in shape and support, in addition to several other functions, the most important of which is the formation of blood cells in the bone marrow. (William Morrison, 2018).

In addition to being the basic structure of the body, it is not considered a fixed frame, but rather a dynamic structure that facilitates the process of movement and flexibility, in addition to adapting to movement requirements such as expansion and contraction, as the complex balance between rigidity and flexibility in the skeletal system represents one of the most important basic matters for understanding the process of maintaining the body's vital functions. (Warren Andrew, 2024).

Bone is an essential and multifunctional organ with roles ranging from providing weight-bearing sustainment and assisting locomotion, to the generation of blood cells (hematopoiesis), physical protection of vital organs like the brain or heart, and storage of minerals and growth factors. Therefore, the development of effective bone tissue engineering strategies is of paramount importance (Filippi et al., 2020a), as a promising solution to the limitations related to traditional bone grafting procedures (Dimitriou et al., 2011) . Bones are composed of dense connective tissue that achieves several functions. It communicates with the body's mineral reserves and provides protection and support for delicate tissues (Florencio-Silva et al., 2015). It is possible to distinguish between the auxiliary bone and the basic bone from a microscopic point of view. The bone tissue that is necessary for embryonic development and for repairing fractures is known as essential bone. In contrast to the well-organized lamellar way of collagen inside the supporting bone, it is characterized by an uneven mien of tiny collagen fibers. In adults, supplementary bone tissue often replaces basic bone tissue, with the exception of a relatively small number of locations inside the body (such as inside the tendons' incorporations). To sum up, compared to helper bone tissue, basic

bone tissue has a lower mineral content and a better, higher, stronger degree of osteocytes. The assistant bone is made up of the trabecular bone, which has tall porosity ranging from 30% to 95%, and the cortical bone, which makes up 80% of the skeleton and is characterized by low porosity (5–30%) (Perić Kačarević et al., 2020) (Morgan et al., 2018) . The osteon, also known as the Haversian system, is the valuable unit of the created bone. Its center canal, known as the Haversian canal, is where the blood vessels are gathered. Its form is empty and round. Haversian system dividers consist of concentric lamellae. In cortical and trabecular bone, lamellae are arranged in parallel totals or proliferate randomly. Cells and intercellular material make up lamellae. The last specified is made up of mineralized layers that interject into the typical ones, so acting as a cushioning agent; 77% of the general cross-section is made up of the inorganic division, which is composed of calcium carbonate (10%) and calcium phosphate (90%). The normal division, also known as the "osteoid," is made up mostly of collagen type I (the first of the approximately 29 types of collagens found in the human body) fibers that are 90% evacuated in an ambiguous grid, along with a few additional proteins. Bone is composed of 36% inorganic and 36% characteristic components by volume, and 28% water. Even while common fibers provide strength, the inorganic division ensures that the bone is hard. In step, the water substance gives the bone its viscoelasticity (Monesi & Adamo, 1975) (Mohamed, 2008) .

1.1.1 Cellular component. Osteoblasts, osteoclasts, bone lining cells, and osteocytes are the four different cell types that are found in bone**.**

The primary three cell types are situated on the surface of bones and arise from nearby mesenchymal progenitor cells. Osteocytes penetrate the inside of the bone by the combination of precursor cells from the bone and mononuclear blood (Mohamed, 2008).

Mesenchymal cells divided into osteoprogenitor cells during the organization of bone tissue, which subsequently separated into osteoblasts. These last-mentioned cells must ultimately be osteocytes since they include distinctive elements of the bone grid (Monesi & Adamo, 1975). Osteocytes are the first cells that make up the majority of bones (90–95%) and are distinguished by having a life expectancy of up to 25 years. They are located within calcified grids, contained within internal lacunae, and their shape is tissue-specific subordinate:

Compared to those from trabecular bone, osteocytes from cortical bone have a longer morphology (Florencio-Silva et al., 2015). Osteocytes function as mechanosensory coordinating the osteoblasts and osteoclasts in bone remodeling because of their ability to detect mechanical weight and stack (Florencio-Silva et al., 2015). The progenitors of osteoclasts, which are polynucleate cell types that are derived from hematopoietic stem cells, are related to monocyte macrophages. The task of osteoclasts is to maintain the cross-section of bones (Monesi & Adamo, 1975). The three main phases of this preparation are the enzymatic absorption of the natural structure, the destructive breakdown of the mineral framework, and the attachment of the osteoclast to the grid. Finally, the smooth, flat cells that line the surfaces of bones are called osteoblasts. Even if their exact functions are yet unknown, it is known that they are a part of the osteoclast partition and function to prevent the coordinated interaction between the osteoclasts and the bone system (Florencio-Silva et al., 2015).

1.1.2 Bone mechanical properties .In common, bone tissue carries on as an anisotropic texture, characterized by the flexible modulus of 18 GPa in case of significant oblige application, 12 GPa in transverse stack condition and, at long final, reacts with because it were 3.3 GPa to shear extend (Karpiński et al., 2017). In expansion, bone mechanical reaction changes as well concurring to the versus of the solicitation, appearing a compressive strength that ranges from 12.56 to 16.89 kg/mm2, and on a mouldable strength of 10–12 kg/mm2 (Karpiński et al., 2017). The specific bone mechanical properties are essentially affected by both the inorganic/organic arrange extent, careful for hardness and elasticity, and the cortical/trabecular one (Karpiński et al., 2017). In truth, in show disdain toward of the closeness in terms of materials and morphological highlights between the cortical and trabecular bone, they have unique mechanical qualities because of the differences in porosity. To be more precise, the trabecular bone strength is one or two orchestrates of significance less (2 to 12 MPa), whereas the cortical bone is marked by towering compressive quality (100–230 MPa) (Perić Kačarević et al., 2020) (Karpiński et al., 2017). Conversely, the trabecular bone exhibits a tall imperative capacity potential, which is reflected in increases in length up to a significance level higher than the cortical one (50% vs. 2%) (Karpiński et al., 2017) .

1.1.3 Homeostasis. The bone constantly changes throughout life, demonstrating contempt for the fact that adult bone changes at a somewhat slower rate. These forms are susceptible to homeostasis which is regulated by both metabolism and mechanics. This homeostasis maintains the mechanical capacities and synchronizes the calcium concentration within the plasma. Osteoblasts evacuate unused bone when an external force results in a strain state greater than 2500μ strain (0.25formation). Conversely, bones are unable to heal themselves in cases of well-known strain (5 cm) absconds (such as non-union breaks, tumor ablations, maxillofacial damage or degeneration), necessitating the use of reconstructive techniques and cellular medications (McEwan et al., 2018) (Mehta et al., 2012).

1.1.4 Bone tissue pathologies and customary treatments. In development to the damage, other sicknesses influence the bone tissue such as Tumors, infections, osteopetrosis, pseudoarthrosis, osteoporosis, and others (Morgan et al., 2018) (McEwan et al., 2018) (Zanker & Duque, 2019) (Catanzano Jr & Fitch, 2018). Standard medications for osteoporosis and osteopetrosis consolidate both non-pharmacological and pharmacological approaches. Non-pharmacological rules join fitting calcium and vitamin D confirmations, weight-bearing workouts, smoking cessation, obstacle of alcohol/caffeine utilization, and fall-preventing procedures (Tu et al., 2018). Instep, the pharmacological approach incorporates antiresorptive drugs and anabolic solutions pointing at the same time reducing the bone reabsorption and progressing bone course of action, independently. In any case, antiresorptive masters and hormones organization are the cause of cardiovascular, intestinal, renal, and urinary system side impacts. In this setting, long-term drug-delivery materials may talk to an elective method to ensure adjacent release of such arrangements. Other than, in case of breaks, major degree bone surrenders or pathologies requiring bone surgical resection (i.e., osteosarcoma, chondrosarcoma) are ordinarily treated with outside obsession, metallic prostheses or bone unites (Rajani & Gibbs, 2012) (Oryan et al., 2014) . All things considered, many impediments related to extending ensuring circumstances, sensitivities, septic and aseptic mobilization, periprosthetic osteolysis, assistant, and shortcoming subsidence make scaffold-based regenerative pharmaceutical one of the preeminent promising strategies (Asnaghi et al., 2011) . In this circumstance, the nonstop increment in bone-related sicknesses due to people

developing talks to an additional boost in making made stages able to substitute the physiological ones (autograft, allograft), whose availability is limited.

Furthermore, a group of Researchers conducted a comprehensive systematic review and meta-analysis to investigate the prevalence of osteoporosis worldwide. Following methodological approaches, 86 papers were selected for the meta-analysis, with 103,334,579 people in the age group of 15 to 105 years making up the study sample size. According to the study, the prevalence of osteoporosis is 18.3% worldwide, which represents about 1.5 million osteoporosis-related fractures every year. The results also showed that women are more susceptible to osteoporosis than men, as the incidence of osteoporosis in women reached 23.1%. Compared to 11.7% for men. The study concluded that giving significant appreciation to this problem is necessary and that a robust and comprehensive analysis of the global osteoporosis epidemic is needed to help develop policies and plan for the health system, as well as to ensure that those who need it receive the necessary care to reduce the significant risks that lead to mortality. associated with fractures, and monitoring the incidence of osteoporosis around the world (Salari et al., 2021).

1.2 Importance of Bone Tissue Engineering

There is no doubt that the field of tissue engineering is one of the most developing and advanced fields in recent years, especially in the field of bone development, as these studies are based on evaluating the regeneration of cells of bone tissues. The field of bone tissue engineering is one of the fields that has witnessed remarkable development in order to produce new and effective strategies to treat damaged bone tissue, whether through replacement or repair.

Due to their extraordinary significance, conventional bone uniting strategies, counting autografts, allografts, and xenografts, have been considered the most common choice within the treatment of bone distortions and wounds in common. In any case, these strategies come with noteworthy confinements that influence their adequacy and appropriateness. Indeed autografts, considered the perfect because of their osteoconductive, osteogenic, and osteogenic properties, are restricted by giver location horribleness and the restricted sum of accessible giver tissue. On the other hand, allografts and xenografts, whereas dodging horribleness at the benefactor location, posture dangers of cross-infection, illnesses, resistant responses, and

modified resorption rates result in need of compatibility with the host bone (Bhattarai et al., 2010) (Dimitriou et al., 2011).

Enhancing modern and present-day procedures within the field of bone tissue designing can put a conclusion to the confinements, as these advancements point to create biomimetic materials that have the capacity to improve the bone regeneration process without the restrictions related with conventional joining strategies. One of the foremost imperative key developments in this field is the utilization of scaffolds that are profoundly biocompatible, development variables, and stem cells. Tissue-building approaches also look for to form a reasonable environment for the bone recovery preparation, which gives the plausibility of treating bone abandons of different sorts that speak to a major challenge for traditional joins (Hollister, 2005).

1.3 Potential of Tissue Engineering to Revolutionize Bone Regeneration and Repair Processes

Bone tissue engineering is considered one of the promising areas for revolutionizing the recovery and repair of bone tissue, as this is often done through the development and advancement of biomaterials and unused biotechnologies. The utilization of scaffolds or Hydrogels made of engineered polymers such as poly (lactic-co-glycolic acid) (PLGA) or normal polymers such as collagen provides an environment that mimics the environment within the extracellular matrix of bone, upgrading biocompatibility forms such as cellular separation, cellular expansion, and cellular connection (Schantz et al., 2003) , (Lienemann et al., 2012) .

Furthermore, The improvements taking put within the field of tissue and bone engineering including the method of including development variables to scaffolds and hydrogels, and vital development components (BMPs) are critical, as tests have demonstrated its capacity to improve the stimulatory properties of designing structures and works on the separation of forebear cells into bone cells and is considered a major catalyst within the arrangement prepare Bone (Bessa et al., 2008).

Integrating stem cells, particularly mesenchymal stem cells (MSCs), into tissue-engineered builds encourages increases in the regenerative potential by giving a source of cells capable of osteogenic separation (Pittenger et al., 1999) .

Progressions in creation advances like 3D printing have empowered the creation of exceedingly customized and complex platform geometries that closely reproduce

the local engineering of bone tissue. This customization guarantees that built bone builds can fit absolutely into deformity locales, encouraging integration with encompassing bone and improving useful reclamation (Datta et al., 2017).

1.3.1 Hydrogel and techniques in bone tissue engineering.

1.3.1.1 Overview of Hydrogel. Hydrogels are three-dimensional network-structured materials made up of hydrophilic polymers that empower them to retain and hold huge amounts of fluid. This unique include gives hydrogels closeness to normal soft tissues, making them key materials for a wide extend of applications in different areas, particularly within the field of tissue engineering (Agrawal & Hussain, 2023).

Hydrogels are classified into three categories based on their constituent materials. The choice of hydrogel fabric significantly influences its biological properties, such as biocompatibility and mechanical properties of the ultimate structure. Analysts have to carefully consider these variables when selecting a hydrogel fabric for their particular application to guarantee ideal execution and crave comes about. It is worth noticing here that hydrogels can be classified based on crosslinking strategies as well. On the premise of composition, hydrogels can be normal or synthetic engineered hydrogels (Agrawal & Hussain, 2023).

1.3.1.2 Role of Hydrogels in Bone Regeneration. Hydrogels' intriguing characteristics, which mimic those of the extracellular matrix (ECM), have made them a crucial element in bone tissue creation. Because of their ability to successfully deliver cells and bioactive chemicals, as well as their biocompatibility and tuneable mechanical properties, these water-containing polymers can provide an environment that is favourable for cell expansion and separation. Hydrogels can be derived from synthetic polymers like poly (ethylene glycol) (PEG) and poly(vinyl liquor) (PVA), as well as from natural polymers like collagen, alginate, and hyaluronic acid. This allows for a broad range of applications in bone regeneration (Drury & Mooney, 2003; Lee & Mooney, 2001).

1.3.1.3 Advancements in Hydrogel Fabrication. *The functionality and utilization of hydrogel generation procedures in bone tissue engineering have been incredibly moved forward by later breakthroughs within the field. The mechanical qualities, flexibility, and stimulus-responsiveness of hydrogels have been improved by advancements including self-healing, interpenetrating network (IPN), and stimuli-responsive hydrogels. These characteristics are basic for building scaffolds that can support the mechanical strains set on bone structures and discharge development components and other bioactive substances in a regulated manner to help with the patching of broken bones (In & Türkei, 2017; Kopeček, 2007).*

1.3.1.4 Integration with Other Technologies. To progress bone recovery, hydrogels are being utilized with other tissue engineering innovations increasingly. As an example, complex, bespoke structures that absolutely coordinate the issue location can be made by combining hydrogel scaffolds with the utilization of 3D printing. Moreover, including nanoparticles or nanostructured materials in hydrogels has illustrated the potential for improving their osteoconductivity, mechanical quality, and restorative adequacy. These crossbreed materials can combine the focal points of hydrogels with the moved-forward usefulness of nanoparticles to create a synergistic impact (Shin et al., 2013).

1.3.1.5 Clinical Implications. The progressions in hydrogel strategies hold critical clinical suggestions for bone tissue design. The capacity to form more strong, useful, and customizable scaffolds can lead to progressed results for bone regeneration applications. Hydrogels that can successfully deliver stem cells, development components, and other regenerative operators specifically to the bone deformity location offer a promising approach to quickening bone repair and recovery in patients, possibly lessening recuperation times and progressing the quality of life for those influenced by bone wounds or diseases (Park, 2011).

(Qiu et al., 2020) study, they produced and characterized a hydrogel determined from porcine decellularized periosteum. It advanced macrophage chemotaxis and M2 polarization related to useful bone remodelling and did not trigger antagonistic resistant responses. It moreover advanced the arrangement and relocation of blood vessels, osteogenic separation, and bone biomineralization. Polyelectrolyte Multilayer

(PEM) hydrogels take part powerfully in all stages of the bone break repair handle and advanced bone recovery in vivo. Collectively, their comes about recommended that periosteal extracellular matrix (PEM) hydrogels are promising biomaterials for bone deformity repair.

1.3.2 Scaffolds and techniques in bone tissue engineering.

1.3.2.1 Overview of Scaffolds. An artificial, temporary platform used to support, fix, or improve a structure's performance is referred to as a "scaffold." Depending on the form and purpose, this can be done on different length and size scales with different kinds of support. To investigate cell–biomaterial interactions, two-dimensional studies of biomaterial substrates are typically conducted. Nonetheless, the scaffold is required to replace the deficiency or create a three-dimensional model of the organs or tissue structures in order to guarantee the functions of the injured tissues (Olivares & Lacroix, 2012). Mechanical properties, pore size, porosity, dimensionality, biocompatibility, and biodegradability (Olivares & Lacroix, 2012). The main design parameters for the scaffold include biocompatibility, biodegradability, mechanical properties, pore osteoinductivity, osteoconductivity, osteogenesis, and osteointegration. Porosity is also considered in this process (Giannitelli et al., 2014)(Prasadh & Wong, 2018).

Some basics of scaffolds used in tissue engineering are important factors in scaffold design. Once integrated into the body, the scaffold must strive to become a reliable tool for tissue engineering. When a scaffold is used in the body, it must be a structure responsible for cell adhesion, proliferation, and differentiation a biomechanical environment necessary for coordinated tissue regeneration; The layer allows and creates the biomechanical environment necessary for the regeneration and coordinated distribution of nutrients and oxygen, enables the encapsulation of cells and tissues, allows for the delivery of nutrients and oxygen, and allows for the release of cells containing growth factors (Morouço et al., 2016)**.**

1.3.2.2 Importance of Scaffolds in Bone Regeneration. Scaffolds are fundamental to bone tissue engineering, serving as the framework upon which new bone tissue can form. They not only support the physical structure of the developing tissue but also influence cellular behaviour, including adhesion, proliferation, and differentiation. The ideal scaffold should be biocompatible, osteoconductive, possess suitable mechanical properties, and be biodegradable at a rate matching new tissue formation. Materials commonly used for scaffolds include polymers (both natural like collagen, and chitosan, and synthetic like PLGA, and PCL), ceramics (hydroxyapatite, tricalcium phosphate), and composites combining the benefits of different materials (Hutmacher, 2000) (Rezwan et al., 2006).

1.3.2.3 Advanced Fabrication Techniques. Recent advancements in scaffold fabrication techniques have greatly expanded the possibilities for creating structures that closely mimic the natural bone matrix. These include:

3D Printing (Additive Manufacturing): This technique allows for the precise fabrication of complex, patient-specific scaffold geometries directly from digital models. It enables the creation of scaffolds with customized shapes, sizes, and porosities to fit specific defect sites, significantly enhancing the integration and regeneration of bone tissue (Hollister, 2005) .

Electrospinning: Producing fibrous scaffolds with ultrafine fibers, electrospinning can create structures that closely resemble the extracellular matrix of bone tissue. These fibrous scaffolds provide a high surface area for cell attachment and proliferation and can be engineered to deliver growth factors or other bioactive molecules gradually (Lienemann et al., 2012) .

Sol-gel Techniques: Used primarily with ceramic materials, sol-gel processes can produce highly porous and bioactive scaffolds that support the attachment and growth of osteoblasts. These scaffolds are particularly useful in applications requiring high mechanical strength and osteoconductivity (Jones, 2013) .

Solvent casting / particular leaching: The scaffold product process entails dissolving a polymer in a detergent to form a polymer result, which is latterly poured into a Mold using the solvent casting/ particulate leaching method. Particulate filtering is an approach that increases the porosity of the scaffold by adding porogens, similar as swab patches, to the polymer result. After the solvent evaporates, the scaffold

becomes solid and the porogens are removed by immersing the scaffold in water or another solvent, performing in the formation of a porous structure. The application of this technique enables the regulation of the confines and arrangement of pores, a critical factor for the growth of tissue and the dissipation of nutrients (R. Zhang & Ma, 1999b).

1.3.2.4 Scaffold Degradation and Remodelling. The development of scaffolds with controllable degradation rates matching the rate of new bone formation is essential for successful bone regeneration. This ensures that the scaffold provides temporary support while gradually transferring load-bearing responsibilities to the newly formed bone, facilitating its integration into the surrounding tissue (O'brien, 2011a).

These advancements in scaffold techniques underscore their pivotal role in bone tissue engineering, offering innovative solutions to enhance bone regeneration and repair processes.

1.4 Polymers used for Bone Tissue Engineering

1.4.1 Overview of PLGA as biomaterials in bone tissue engineering. PLGA is one of the polymers that has gotten incredible consideration within the field of bone tissue engineering because of its brilliant natural properties.

PLGA is an FDA-approved copolymer. It is utilized in a wide run of helpful fields for a few reasons, the most critical of which is its capacity to be biodegradable in expansion to its biocompatibility (Zhi et al., 2021)), because it does not cause any negative problems when transplanted interior the body of a living organism, and usually due to its compatibility with living tissues (Gentile et al., 2014a), in expansion to appearing the potential of Impressive as a carrier for medicate delivery and as a platform for tissue engineering, it has been broadly examined as a vehicle for the delivery of drugs, proteins and numerous other atoms such as DNA & RNA and peptides (Bouissou et al., 2006) (Jain, 2000a) (Ruhe et al., 2003).

As an engineered polymer, it is composed of lactic acid and glycolic acid polyesters, and is synthesized through numerous condensation responses of lactic corrosive and glycolic corrosive to create piece copolymers PLA and PGA (Gao et al., 2002) (Fukuzaki et al., 1989), or is synthesized through ring-opening polymerization

responses of lactide and glycolide. (Gilding & Reed, 1979) (Deasy et al., 1989) To fabricate it as a tall atomic-weight PLGA polymer, ring-opening polymerization forms can be utilized (Bendix, 1998) whereas the polycondensation preparation is more appropriate for the fabrication of moo atomic-weight polymers (Lunt, 1998) compared to homopolymers, PLGA polymer is favoured for the fabricate of bone substitution structures because it has the property of being able to change the proportion between its monomers and the proportion of these monomers can be balanced to suit the physical, chemical and natural properties of the polymer, making it a flexible fabric for different restorative applications, especially in tissue engineering. Osteoarthritis in this way gives a fabulous result with its hydrolysis property (Lanao et al., 2013).

1.4.1.2 Characteristics and features of PLGA to Bone Tissue Engineering. Biodegradability and biocompatibility: biocompatibility is the main reason for the widespread use of PLGA in bone tissue engineering. In addition, the biodegradability property is changeable, as PLGA decomposes into lactic acid and glycolic acid, which are obtained naturally in the body by Metabolic processes, thus avoiding the complications of reflexive immune reactions. This degradation process can be controlled precisely by changing the proportions of the compositions in the polymer, which facilitates the possibility of obtaining scaffold designs that are compatible with the dynamic nature of the healing processes (Jin et al., 2021) .

Mechanical properties: by precisely controlling the proportions of the polymer, we can obtain the required mechanical properties of PLGA to closely match those found in natural bone tissue and obtain the main factors supporting bone integration and regeneration. Scaffolds based on PLGA can be obtained with variable degrees of in terms of strength, rigidity and flexibility, which are necessary to withstand physiological loads (Gentile et al., 2014a) .

Manufacturing: PLGA polymer can be manufactured in more than one way, such as 3D printing, solvent casting, and electrospinning, which provides basic solutions to the problem of manufacturing scaffolds with complex shapes and structures. These scaffolds can be created with designs that have a highly porous structure, enhancing vital processes such as reproduction and differentiation. In addition to the possibility of modifying pores to facilitate food transport and waste disposal, providing an environment that mimics that of bone cells (Jin et al., 2021) .

Surface adjustment property: although PLGA contains moderately few ionic molecular groups compared to natural polymers, its surface can be effectively adjusted to progress its interaction with biological molecules and cells. This adjustment improves cell adhesion, proliferation, and differentiation on PLGA scaffolds. Methods such as plasma treatment, coating with natural polymers (e.g., collagen), or incorporation of bioactive molecules (e.g., growth factors) can essentially increment the bioactivity of PLGA-based materials, advancing osseointegration and bone tissue recovery (Gentile et al., 2014a) .

So, from over we can say PLGA stands out as a prime candidate for bone tissue engineering applications due to its biocompatibility, adjustable biodegradability, mechanical tunability, manufacture flexibility, and surface adjustment potential. These properties empower the plan of scaffolds that not as it were support but also effectively advance the recovery of bone tissue. As investigative advances and manufacturing technologies progress, PLGA-based biomaterials proceed to advance, advertising promising arrangements for bone repair and recovery.

1.4.1.2 The Applications of PLGA in Bone Tissue Engineering. PLGA could be a flexible fabric broadly utilized in bone tissue engineering applications. It serves as a foundational scaffold material, supporting cell attachment and multiplication for bone regeneration (Hines & Kaplan, 2013). PLGA's flexibility permits for the controlled delivery of drugs and development factors, quickening bone healing forms (Rezwan et al., 2006a) . By combining PLGA with inorganic materials, composite scaffolds with improved mechanical properties are made, advertising predominant support for bone tissue regeneration (Rezwan et al., 2006b) . Moreover, the customizable nature of PLGA enables custom fitted alterations to meet particular requirements, such as degradation rates and mechanical quality, in planning personalized scaffolds for diverse bone tissue engineering applications (Hines & Kaplan, 2013). Moreover, PLGA microspheres serve as viable carriers for delivering cells to focus on bone deformity destinations, encouraging cell-based treatments for proficient bone regeneration (Hines & Kaplan, 2013). These applications emphasize the critical part of PLGA in progressing the field of bone tissue engineering.

1.4.2 Overview of collagen as a biomaterial in bone tissue engineering. Collagen, of its types is considered the main structural component of mammalian bones, especially (Type I), as it constitutes the largest percentage of the organic components of the extracellular bone matrix, 90% With this percentage, it is the most abundant protein in the body and has It plays a major role in supporting tissues and structural integrity (Fan et al., 2023) . Studies have also shown the emergence of collagen as a promising vital material for the regeneration process. Bones are due to their compatibility with the body (Y. Li et al., 2021) .

1.4.2.1 Role of Collagen in Bone Tissue Engineering. Despite the critical structural commitment that collagen plays within the extracellular matrix, but its significance within the field of bone tissue building is much more prominent. As the essential component of the bone matrix, collagen type I serves as a platform for bone formation and mineralization, making a conducive environment for cell attachment, proliferation, and differentiation (Rico-Llanos et al., 2021). Its interesting capacity to mimic the characteristic bone microenvironment makes collagen a perfect substrate for advancing osteogenesis and encouraging the recovery of harmed or lost bone tissue (Rico-Llanos et al., 2021).

Besides, collagen-based materials have been instrumental in progressing synthetic bone substitute materials, advertising a practical elective to autologous grafting in orthopedic investigation and clinical practice (Fan et al., 2023). By tackling the regenerative potential of collagen, analysts have been able to develop imaginative biomimetic scaffolds that support bone development and integration, eventually improving the results of bone tissue-building strategies (Y. Li et al., 2021).

Moreover, the application of collagen in bone tissue engineering expands to recombinant human collagen, which presents a promising road for overcoming immunogenicity concerns related to common collagen sources (Cao et al., 2024). Recombinant human collagen holds the biological properties of local collagen whereas tending to issues related to immunogenic responses, clearing the way for improved bone regenerative engineering techniques (Cao et al., 2024).

In quintessence, collagen stands as a foundation in bone tissue engineering, advertising a flexible stage for the improvement of advanced biomaterials that advance successful bone recovery and repair (Rico-Llanos et al., 2021) . Its multifaceted role in giving basic support, bioactivity, and biocompatibility underscores its importance

as a key player within the journey for imaginative solutions in bone tissue engineering (Fan et al., 2023) .

1.4.2.2 Characteristics and features of Collagen to Bone Tissue Engineering. As the foremost protein within the extracellular matrix of animals, it is broadly known for its advantageous properties as a prebiotic. These point-by-point highlights emphasize the significance of collagen as a biomaterial, and highlight the interesting properties that make it a profitable fabric for regenerative medication,

Biocompatibility: Collagen appears tall biocompatibility with human tissues, making it a perfect choice for different biomedical applications. Its normal root and compatibility with the body contribute to diminishing the chance of hurtful responses (Rezvani Ghomi et al., 2021).

Regenerative Properties: Collagen-based biomaterials play a crucial part in tissue recovery and improvement within the areas of medication and dentistry. The properties of collagen back the recovery of harmed tissues, encourage the development of unused cells, and advance mending forms (Khan R, 2013).

Auxiliary Differences: The multiple progressive structure of collagen gives extraordinary basic differences, permitting it to imitate the complexity of characteristic tissue. This highlight improves their natural execution and reasonableness for a wide run of biomedical applications, counting tissue building and medicate conveyance frameworks (Q. Chen et al., 2023).

Characteristic origin: Collagen is determined from characteristic sources, such as creatures, and has been utilized in biomedicine for centuries. Its common origin and long history of clinical applications make it a dependable biomaterial in different restorative intercessions, counting wound recuperating, tissue repair, and restorative strategies (Troy et al., 2021).

1.4.2.3 The Applications of Collagen in Bone Tissue Engineering. Collagen, an essential component of the extracellular matrix, has developed as a urgent biomaterial within the domain of bone tissue engineering, displaying different applications over different techniques. Collagen-based biomaterials have been tackled in scaffold-based approaches, cell delivery systems, and development figure conveyance systems, illustrating their flexibility and viability in advancing bone regeneration and repair (Amirthalingam & Hwang, 2023)Outstandingly, collagen scaffolds play a significant role by giving a three-dimensional system that encourages cell connection and tissue ingrowth, serving as a scaffold for new bone arrangement and directing the recovery process (Y. Li et al., 2021). Additionally, collagen shows extraordinary potential as a carrier for cells, growth variables, and bioactive particles, empowering improved maintenance and localized delivery at the specific location of bone defects, subsequently expanding the restorative results in bone tissue designing applications (Y. Li et al., 2021). The multifaceted properties of collagen emphasize its importance as a foundation in progressing the field of bone tissue engineering, advertising promising roads for inventive and viable regenerative treatments.

1.5 Problems of the study

Lack of surface chemistry to encourage cell adhesion, low mechanical strength, fast degradation rates, and restricted cell attachment are some of the issues with the materials used to build scaffolds that need further investigation. These problems may have an impact on the scaffolds' efficacy in tissue engineering applications**.**

We have trouble striking a balance between the mechanical characteristics and porosity of the scaffold that will be created with (Solvent casting / particular leaching) method when it comes to synthetic polymer applications. However, employing natural polymers presents challenges with poor stability, challenging manufacturing processes, and insufficient mechanical properties. casting scaffolds with the proper shape and porosity for tissue regeneration, balancing mechanical strength and compliance, enhancing degradation rates, managing stress protection, and enhancing surface properties for cell adhesion are some of the significant issues that we will encounter in our research. As a result, the primary challenge is supplying the required mechanical strength and the suitable macroenvironment. for the development and renewal of bone cells.

1.6 The Purpose of the Study / Study Hypothesis

The hypothesis that we adopt in this study is a comparison between artificial bone models based on collagen and PLGA as scaffolds, through bone tissue engineering techniques. Which gives advantage to the properties of PLGA over collagen in providing a better environment for the growth of bone cells due to its biodegradability and biocompatibility, as well as its capabilities as a drug delivery system. **By evaluating the performance of PLGA models with collagen-based artificial bone models, we aim to gain a comprehensive understanding of the properties of each in supporting bone cell growth.**

Chapter 2

Literature Review

The creation of artificial bone models that develop useful tools for research in bone regeneration, biomaterials research, and tissue engineering techniques is at the heart of advances in the field of bone tissue engineering in recent years due to the urgent need to find effective treatments for bone injuries and defects. One of the most important developments occurring is the use of synthetic and natural polymers as basic building blocks for artificial bone models, in addition to studying their effects on bone cell growth and proliferation, By reviewing the literature, we aim to develop a current knowledge on the benefits and drawbacks of using synthetic against natural polymers in creating artificial bone models. Specifically examine how these polymers affect the bone cell growth, proliferation, and differentiation within the scaffold and hydrogel microenvironment. Regarding the questions related to the study, there are many questions related to our study, but we summarized and selected the questions based on the principle of importance, as the following questions will be answered through the study:

1- How can the structure and composition of artificial bone models influence bone cell growth and proliferation ?

2- What are the optimal fabrication techniques for creating artificial bone models using synthetic and natural polymers ?

3- How can we assess and compare the effectiveness of artificial bone models in supporting bone cell growth and tissue formation ?

The study done by Khasnis et al. 2020 explores the development of a composite scaffold using collagen and Poly (lactic-co-glycolic acid) (PLGA) for bone tissue engineering. While the research highlights promising results in enhancing scaffold properties crucial for bone cell growth, several gaps remain in the existing literature.

How does the scaffold behaviour translate from in vitro to in vivo settings, and what are the implications for scaffold efficacy and biocompatibility in clinical applications?

Additionally, what are the long-term stability and degradation kinetics of collagen/PLGA composite scaffolds, and how do these factors impact scaffold performance over extended periods?

Furthermore, what comparative studies exist with alternative scaffold materials and fabrication techniques, and what insights do they provide into the relative advantages and disadvantages of different approaches (Khasnis et al., 2020)?

Lastly, what are the regulatory considerations and scalability challenges associated with the clinical translation of collagen/PLGA composite scaffolds, and how can these be addressed to facilitate their adoption in clinical practice?

The study conducted by Lai et al.2019 highlights how the shape and size of pores in scaffold accoutrements can impact how cells behave, especially in cartilage tissue engineering. However, there is a lack of research directly comparing PLGA pullets with collagen-based alternatives in bone tissue engineering operations. Similar research could help identify the best-supporting accoutrements to promote bone cell growth and isolation. Collagen is a natural polymer with bioactivity and biocompatibility that almost mimics the extracellular matrix. It has great potential to improve cellular interactions in bone regeneration. Studies on collagen-based pullets suggest that there is a need for further discussion of altar design parameters that maximize bioactivity while maintaining mechanical stability. Thus, a comprehensive comparative analysis of PLGA and collagen applications is required to fill these gaps in the literature. This analysis could help develop better scaffold accouterments for bone tissue engineering operations (Lai et al., 2019).

In 2022, Zhang and Zhang conducted a study on bone tissue engineering, which significantly contributed to the field. The study focused on developing and characterizing collagen scaffolds that promote bone rejuvenation. The authors provided detailed insight into the unique features and performance criteria of collagen

scaffolds, demonstrating their potential as effective platforms for bone tissue regeneration. The study explored various fabrication methods and characterization techniques, explaining the intricate details of collagen scaffold design and highlighting its crucial role in facilitating successful bone tissue rejuvenation processes. However, despite the valuable insights provided by Zhang and Zhang's study, significant gaps in the current literature still need further exploration.

One such gap pertains to the lack of a comparative analysis between collagen scaffolds and other materials, especially synthetic polymers like PLGA, commonly used in bone tissue engineering. A comparative examination of different scaffold materials could provide invaluable insights into their relative efficacy, mechanical properties, and biocompatibility, helping researchers and clinicians make informed decisions regarding material selection for specific tissue engineering operations.

Moreover, further research efforts are necessary to optimize collagen scaffold design parameters, such as pore size, porosity, and mechanical properties. By fine-tuning these parameters, scientists could develop scaffolds with improved performance and regenerative capabilities, eventually bridging the gap between current scaffold capabilities and the evolving demands of bone tissue engineering (Sun et al., 2022) .

The study by Wu, Wang, and Liu in 2021 involves using naringenin, a natural compound, to modify poly (co-glycolic acid) (PLGA) scaffolds. This modification aims to promote bone regeneration by targeting the NF-κB signaling pathway. Through careful experiments and analyses, the study provides valuable insights into the effectiveness of naringin-modified PLGA scaffolds in promoting bone tissue regeneration. However, there are still significant gaps in our understanding of these scaffolds, particularly in terms of their long-term stability and biocompatibility, as well as their potential use in clinical settings. Further research is necessary to address these gaps and explore the therapeutic potential of PLGA scaffolds modified with naringin or another compound and the fundamentals of scaffold composition in terms of ratios and compositions in bone tissue engineering and regenerative medicine applications (Reddy et al., 2021) .

The study by Mustafa and Koneru (2020) provides a comprehensive review of collagen-based biomaterials and their applications in the field of bone tissue engineering, covering various aspects including fabrication techniques, properties and

applications. Despite the comprehensive examination presented in the study, there are still significant gaps in the existing literature, especially with regard to improving scaffold design standards, detailed assembly, and translating research results into clinical practice. These gaps underscore the need for further research endeavors aimed at improving scaffold design and facilitating a smooth transition of collagen-based biomaterials from bench to bedside, ultimately enhancing their effectiveness in bone tissue engineering and advancing patient care (Donnaloja et al., 2020) .

O'Brien's study from 2016 provides a comprehensive look at recent developments in polymeric materials widely used in tissue engineering applications, covering a variety of topics such as scaffold design, manufacturing techniques, and biomedical applications. While the research provides valuable insights into the use of polymeric materials to increase and enhance tissue regeneration, there are still some gaps in the existing literature, specifically when it comes to their application in bone tissue engineering. Although polymeric materials are widely covered, there is a lack of in-depth research into polymeric scaffolds such as PLGA and collagen, specifically in bone regeneration applications due to their great importance. More research is needed to understand the effectiveness of polymeric scaffolds in bone tissue engineering, focusing on factors of interest such as mechanical properties, biocompatibility, and degradation kinetics. In addition, comparative studies evaluating the performance of polymeric scaffolds against conventional materials can provide valuable insights into their clinical applicability and guide the development of more effective scaffold designs for bone tissue engineering applications (Dong & Lv, 2016).

Abaci, Guvendiren, and Tasoglu (2020) provided a comprehensive review of recent developments in collagen bio ink's for bone tissue engineering processes. The study explores the bio ink's composition, diffusion methods and processes in tissue regeneration. Although the exploration provides valuable insight into bio ink's expression and printing methods, there is still a gap in the literature regarding the specific functionalization of collagen bio ink's in bone tissue engineering. The study also lacks an in vivo comparison with traditional 3D printing. Therefore, further exploration is needed to enhance collagen inks for bone regeneration processes, including exploring their mechanical strength, biocompatibility, and implication to support bone cell growth and sequestration. Likewise, comparative studies evaluating the performance of collagen-based inks versus other scaffold preparations in bone

tissue engineering texts can give valuable insight into their relevance to clinical application. Similar studies could help develop more effective scaffold designs for bone tissue engineering (Filippi et al., 2020b).

Smith and Jones (2023) conducted a comprehensive review of biocompatible and bioabsorbable polymers used in bone tissue engineering. This study covers various aspects such as polymer properties, production methods, and applications in bone regeneration. Although this study provides valuable insight into the potential of these polymers to promote bone tissue regeneration while being compatible with the body's natural processes, gaps remain in our understanding of how to optimize these polymers for specific bone regeneration applications. Further research is needed to explore the mechanical properties, degradation kinetics, and biocompatibility of these polymers in the context of bone tissue engineering, with a particular focus on tailoring their properties to meet the requirements of different bone regeneration situations. also, comparative studies comparing the performance of different biocompatible and bioresorbable polymers in relevant bone tissue engineering models could provide valuable insights into their effectiveness and guidance in selecting optimal scaffold materials for specific bone regeneration applications (X. Li et al., 2023).

Kondo et al. (2013) present a detailed overview of polymeric scaffolds utilized in bone regeneration, which covers scaffold fabrication techniques, their properties, and applications in bone tissue engineering. The study offers valuable insights into the potential of polymeric scaffolds in promoting bone regeneration and repairing bone defects. However, there are still gaps in the optimization of these scaffolds for specific bone regeneration applications, as well as in the knowledge of the optimal techniques for fabricating bone scaffolds. Additionally, there is a lack of comparative studies. Further research is necessary to investigate the mechanical properties, biocompatibility, and degradation kinetics of polymeric scaffolds, as well as their ability to support bone cell growth and differentiation in vivo. Moreover, comparative studies evaluating the performance of different polymeric scaffolds in relevant bone tissue engineering models can provide valuable insights into their effectiveness and guide the selection of ideal scaffold materials for specific bone regeneration applications (Khang et al., 2013).

Zhang et al. (2020) conducted a review of polymeric scaffolds for bone regeneration. Their study covers various aspects of scaffold design, fabrication

techniques, properties, and applications in bone tissue engineering. It offers valuable insights into the advancements and challenges in the field, laying the foundation for understanding the role of polymeric scaffolds in bone regeneration. However, there are still some gaps in the research. For instance, the long-term stability and durability of polymeric scaffolds need to be investigated, as most studies only focus on short-term outcomes. It's essential to understand the host response to polymeric scaffolds, including their immunomodulatory properties and potential adverse reactions.

Additionally, there is a need to bridge the gap between benchtop research and clinical translation, which requires addressing regulatory hurdles and facilitating commercialization. Exploring the incorporation of bioactive molecules into polymeric scaffolds to enhance their regenerative capacity is another promising research avenue. Finally, personalized approaches to scaffold design are necessary to account for the variability in patient demographics, anatomy, and disease conditions. Interdisciplinary research efforts are necessary to address these gaps and advance the field of bone tissue engineering, ultimately realizing the full potential of polymeric scaffolds for clinical applications (P. Chen et al., 2019).

Liu et al. (2017) present a groundbreaking study that demonstrates the potential of multiphoton microscopy in visualizing angiogenesis in vivo. Their research showcases the remarkable capabilities of this technique in capturing high-resolution, three-dimensional images of angiogenic processes in real time, using genetically encoded fluorescent proteins to label endothelial cells and vascular structures. The study offers a valuable methodology for studying angiogenesis dynamics, providing insights into the complex process of blood vessel formation in vivo. However, while this technique has shown great promise in visualizing angiogenesis, further research is needed to explore its application within the context of tissue engineering and regenerative medicine. Specifically, we need to investigate how this technique can be effectively utilized to study angiogenesis within engineered tissues and scaffolds, particularly in relevant in vivo models that mimic physiological conditions. Further opportunities may arise to combine multiphoton microscopy with other imaging modalities or molecular probes to enhance our understanding of vascularization processes in tissue-engineered constructs and advance their clinical translation.

Regarding the 3D-PLGA/nHAp scaffold for calvarial critical bone defect repair, the study introduces an innovative biomaterial approach for bone regeneration. By

fabricating a 3D scaffold composed of poly (lactic-co-glycolic acid) (PLGA) and nano-hydroxyapatite (nHAp), the authors demonstrate promising outcomes in promoting bone regeneration in a critical bone defect model. However, further studies are needed to comprehensively evaluate the efficacy of this scaffold in promoting bone regeneration and its interaction with angiogenesis processes. Specifically, research should focus on assessing the mechanical properties, biocompatibility, and osteogenic potential of the scaffold, as well as its ability to support vascularization and integration with host tissues in critical bone defect repair scenarios. Comparative studies with other scaffold materials and assessment in clinically relevant animal models would provide valuable insights into the scaffold's performance and guide its optimization for bone tissue engineering applications (J. Li et al., 2017).

Chapter 3

Methodology

All experiments were achieved at Bahçeşehir University, Medical Faculty, Biophysics Laboratory

3.1 Polymer Characterization

Differential Scanning Calorimetry (DSC) Analysis

The thermal properties of the samples were evaluated using a Differential Scanning Calorimeter (SHIMADZU, DSC-60 Plus) available in the Laboratory of Biophysics at Bahçeşehir University School of Medicine. The DSC analysis was performed over a temperature range of 20°C to 600°C with a heating rate of 10°C/min. For each sample, approximately 5 mg was weighed and placed in an aluminum pan for analysis. The thermograms were analyzed to determine the melting point, glass transition temperature (Tg), and thermal stability of the samples.

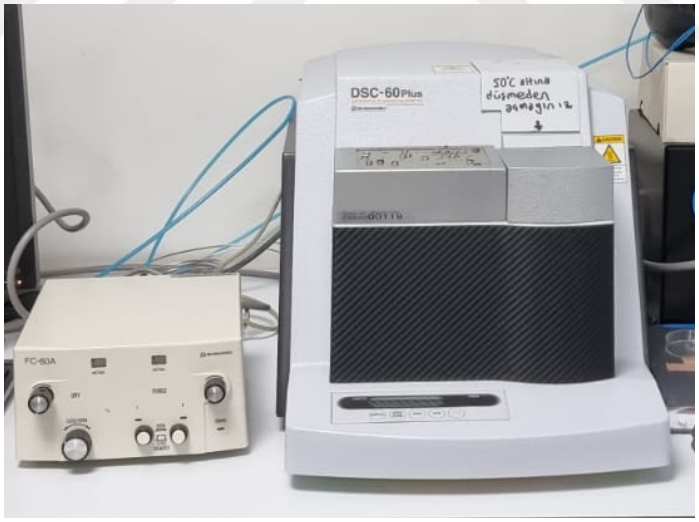

Figure 1. Shimadzu, DSC-60 Plus

3.2 FTIR characterization

The Shimadzu IRAffinity-1S system was used to conduct FTIR spectra measurements within the range of 4000-600 cm^{-1}. Circles with a diameter of 3 mm were placed in the measuring part while taking measurements from the scaffolds.

Figure 2. Shimadzu Iraffinity-1S System

3.3 Collagen Based scaffold

Collagen type 1 (AV8682690033050) was provided by (Aromal Kimya Medikal / Istanbul / Turkey), Chitosan 95% deacetylated was provided by (Biyopol/ Istanbul/Turkey) nHA was provided by (nanografti/Çankaya/Ankara TURKEY). The scaffold was prepared according to these ratios (Collagen 75%, nHA 20%, Chitosan 5%). Glutaraldehyde 25%(1.04239.0250) concentration as crosslinker was provided by (Merck KGaA / Darmstadt / Germany), scaffold prepared by Solvent casting / Particular leaching technique.

The scaffold solution preparation, begin to dissolve a certain quantity of type 1 collagen in 1% acetic acid that was prepared from extra pure Acetic acid 99.5% and diluted in distal water. Use magnetic stirring at a speed of 500 RPM for a duration of 30-45 minutes. To dissolve a certain quantity of chitosan in a 1% acetic acid solution, use magnetic stirring at a speed of 600 revolutions per second for a duration of 1 to 1.5 hours. Next, uniformly distribute the nHA inside the collagen solution by using a magnetic stirrer. Subsequently, chitosan solution gradually incorporated into the collagen-nHA combination while maintaining constant stirring for a duration of 45 minutes, to get a uniform solution. Finally, the porogen NaCl particles (ranging from 200 to 500 micrometers in size) are sieved and thoroughly stirred to ensure uniform dispersion. Transfer the mixture into Petri dishes that have been lined with aluminum strips to aid in the easy removal of the scaffold after fully dried. Ensure that the mixture is distributed uniformly to achieve the appropriate thickness and allow it to dry at ambient temperature until it reaches a semi-solid state. Next, the scaffold was applied for evaporation with 70% ethanol by placing the Petri dish within a container

containing a tiny quantity of ethanol at the base. Following ethanol treatment, thoroughly desiccate the scaffold at ambient temperature, and then subject it to freeze-drying (lyophilization) to enhance its porosity. Furthermore, to extract the scaffold from the Petri dish. Subsequently, the scaffold is cleansed using PBS in order to eliminate the porogen (NaCl) particles, therefore attaining precise pore diameters ranging from 200 to 500 micrometers. Following the leaching process, we fully desiccate the scaffold by subjecting it to air-drying at room temperature for 48 hours. This step is crucial to eliminate any remaining moisture. (figure1)

With the same method, we prepared a scaffold with one extra step which is adding Glutaraldehyde as a crosslinker into the final mixture after casting.

Figure 3. COL-BS artificial bone final extracted shape

3.4 PLGA Based Scaffold

Poly Lactic -co- glycolic acid (PLGA) (Cat: HY-B2247) was provided by (medchemexpress /New Jersy / USA), with same method the scaffold solution preparation, beginning by adding a certain quantity of PLGA solution to chloroform and stirring it with a magnetic stirrer at a speed of 500 RPM until the PLGA is fully dissolved to achieve 20% PLGA solution. Subsequently, incrementally introduce nHA into the PLGA/chloroform solution while stirring with a magnetic stirrer to achieve uniform dispersion. then, while stirring, add CS into the PLGA/nHA/chloroform solution to get a homogeneous mixture. Inspect the mixture to verify that the nHA and CS are evenly distributed throughout the PLGA solution. At this point, the solution should have a viscosity that is easier to work with. Subsequently, the NaCl porogen (with a particle size ranging by sieving from 200 to 500 mm) entered the

PLGA/nHA/CS/chloroform combination progressively. Stir the mixture to guarantee even dispersion of the porogen particles throughout the solution. after ensuring an even dispersion Place the scaffold solution into the mold and let it dry at a typical temperature of 25°C for 24 hours. Upon drying, the scaffold becomes firm. The scaffold was removed from the mold that had been equipped with aluminum foil to facilitate the removal of the scaffold out of the mold after completely dried. by washing the scaffold with PBS, achieving a porous scaffold, and proceeding to thoroughly dry and sterilize it (Figure 2).

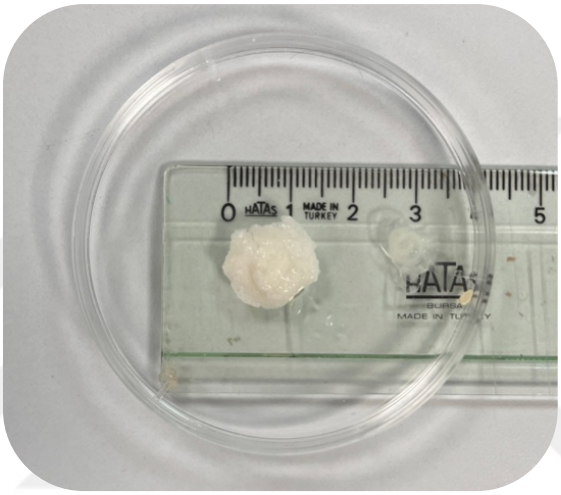

Figure 4. PLGA-BS final extracted shape

3.5 Cell Culture

L929 the fibroblast cell line from a mouse was used. The Cell Culture Laboratory of the Department of Biophysics at Bahçeşehir University School of Medicine expanded the cell line that was originally acquired from (ATCC in Manassas, VA, USA). The cells were placed in cryogenic vials and frozen in liquid nitrogen(-290°C) for future use. It transferred cells from liquid nitrogen to fresh Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin after thawing swiftly at 37°C. Cell culturing was done at 37°C in a humidified environment containing 5% CO_2 until they attained a density suitable for experimental use.

Firstly, the cells were gathered by using a cell scraper and then centrifuged at 1500 rpm for 5 minutes once they reached an adequate density for the experiments.

after that, the pellet that was formed was then mixed with 1 milliliter of medium. A hemacytometer was used to count cells in a 10 µl suspension sample placed in a Thoma counting chamber. In our study, we used a 12-well plate, $5x10^4$ cells were seeded into each well of a 12-well plate with the samples that were divided into four groups (control group, COL/nHA/CS group, COL/nHA/CS/GL group, PLGA/nHA/CS) as shown in the (figure .3) . The cell culture medium was supplemented with sterile samples after 24 hours of culture. To determine the cells' adherence ability, the samples were incubated for 24 hrs.

Finally, after the incubation, the cells' attachment and spreading on the sample surfaces were examined by an inverted microscope (Leica, MC120 HD), and through microscopic images, the morphological characteristics of the cells on the samples and their interactivity with the surface were examined.

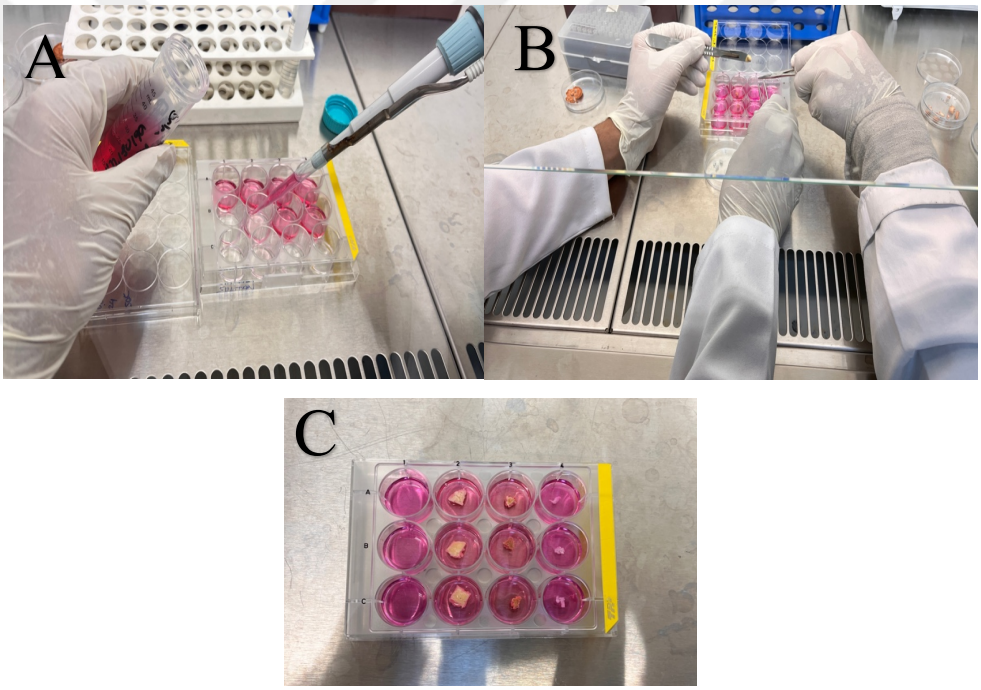

Figure 5. 12-well plate with the samples that were divided into four groups(control group, COL/nHA/CS, COL/nHA/CS/GL, PLGA/nHA/CS), (A) filling the well with cells, (B) cutting the scaffolds samples into equal pieces with surgical scalble and imerge them gently into wells according to the arrangment, (C) final 12- well plate contain samples imerged with cells and ready for incubating.

3.6 Mechanical Test (Compression Test)

The mechanical tests were performed using an MTS Criterion™ Model 41 machine, for which parameters were chosen and set in order to perform the test in conditions that were distinct and accurate. The initial distance to be maintained between the compression plates has also been set at 1.600 mm to handle the thickness of the scaffold' plates prior to the deformation. A test speed of 0.50 mm/min was applied so as to compress gradually and the strain endpoint set at 50 percent level which meant that the height of the scaffold was reduced to 50 percent. Since the testing aimed to add a hose monitor to obtain motion data of load and that of displacement of 20.0 Hz was taken every 5 seconds, as a result, very good data for analysis was obtained. The amounts of load were applied until either of the limits of 10 N was attained or the specified limits of the elongation of the scaffold was found. The test sample was 6.000 mm in diameter, which was the same for all samples tested to eliminate variability.

3.6.1 Compression test analysis method. Test Setup and Preparation:

The compression test was conducted on PLGA and collagen-based scaffolds with a diameter of 6.000 mm. The initial height between the compression plates was set to 1.600 mm, corresponding to the scaffold's thickness before deformation. The scaffold was carefully centered between the compression plates to ensure uniform force distribution during testing.

Compression Test Execution:

The test was performed using an MTS Criterion™ Model 41, with the crosshead speed set to 0.50 mm/min. This setting facilitated a gradual and controlled compression of the scaffold. The machine applied a continuous compressive load until reaching the load endpoint of 10 N or achieving a 50% strain, indicating a 50% reduction in height.

Data Collection:

Data acquisition occurred at a frequency of 20 Hz, capturing load and displacement values every 0.05 seconds to ensure high-resolution data. The primary outputs recorded during the test included force (in Newtons) and displacement (in mm).

Result Interpretation:

The stress-strain data obtained from the test was analyzed to evaluate the mechanical properties of the scaffold, including the compressive modulus, failure point, and energy absorption capacity. The results were plotted on a stress-strain curve to assess the scaffold's performance under compression.

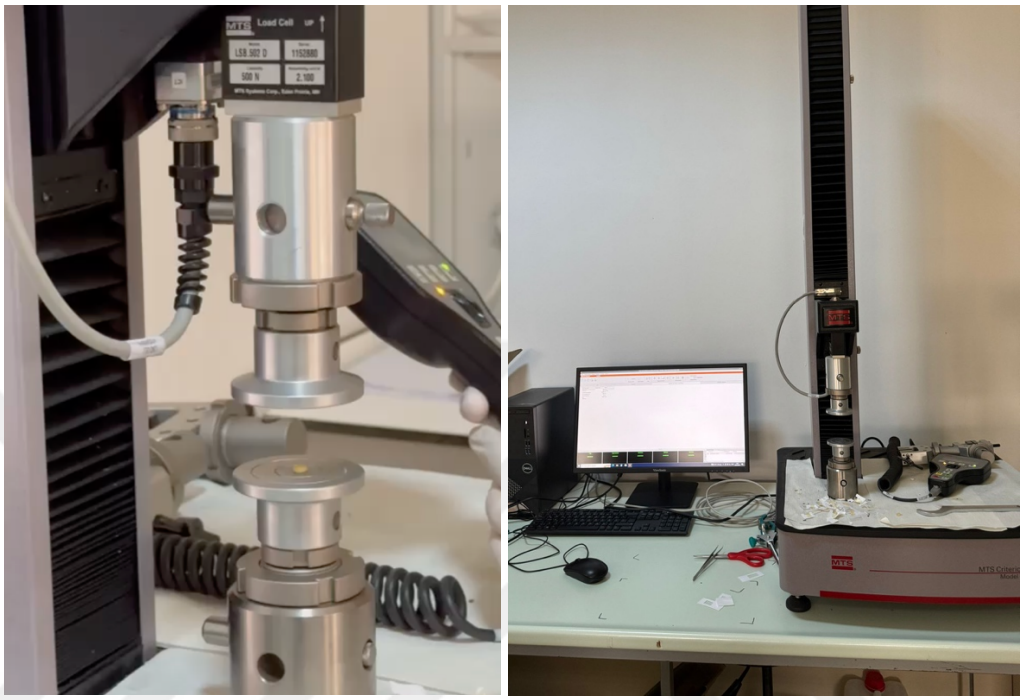

Figure 6. MTS Criterion™ Model 41 machine

Chapter 4

Results

4.1 Polymer Characterization

4.1.1 Poly (lactic-co-glycolic acid (PLGA). A PLGA sample with a 50:50 rate of lactic acid to glycolic acid was analysed, and DSC curve illustrates (Figure 4) its thermal characteristics which align significantly with the literature. The glass transition temperature (Tg) of approximately 55.02 °C falls within the typical range of 45 °C to 55 °C for PLGA with a 50:50 ratio, as reported by (Anderson & Shive, 1997a) (F. Zhang et al., 2001). Additionally, the DSC curve shows a peak indicating an increase in temperature between 281.97 °C and 294.94 °C. The midpoint of this peak is at 287.90 °C, which corresponds to the melting of crystalline regions or declination of the polymer. The findings are consistent with the research conducted by (Chasin & Langer, 1990). The observed thermal declination or melting behaviour is characteristic of PLGA with this composition, indicating the material's amorphous nature(F. Zhang et al., 2001) (Anderson & Shive, 1997a) (Arifin et al., 2006).

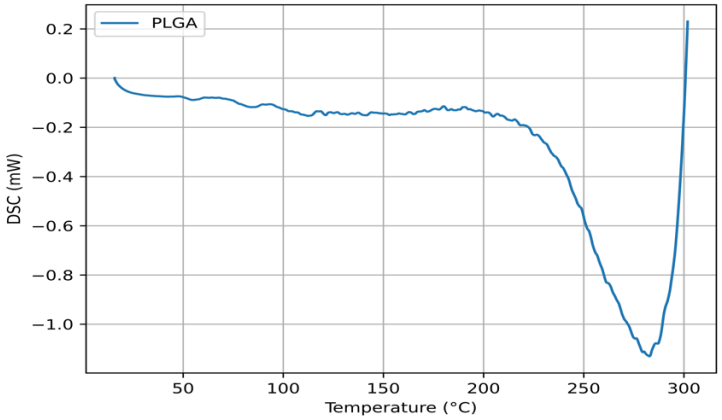

Figure 7. DSC of PLGA

4.1.2 Collagen. Collagen's DSC curve displays several thermal events that are consistent with the literature. The value of Tg (around 75.53 °C) is related to small transition, and typically ranges between 50 °C to 75 °C depending on hydration among other factors (Miles et al., 2005). These values imply that the sample may possess low levels of hydration together with an amorphous structure that is stable. Degradation begins at around 100 °C - 350 °C, whereas degradation of the collagen triple helix comes in at about 107.90 °C which is seen as peaks(Miles & Ghelashvili, 1999). Moreover, big anomalies in the curve such as those at 174.07 °C and the broad peak at 324.78 °C represent a consecutive breakdown of collagen to lesser structures through decomposition steps (Chattopadhyay & Raines, 2014a). In total, it shows that this DSC curve demonstrates thermal behaviour characteristic of collagen showing that the collected specimen performs just like any other collagen investigated under similar conditions.

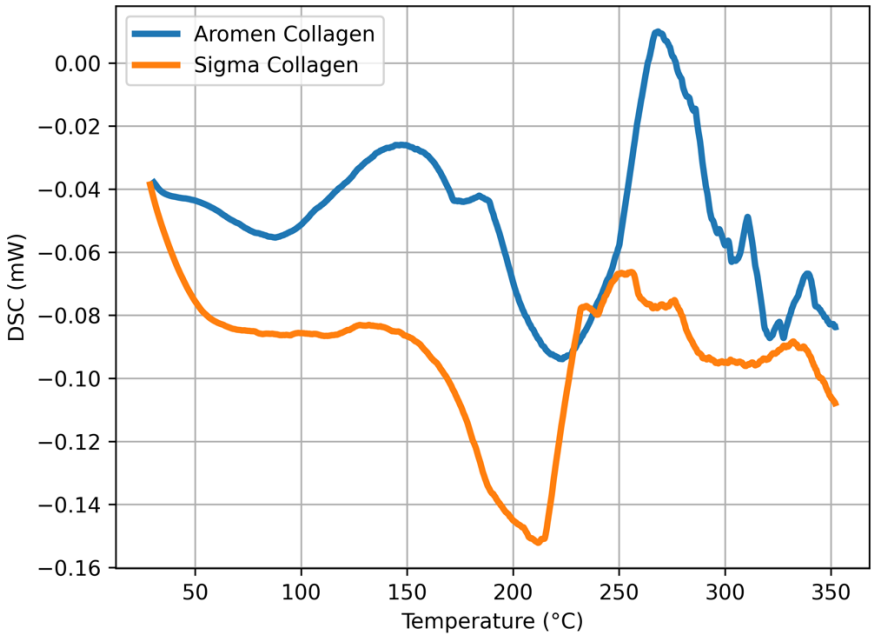

Figure 8. DSC of collagen

4.1.3 Nano-Hydroxyapatite (nHA). More than one thermal event is visible in the DSC analysis of nHA, and the results we obtained are consistent with those reported in the literature.

Starting with dehydration, which typically occurs between 100 °C and 200 °C, though it might not be noticeable in every sample (Suchanek & Yoshimura, 1998a).

Secondly, the point of dehydroxylation, which is frequently observed between (200 and 400°C), this is can result in the loss of hydroxyl groups and structural water (Koutsopoulos, 2002). The phase transformation point during crystallization may occur between 400 °C and 600 °C (Suchanek & Yoshimura, 1998b) and in the DSC curve, we can observe the following:

Endothermic peaks: These are related to phase transitions or dehydroxylation, with peaks observed at (327.73°C, 357.57°C) and (364.45°C), (Yubao et al., 1994).

Exothermic Peaks: the peaks at 466.44°C and 485.67°C may indicate of crystallization or other structural alterations (Gibson & Bonfield, 2002).

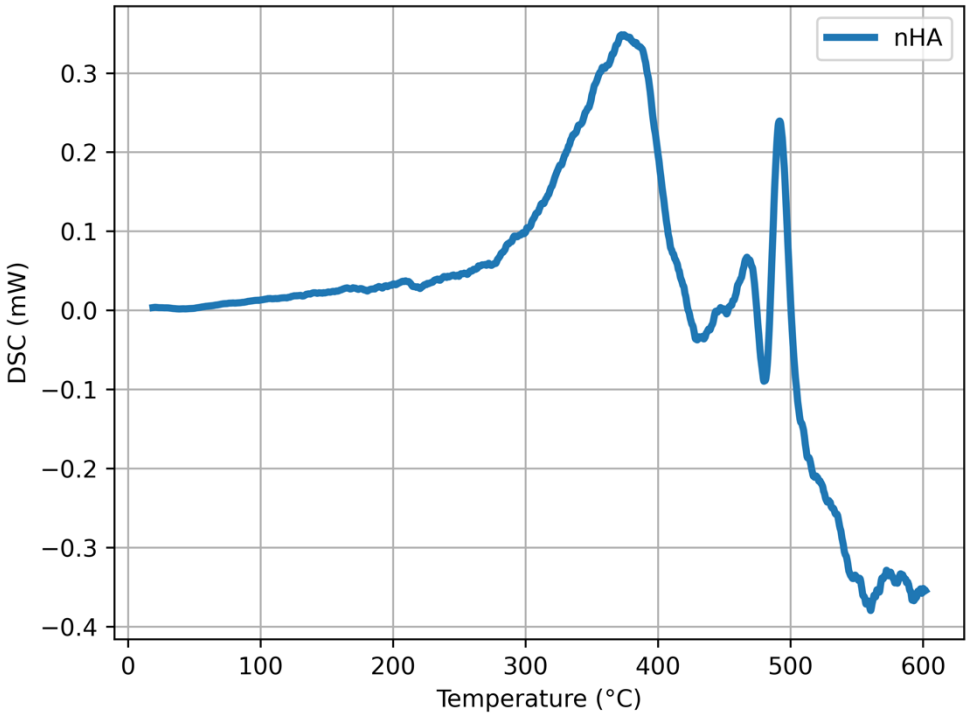

Figure 9. DSC of nano-hydroxyapatite

4.1.4 Chitosan (CS). DSC inspection of chitosan usually discloses a glass transition (Tg) temperature of around 50-60°C, which is necessary for the comprehension of its structural features and applications (Kittur et al., 2002). moreover, endothermic peaks coupled to dehydration and decomposition are noticed between 150°C and 250°C, appropriate for its thermal stability (Rinaudo, 2006). The degree of deacetylation significantly affects these thermal features, with higher deacetylation enhancing thermal stability, making it crucial for chitosan's application

in biomaterials (Kumar, 2000). and These thermal characteristics are crucial for tailoring chitosan's use in various engineering and biomedical applications submitting that the sample behaves normally and is the same as Chitosan samples studied under similar conditions.

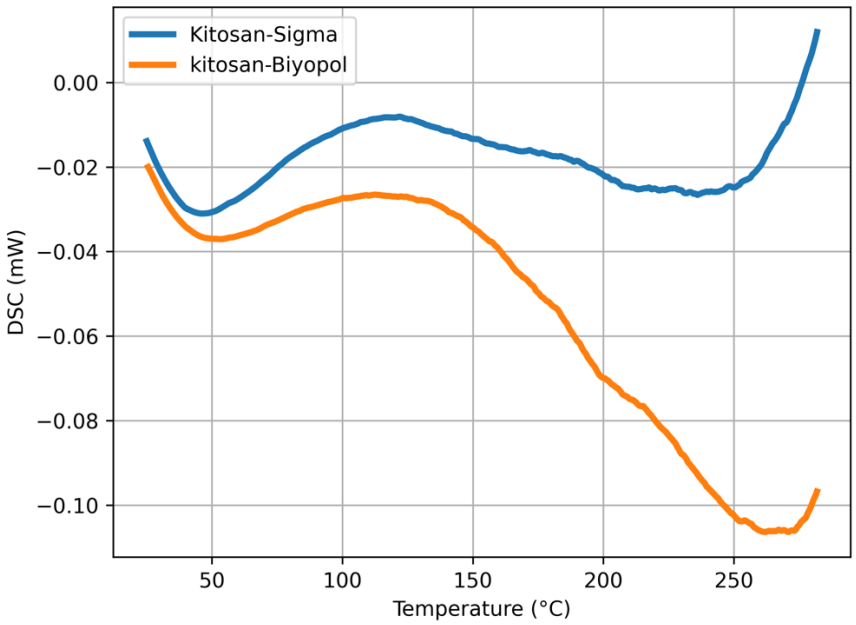

Figure 10. DSC of chitosan

4.1.5 PLGA based scaffold. The PLGA-BS scaffolds DSC (Figure 8) detected various crucial thermal proceedings. The (Tg) was noticed at 75.28°C, and this is higher than representative PLGA values (45-55°C) (Makadia & Siegel, 2011a), submitting intense interactivity between the scaffold materials. This rise in Tg is harmonious with the PLGA/nHA literature blend, where the rise in Tg values has been assigned to limited polymer chain mobility due to the nHA combination (Kouhi et al., 2013). The (Tm) was recognized at 164.40°C, within the predictable range for a PLGA-based mixture (Gentile et al., 2014b). supplementary thermal proceedings were spotted at 156.15°C, 210.26°C, and 243.59°C, suggest a compound multi-phase construction. The condition at 210.26°C may be related to chitosan retrogression, which usually manifests at higher temperatures but can be minimized in composites (*Michelly C G Pellá 2018*, n.d.). The raised Tg and the existence of multiple thermal transformations propose the perfect integration of PLGA, nHA, and CS materials, potentially ruling to amplify mechanical properties and control degradation actions

(Cao et al., 2012). In conclusion, these thermal features are regular with a completely formed combined scaffold, confirming the prosperous incorporation of nHA and CS into the PLGA form, which is beneficial for bone TE applications (Cao et al., 2012).

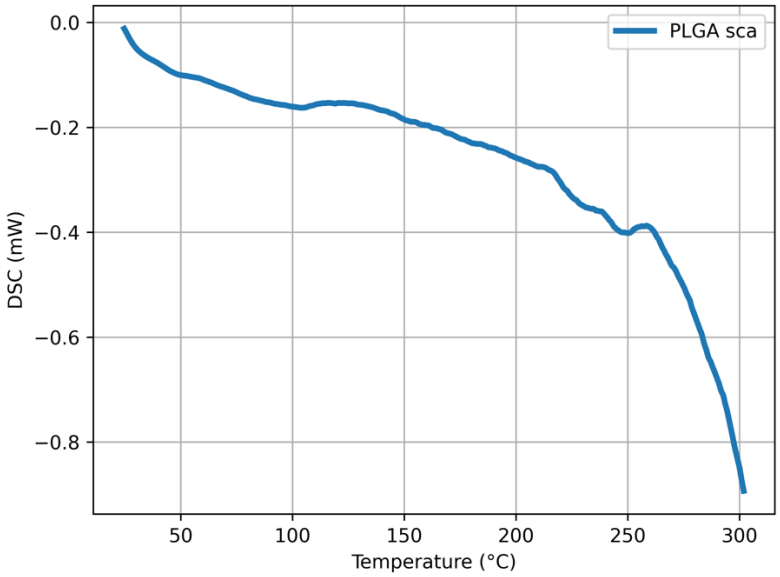

Figure 11. DSC of PLGA-BS

4.1.6 Collagen-based scaffold. The Col-BS (Figure 9) detected sundry basic thermal proceedings, as long as premeditation into its composition and qualities. A wide endothermic peak at 35.64°C coincides with collagen denaturation, which is regular with the ideal range of 35-40°C (Bozec & Odlyha, 2011). The transference at 62.07°C, elevated more than the usual (Tg) temperature of collagen, submitted a reaction between scaffold materials (Teng et al., 2008).

Furthermore, Multiple endothermic proceedings between 136.27°C and 171.19°C refer to a complex multi-phase formation, which is ideal for collagen-based combination (Teng et al., 2008). The status at 208.82°C probably performs CS degradation, transmitted to a minimum temperature according to blend interactions (Martínez-Camacho et al., 2010). The final phenomenon at 278.38°C submitted the existence of thermally resistant phases, maybe according to nHA conjunction (Zhou et al., 2014).

The Col-BS scaffold confirms a complicated thermal profile symptomatic of a skillfully incorporated complex formation. The upraised temperatures of several thermal proceedings contrasted with those of individual ingredients, propose an

impervious reaction between collagen, nHA, and CS. These features reveal improved thermal stabilization and potentially enhanced mechanical characteristics, which are appropriate for bone TE applications (O'brien, 2011a). So, The thermal conductance was declared stratified with a scaffold prepared for planned degradation and structural safety physiologically.

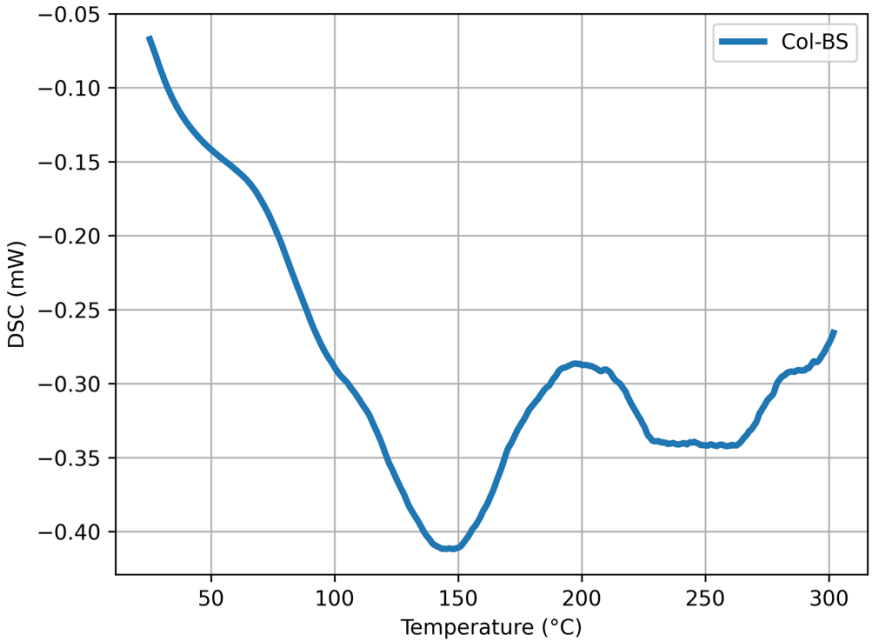

Figure 12. DSC of COL-BS

4.1.7 Collagen based scaffold with glutaradehyde. The Glutaraldehyde-Crosslinked Collagen-Based Scaffold (Col-BS+glu) DSC (Figure 10) shows a compound thermal profile symptomatic of influential crosslinking and promotes thermal constancy. The starting considerable endothermic peak at 90.63°C appears a fundamental carrying from representative collagen denaturation (35-40°C) (Bozec & Odlyha, 2011), which means effective glutaraldehyde crosslinking (Zeugolis et al., 2008). A wide transference at 135.26°C submits restricted water forfeiture or premier ingredient degradation (Teng et al., 2008). The distinguished endothermic state peak at 206.94°C possibly performs crosslinked framework collapse or CS degradation (Martínez-Camacho et al., 2010). carefully spaced proceedings peak at 225.45°C and 229.09°C refers to distinct phases, perhaps performing organic-inorganic reactions (Zhou et al., 2014).

The lack of thermal proceedings below 90°C emphasizes the entire adjustment of collagen's innate construction, implicating modified mechanical features and degradation behavior (Parenteau-Bareil et al., 2010). This advanced thermal profile is considered a competently integrated complex formation with secure inter-ingredient interactions. The raised thermal proceedings submit improved mechanical features and potential for planned degradation, which is critical for bone TE (O'brien, 2011b).

According to these results, the Col-BS+glu scaffold confirms crucially improved thermal stabilization, specifying enhanced formation integrity and potential for extended constancy in physiological situations. This analysis emphasized the scaffold's potential for implementation seeking prolonged mechanical backup and planned degradation averages in bone TE. Nevertheless, the comprehensive adjustment requires an accurate valuation of biocompatibility and cell interaction capacity.

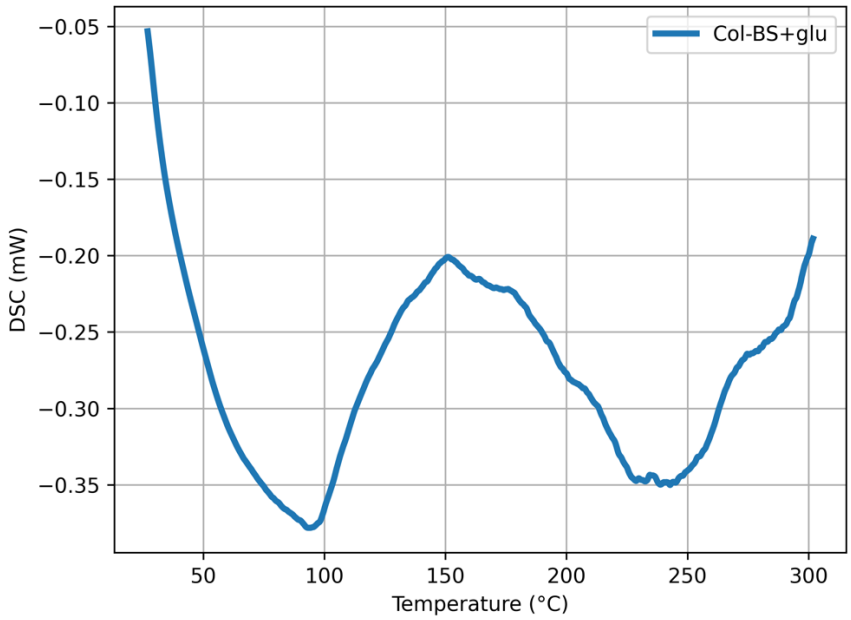

Figure 13. DSC of COL-BS + Glutaraldehyde

4.2 FTIR characterization

4.2.1 Chitosan. The chitosan FTIR shows several characteristic peaks that correspond to different functional groups within the chitosan molecule. Let's analyze the major peaks in the spectrum. There is a broad peak located around 3400 cm^{-1}. O-H and N-H stretching vibrations typically link this broad peak, indicating the presence of hydroxyl (OH) and amine (NH) groups. Because of its polymer structure, chitosan contains both groups. At Peak, around 2884 cm^{-1}This peak corresponds to C-H stretching vibrations, which are common in the aliphatic chains of the chitosan polymer backbone. The peaks range from 1650 cm^{-1} to 1550 cm^{-1}. The peak near 1650 cm^{-1} is generally attributed to the C=O stretching vibration of amide I, which is indicative of the acetylated units (N-acetylglucosamine) in chitosan. The peak around 1550 cm^{-1} is assigned to the N-H bending vibration combined with C-N stretching (amide II band), confirming the presence of the amide group.in Peak, around 1380 cm^{-1} this peak is associated with the bending vibration of the C-H bonds, particularly those in the methyl groups of the acetylated units. when coming at a peak of 1150 cm^{-1} can be attributed to the asymmetric stretching of the C-O-C bridge, which is part of the glycosidic linkage in chitosan. The peak near 1080 cm^{-1} is due to the C-O stretching vibrations in the polysaccharide structure. after that at a peak of around 896 cm^{-1} This peak is associated with the β-glycosidic linkages between the sugar units in the chitosan polymer chain. while around 600-400 cm^{-1} This region shows some minor peaks that can be associated with skeletal vibrations of the chitosan molecule, but these are less significant for functional group identification. In summary, the FTIR spectrum of chitosan exhibits characteristic peaks corresponding to hydroxyl and amine groups, amide I and II bands, and various C-H and C-O vibrations, which are all consistent with the chemical structure of chitosan.

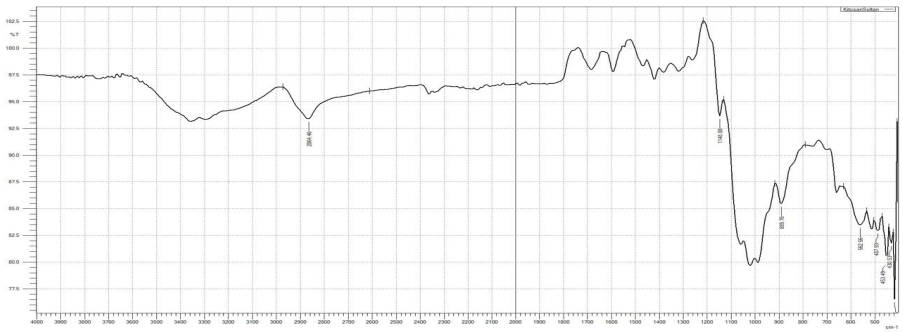

Figure 14. FTIR of chitosan

4.2.2 Collagen. The FTIR of collagen spectroscopy, identified which recognized specific absorption bands that confirm the sample's structural integrity and purity. Firstly, the existence of hydrogen bonding within the collagen triple helix structure is recommended by the broad absorption peak at approximately 3277 cm^{-1}, which is mean that attributed to the N-H stretching vibration of the amide A band (Kavya et al., 2013). Furthermore, the important amide I band, detected at a wavenumber of 1634.92 cm^{-1}, originates from the stretching vibrations of the C=O bonds in the peptide bond. while This band is a remarkable indicator of the secondary structure of collagen, specifically its triple-helical conformation (M. Li et al., 2005). on the other hand, to submit to the integrity of collagen's secondary structure, the existence of the amide II band at 1521.27 cm^{-1} indicates the appearance of N-H bending and C-N stretching vibrations (Muyonga et al., 2004). Moreover, the amide III band, which is crucial for confirming the organized structure and triple-helical form of collagen, was noticed at a wavenumber of 1245.67 cm^{-1} (Kavya et al., 2013). also there are Other remarkable peaks noticed were the amide B band at 2923.35 cm^{-1}, which is in agreement with the asymmetric stretching of CH2 groups, a C-O stretching vibration at 1039.20 cm^{-1} coupled to carbohydrates, and a C-H bending peak at 1408.04 cm^{-1}. The spectral characteristics noticed in the inspected sample are regular with values declared in the literature. This provides intense confirmation that the sample contains structurally entire and pure collagen.

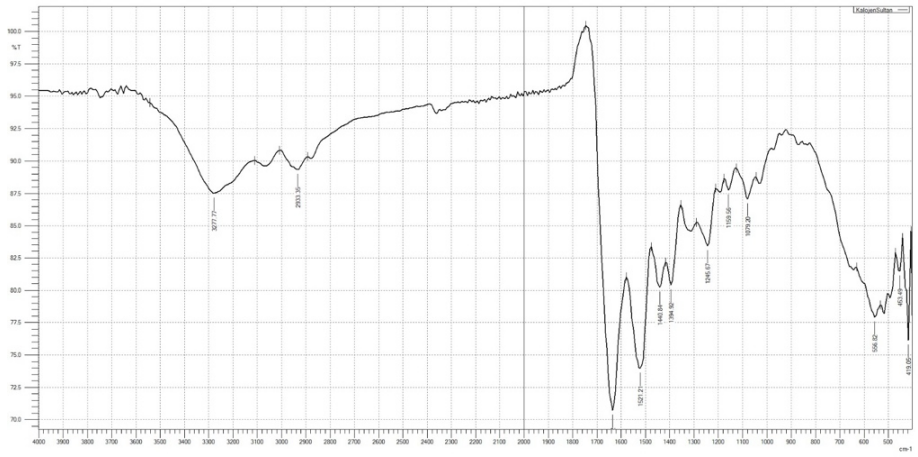

Figure 15. FTIR of collagen

4.2.3 Nano-hydroxyapatite. According to previous studies, the FTIR spectra of the (nHA) sample exhibit marked absorption bands. One main characteristic of hydroxyapatite (HAp) is be stretchable vibration of the phosphate group (PO_4^{3-}), which is thrown back by the powerful band around 1022.71 cm^{-1} (*LeGeros RZ 1991*, n.d.). The bending and symmetric stretching modes of the carbonate group are responsible for the peaks at 878.28 cm^{-1} and 984.39 cm^{-1}, respectively, suggesting the presence of carbonate-substituted hydroxyapatite (LeGeros, 1993). Additionally, the HAp structure is stiffened and further supported by the broadband at 602.72 cm^{-1}, corresponding to the phosphate group's bending vibration. Furthermore, hydroxyapatite's crystalline phase is distinguished by the O-P-O bending mode, typically associated with the peak at 552.05 cm^{-1} (Rehman & Bonfield, 1997).

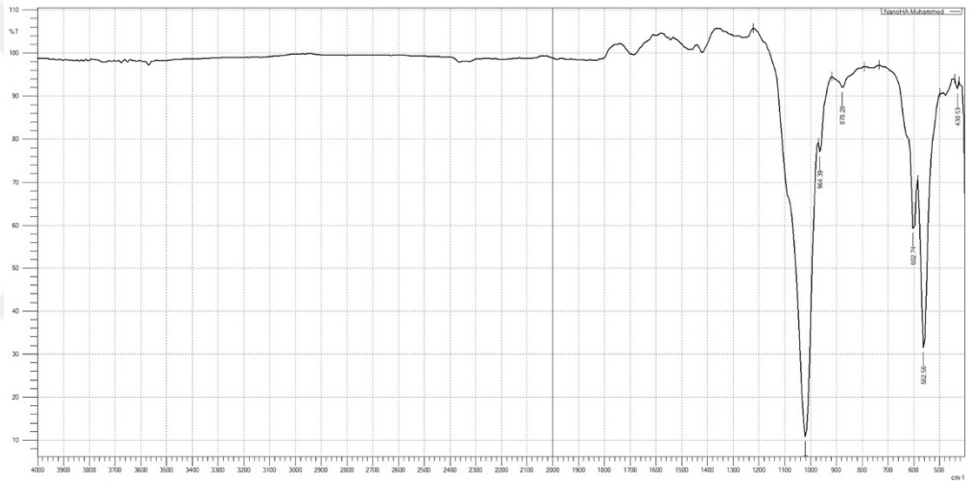

Figure 16. FTIR of Nano-Hydroxyapatite

4.2.4 Collagen-based scaffold. Interpretation of the Peaks:

3700 - 3500 cm c1 (O-H stretch): This category is typically associated with hydroxyl for the determination of the presence of water or alcohol.

2900 - 2800 cm c1 (C - H stretch): This group of peaks is associated with aliphatic hydrocarbons and can be related to fatty acids or some other organic materials.

1700 - 1600 cm c1 (C=O stretch): Carbonyl Group the basic stretching frequency which is suggestive of aldehydes, ketones or esters.

1400 - 1300 cm c1 (C-H bending): Methyl groups bending movement practiced in hydrocarbon.

1000 - 1100 cm c1 (C-O stretch): This peak notes the presence of oxygen based functional groups such as alcohol, ester or ether may appear.

These peaks suggest the present of important functional groups such as hydroxyl, carbonyls and methyl group which are normally found in collagenic material.

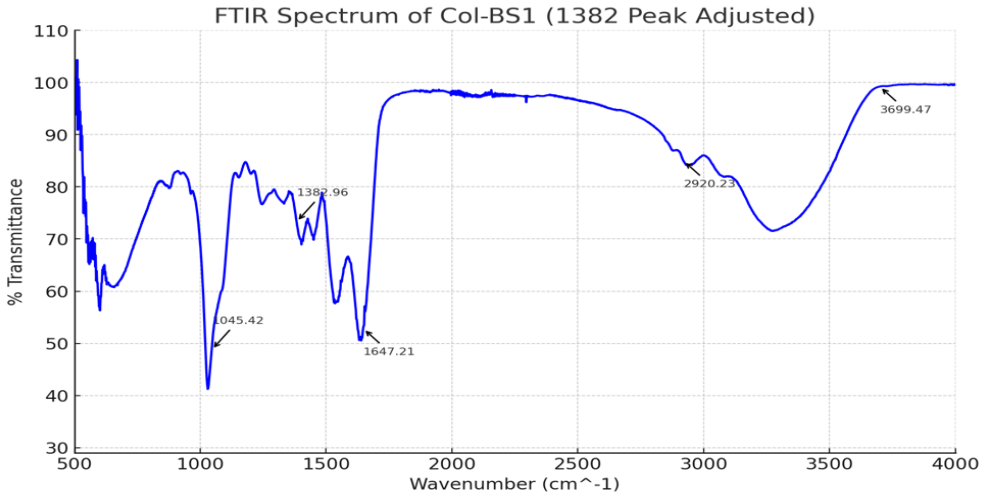

Figure 17. FTIR of Col-BS

4.2.5 Collagen-based scaffold + glutaraldehyde. Interpretation of the Peaks:

3718 cm c1 (O-H stretch): This peak is attributable to hydroxyl groups which means that these are usually moisture or alcohols.

3320 cm c1 (N-H stretch): Suggests the occurrence of amine or amide compounds which indicates the presence of protein or substances with nitrogen.

2920 cm c1 (C-H stretch): Describes C-H stretching which is more apon p-m body stretches associated with aliphatic hydrocarbons as in fatty acids or organic materials.

1647 cm^{-1} (Amide I C=O functional vibrations) – These are due to peaks attributed to stately carbonyl moieties occurring, eg: in aldehydes or in proteins.

1383 cm^{-1} (C-H bending) - Focuses on bending of the easily heat' s of methyl groups characteristic of organic compounds.

1044 cm^{-1} (C-O stretching): The functional group appears with oxygen that carries alcohol or esters, diethyl ether, triethyl ether for example.

878 cm^{-1} (C-H bending): This peak reveals the occurrence of C-H bending which is out of the plane in addition to plane C-H bonding which is a feature mainly of unsaturated and aromatic compounds.

Such peaks where arise from the absorbed band spectra are promising for simplicity in hydroxyl, carbonyl and amine's moieties which are characteristic of structures like that of glutaraldehyde.

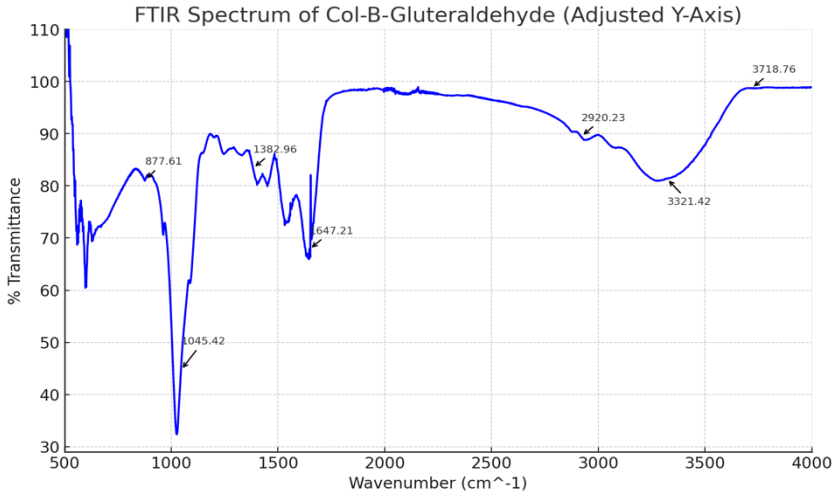

Figure 18. FTIR of Col-BS+ Glutaraldehyde

4.2.6 PLGA-based scaffold. Interpretation of the Peaks:

700–800 cm^{-1}: This envelope is appendant to vinyl C-H out of plane and C-C in plane melting bending oscillation of aromatic moiety. Such peaks are present in alkenes or aromatic rings.

1000–1250 cm^{-1}: Its functional groups and their combinations are employed mainly in the polyvinyl alcohol moieties prepared by UV irradiation polymerisation. Well, the peaks in PLGA are most likely related to the ester linkages present in the polymer chains, probably, due to the o-glactic and lacitic acid components inside plastics.

1450 – 1500 cm -1: This region comprises mainly C-H bending vibrations of differing types of -CH2 or -CH3 groups that more often their hydrophobic tail or hydrophobic tails in general. Such peaks are often related to the alkyl side chains that are incorporated into the polymer compound such as PLGA.

1750 cm-1: This band appears to be quite prominent and is assigned to C=O (carbonyl) functional groups and corresponding carbonyl stretching. This is a part of the carbonate moiety in polymers and is particularly relevant here in the context of PLGA side chain. This ester micromer polymer useful structure leans on this structure.

2800-3000 cm[1]: These peaks are associated with C-H stretching associated with the aliphatic hydrocarbons C-H bond. Here the symmetric and asymmetric stretching modes of -CH2 and -CH3 groups are seen indicating the presence of alkyl chains in the polymer.

3500-3200 cm: The peaks in this region are usually attributed to simple O-H stretching probably due to the presence of hydroxyls introduced by moisture or residual alcohols in the polymer.

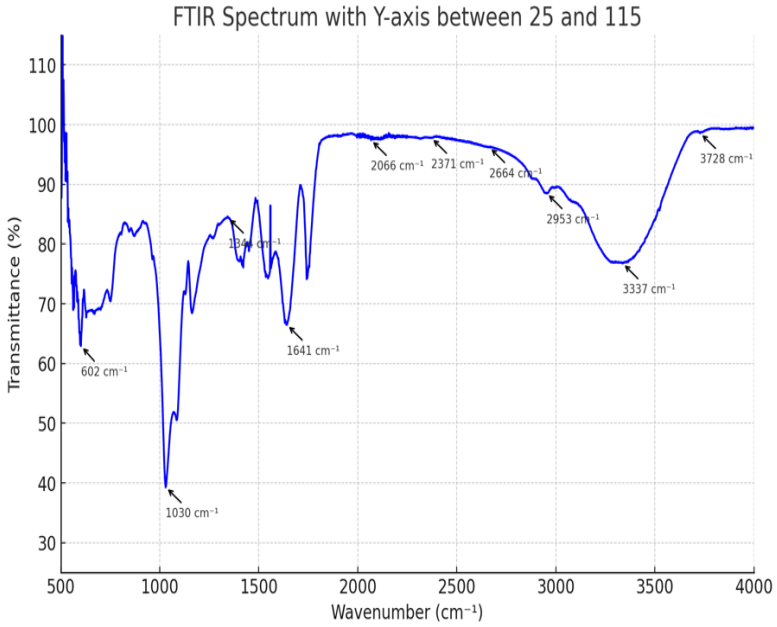

Figure 19. FTIR of PLGA-Based Scaffold

4.3 Cell culture assays

4.3.1 Collagen-based scaffold. The cytotoxicity of the scaffold, composed of 75% collagen (Col), 20% nano-hydroxyapatite (nHA), and 5% chitosan (CS) without a crosslinker, was evaluated through direct observation of L929 fibroblast cells using an inverted microscope (Leica, MC120 HD). The L929 cells undergo considerable morphological qualifications after exposure to the scaffold, as confirmed by the images captured (Figure 14). Cell shrinkage, membrane blebbing, and a loss of standard fibroblast morphology are the most significant changes noticed, indicative of apoptosis or cytotoxic stress (figure 15). This scaffold may induce cellular destruction, potentially by way of mechanisms like oxidative stress, which has been linked to nanomaterials like nHA (Oberoi et al., 2018). Additionally, chitosan has the potential to cause cell membrane disruption and induce cytotoxic effects, depending on its concentration and molecular weight, as reported in various studies. These inspections are regular with prior research that has demonstrated cytotoxic effects in specific cell lines when composite scaffolds containing nanohydroxyapatite and chitosan are not properly formulated (Reilly & Engler, 2010).

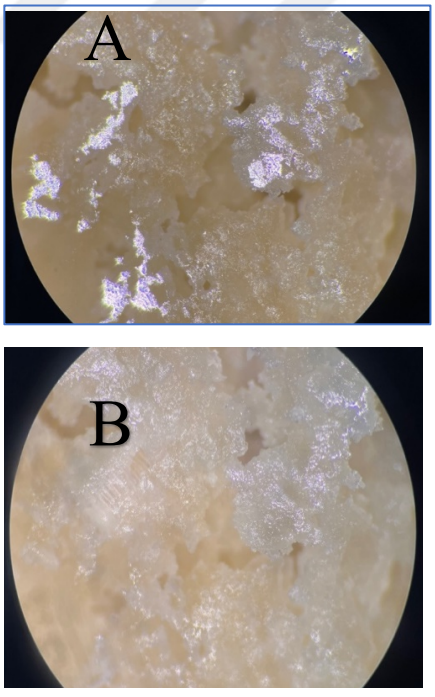

Figure 20. Col-BS indicates a porous structure crucial for supporting microenvironment cell adhesion and proliferation. The surface morphology appears a potential for functional tissue combination.

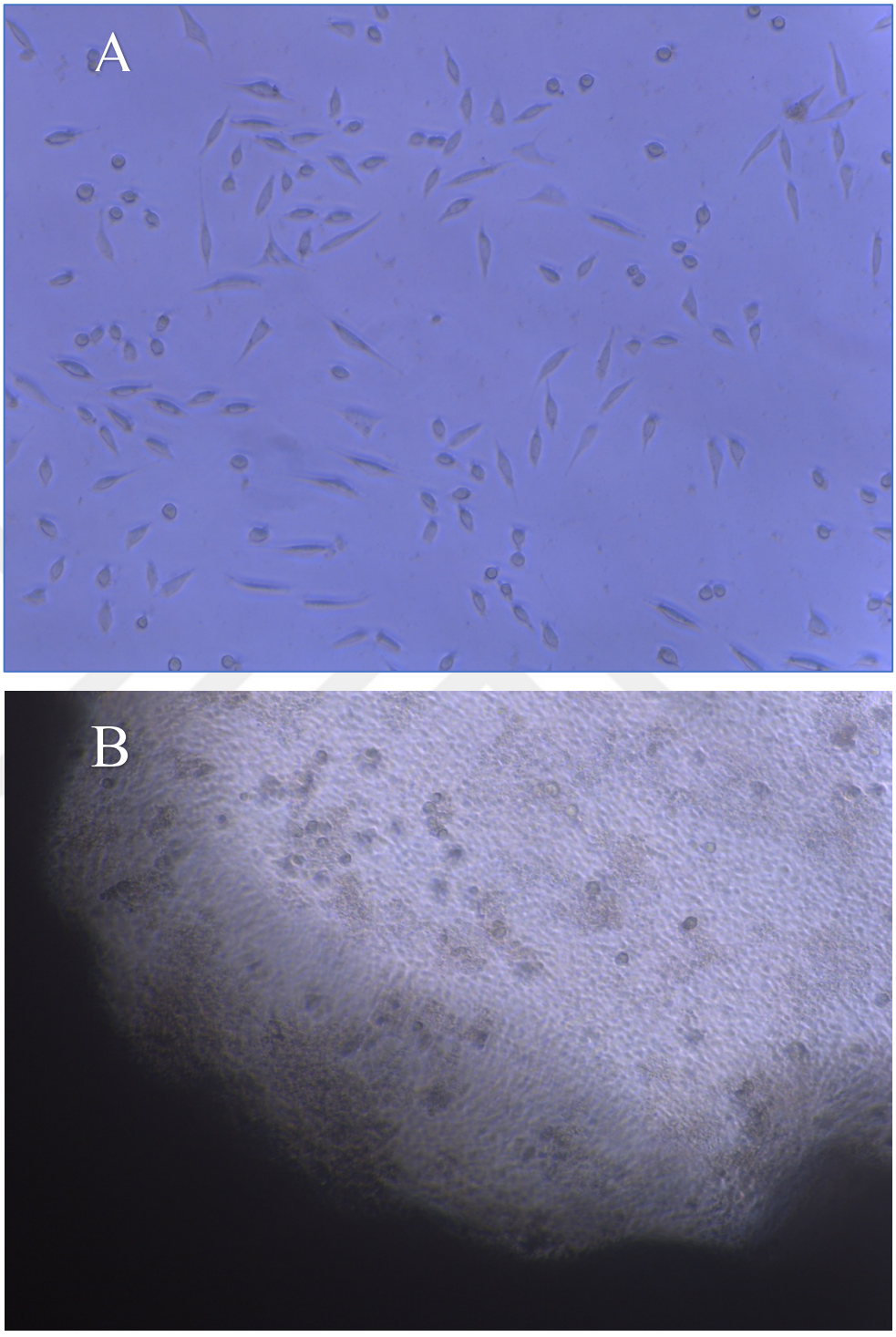

Figure 21. control group (A) and Col-BS group (B) after cell culture

Following the same procedure and ratios, another sample of the COL/nHA/CS scaffold was prepared, this time incorporating a crosslinker (glutaraldehyde) added after casting the solution into molds.

The collagen-based scaffolds composed of collagen 75%, (nHA)20%, and CS 5%, cross-linked with **glutaraldehyde**, were fabricated to evaluate their effects on the adhesion and proliferation of L929 mouse fibroblast cells. The results showed that the duration of the scaffolds was as long as a favorable environment for cell proliferation compared to collagen scaffolds without crosslinker, while also imposed a limit on excessive cell growth. The Microscopic analysis detected very limited cell adhesion, with cells manifesting less proliferation and attachment when compared to control cell cultures on tissue culture plates. Furthermore, the scaffolds supported cell attachment to a very low degree, with fewer cells displaying a well-spread morphology and most cells undergoing apoptosis, as shown in Figure 16 and Figure 17.

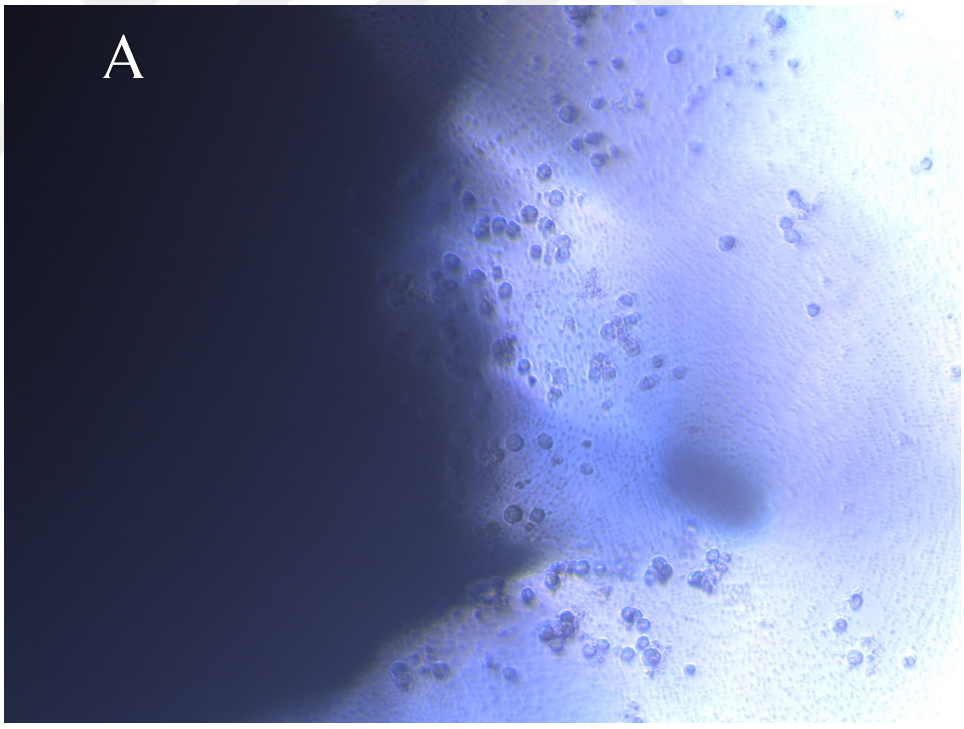

Figure 22. Col- BS + Glutaraldehyde (dead cells)

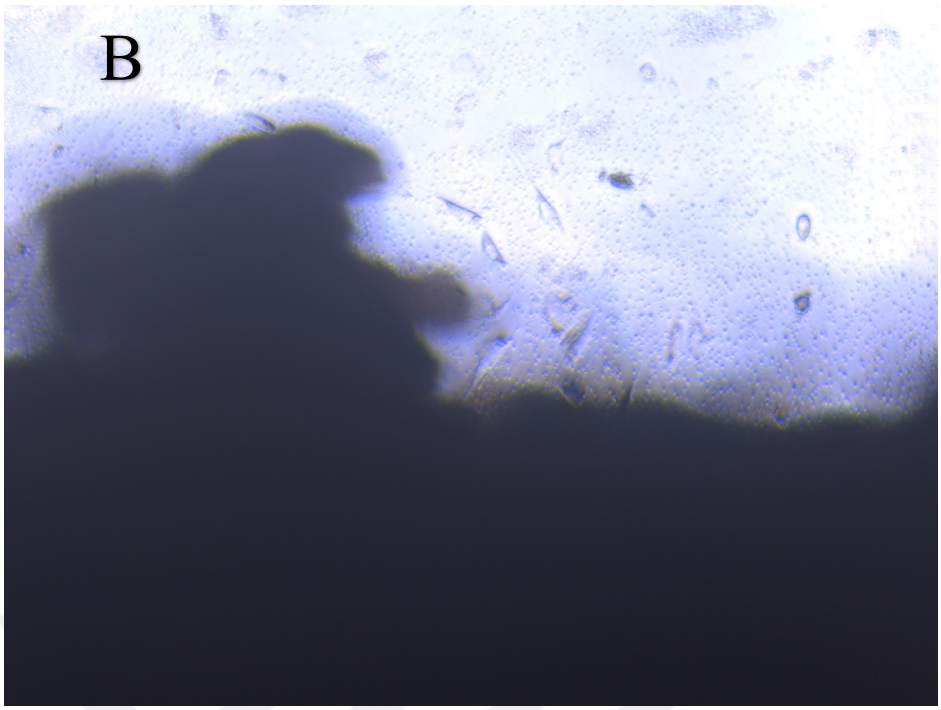

Figure 23. Col- BS + Glutaraldehyde (survival cells)

4.3.2 PLGA-Based Scaffold. (PLGA) scaffolds (Figure 18) were fabricated by SC/PL method without a crosslinker (Glutaraldehyde) and their biocompatibility was evaluated by using the L929 mouse fibroblast cell line. The outcomes demonstrated that L929 cells attached excellently to the PLGA scaffolds and exhibited significant proliferation during the incubation period. Morphological inspections under an inverted microscope revealed that the cells maintained their typical fibroblast-like shape, spreading uniformly throughout the scaffold superficially and entirety.

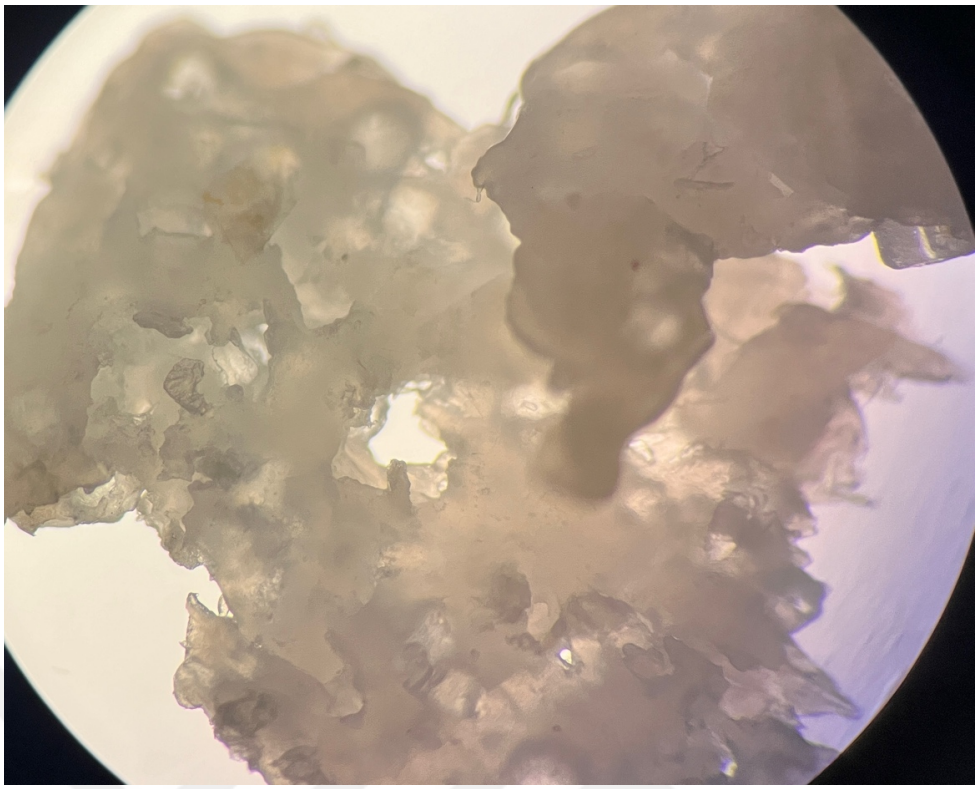

Figure 24. PLGA- BS, The PLGA-based scaffold manifests as a porous, irregular structure ideal for encouraging cell attachment and tissue integration. Importantly, it also mimics the trabecular architecture of bone. The scaffold's morphology suggests it could support various cell types

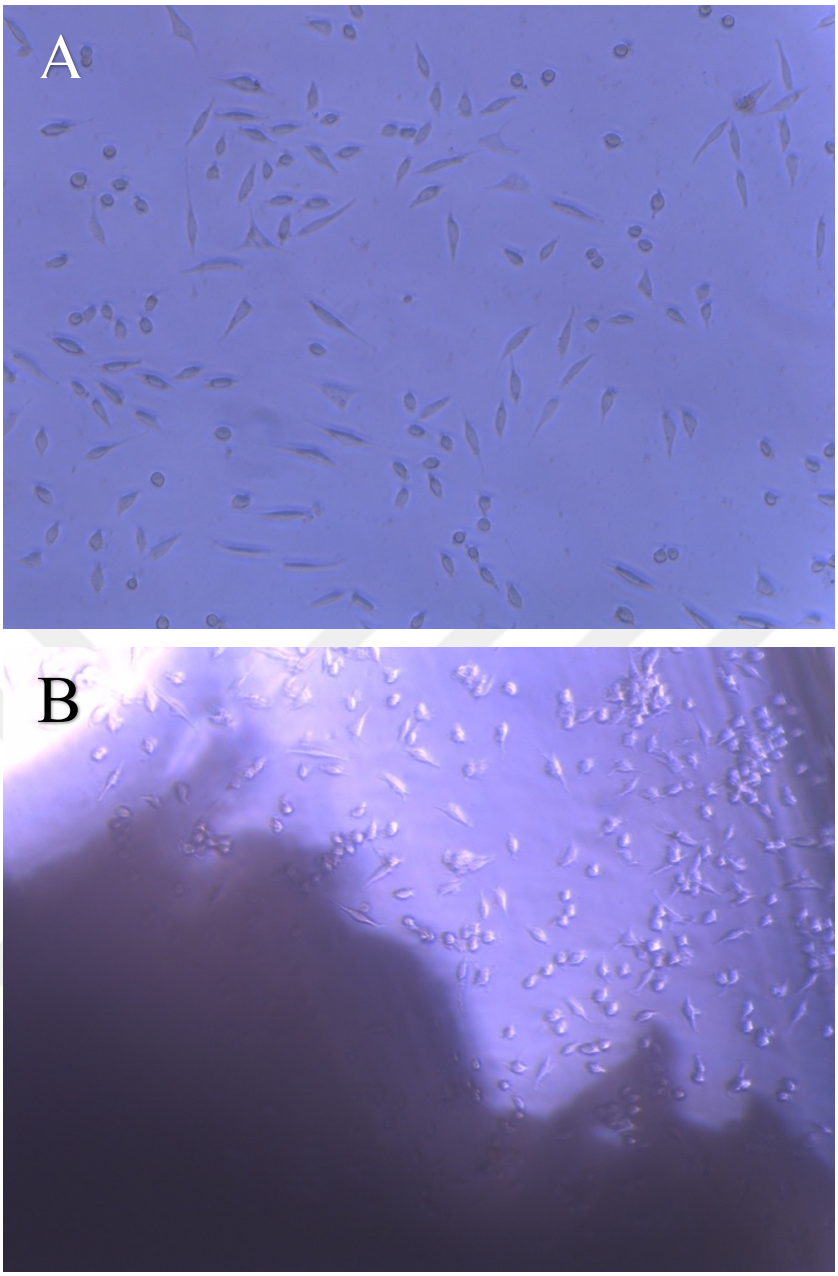

Figure 25. Control group (A), PLGA-BS (B) The outcomes appeared that the cells from L929 tied healthy to the PLGA scaffolds and demonstrated critical proliferation during the incubation period, also the penetration of L929 into eternal parts of the scaffold is obvious, Morphological monitoring under an inverted microscope (Leica, MC120 HD) revealed that the cells preserve their perfect fibroblast-like shape, circulations uniformly and actively throughout the scaffold surface and entirety.

4.4 Mechanical Test

Scaffold Type	Parameter	Results	Literature Data for Comparison
Col-BS with Glu	Peak Stress	5.5 MPa	0.76 MPa (glutaraldehyde treated), 0.35 – 0.6 MPa (other crosslinkers)
	Strain	~30%	Enhanced strain-to-failure characteristics are reported
Col-BS	Peak Stress	3.1 MPa	Hundreds of kPa to a few MPa
	Strain	~20%	Large deformations occur in the hydrated condition
PLGA-BS	Peak Stress	2.9 MPa	2.5-3 MPa
	Strain	20-25%	Expected strain behavior is maintained

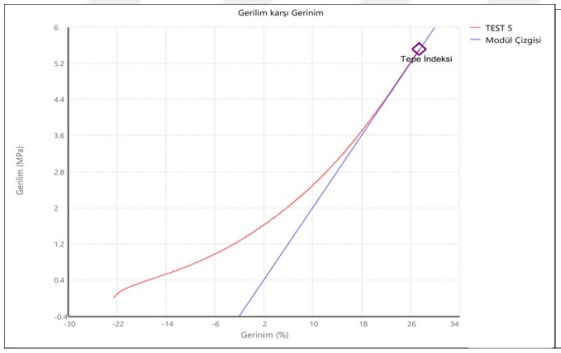

Figure 26. MT of Col-BS + GLU

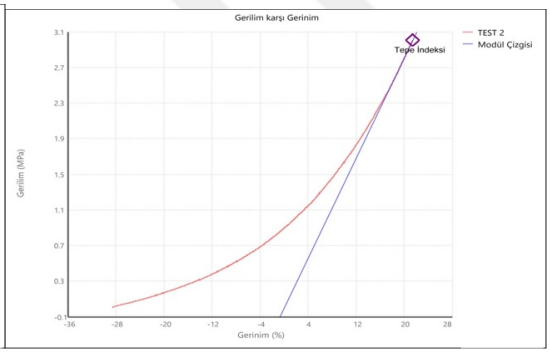

Figure 27. MT of Col-BS

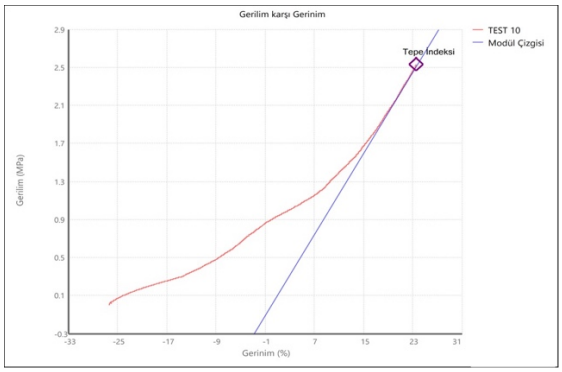

Figure 28. MT of PLGA-BS

Chapter 5

Discussion, Conclusion, and Recommendations

5.1 Discussion

5.1.1 Materials and Ratios. Two different artificial bone scaffolds were prepared as natural polymer and synthetic polymer by solvent casting / particular leaching techniques. The natural scaffold blend contains 75% collagen, 20% nano-hydroxyapatite (nHA), and 5% chitosan (CS), while the synthetic scaffold blend contains 75% PLGA, 20% nHA, and 5% CS, maintaining a consistent ratio of 75:20:5. These ratios were chosen according to our hypothesis to achieve our goal.

Combining nanohydroxyapatite (nHA) and chitosan (CS) with bone scaffolds made of natural or synthetic polymers creates a biomimetic environment that enhances bone regeneration. This integration synergistically combines the unique characteristics of nHA and CS. Nanohydroxyapatite (nHA) has bioactivity, strong mechanical characteristics, and the capacity to stimulate bone production (Roveri & Iafisco, 2011). However, chitosan (CS) has biocompatibility, biodegradability, antimicrobial characteristics, and enhanced cell adhesion (Muzzarelli, 2011; Rinaudo, 2006). The resulting structure closely mimics the natural bone extracellular matrix, facilitating enabling effective and rapid bone repair and regeneration (Jayakumar et al., 2010). This approach underscores the importance of carefully selecting appropriate materials in scaffold construction to optimize bone tissue engineering outcomes (Ripamonti, 2010; Teixeira et al., 2009).

The utilization of the SC/PL method provides numerous benefits in the production of bone scaffolds made from polylactic-co-glycolic acid (PLGA) or collagen with nano-hydroxyapatite (nHA) and Chitosan (CS). The functionality of this system allows for accurate regulation of the permeability of the altar, which is crucial for achieving the best possible infiltration of bone cells and vascularization, as evidenced by the research conducted by (Murphy et al., 2002). It also enables the formation of interconnected networks that are crucial for cell migration and nutrient distribution (Liao et al., 2002). Moreover, the addition of nHA to PLGA-based scaffolds improves their mechanical properties and bioactivity (Rezwan et al., 2006a). Introducing nano-hydroxyapatite (nHA) to collagen-based scaffolds enhances their mechanical durability without

compromising their biocompatibility. The SC/PL ensures excellent biocompatibility by efficiently eliminating detergents and enables controlled biodegradability, which is especially advantageous for PLGA scaffolds (Makadia & Siegel, 2011b). Additionally, it facilitates the regulated discharge of bioactive particles, thereby improving the process of bone rejuvenation (Wei & Ma, 2004). The system's capacity to incorporate components allows for a smooth distribution of nHA and CS within both PLGA and collagen matrices, which is crucial for facilitating bone growth and cell attachment (Peter et al., 2010). When compared to more sophisticated methods, SC/PL is both cost-effective and scalable, making it potentially suitable for use in clinical settings (Salgado et al., 2004). By adjusting the ratio of lactic acid to glycolic acid, the degradation rates of PLGA scaffolds can be precisely controlled. The degradation of collagen scaffolds can be altered by utilizing crosslinking methods (Gentile et al., 2014c). The SC/PL system is highly advantageous in in bone tissue engineering due to its ability to provide a combination and controlled structure. The bone scaffolds, composed of a blend of PLGA (75% PLGA, 20% nHA, 5% CS) and collagen (75% collagen, 20% nHA, 5% CS), provide regulated structure, compatibility with living organisms, and adaptable to various applications.

5.1.2 Collagen-Based Scaffold. The existence of an elevated concentration of collagen in the scaffold could significantly influence the observed cytotoxicity, particularly when it interacts with other scaffold components like nHA and CS. A higher concentration of collagen may lead to a more compact scaffold structure, potentially restricting cell infiltration, nutrient diffusion, and waste removal. This can result in reduced cell viability and increased cytotoxicity (Glowacki & Mizuno, 2008). Collagen plays a vital role as a main constituent of the extracellular matrix, which is important for cell adhesion and signaling. Nevertheless, when adjacent in high quantity, collagen can lead to the scaffold becoming excessively rigid. This rigidity can affect cell behavior and potentially damage cells due to mechanical stress, which causes cytotoxic effects (Discher et al., 2005). The concentration of collagen may have damage on the reactions between collagen and other scaffold materials, such as nHA and CS. Raised concentrations of collagen may overshadow the beneficial impacts of nHA and CS or induce phase separation or uneven distribution enclosed by the scaffold, thereby making worse cytotoxicity (Chattopadhyay & Raines, 2014b). investigations have indicated that although collagen is almost always compatible with

living organisms, it is obligatory to optimize its concentration. raised levels of collagen have been associated with heightened stiffness of the scaffold and reduced cellular functions, potentially causing toxicity in specific cell types, like fibroblasts (Rezwan et al., 2006c). The scaffold's rigidity, determined by collagen concentration, can significantly impact cellular activity. Excessive rigidity can cause stress-induced cell death, particularly in more sensitive cell types like fibroblasts (Levental et al., 2009). The proportion of collagen relative to other materials like nHA and CS is crucial. Excess collagen in the scaffold could hinder the incorporation of other components, thereby affecting overall biocompatibility and functionality (R. Zhang & Ma, 1999a). The cytotoxicity observed in our scaffold is likely due to the high collagen concentration, which resulted in increased scaffold density and rigidity, thereby compromising cell viability. To improve the scaffold's biocompatibility, these effects could be mitigated by either adjusting the collagen concentration or modifying the scaffold preparation process to achieve a balance between physical and biochemical properties.

5.1.2.1 Col-BS Mechanical Test in Comparison To The Literature. Stress-Strain Behavior: The stress-strain behavior of collagen scaffolds has been shown to possess a viscoelastic character with an elastic portion at the start and also a failure zone which is-plastic. This is particularly the case when the scaffold is unstably compressed. Responses to mechanical tests usually start with softening before progressive hardening sets in at higher stress levels as is particularly the case with hydrated conditions as collagen fibers start reorganizing (Shen et al., 2008).

Compressive Modulus: The compressive modulus with respect to specially collagen scaffolds has normally been in the range of several Kpa to several hundred kPa*/in that case, the usability of these scaffolds depends on the cross linking attack and hydration level. For example (Abou Neel et al., 2006). describes dense collagen matrices achieved through repeated compressions to develop Implants with compressive moduli that get higher as collagen fibers compress successively increases (Abou Neel et al., 2006).

Yield Stress & Toughness: There is variation in the yield stress for collagen scaffolds especially due to crosslinking and also the source whereby collagen is extracted from (bovine, porcine, ovine). Certain studies like the one done by

(Ghodbane & Dunn, 2016). have revealed yield stresses in the range of 20-40kPa in cross linked collagen scaffolds (Ghodbane & Dunn, 2016).

Hydration Effects: The tensile properties of collagen-based scaffolds can change a lot depending on the conditions under which they are evaluated whether dry or when moistened. Generally, hydration softens the scaffold as it decreases the stiffness and modulus but increases the extensibility. For instance, in the work done by (McManamon et al., 2020). collagen structures showed much lower stiffness when hydrated (McManamon et al., 2020).

Comparison with The Results:

Peak stress: 3.1 MPa, Strain: ~20%: This shows a relatively high peak stress for a collagen scaffold, which is an indicator of a stiff and strong material. This suggests quite a level of stress tolerance than what is commonly reported which typical peak stresses for collagen scaffolds has been reported to be in the region of several hundreds of kPa sometimes up to 1MPa. This suggests a possibly high degree of crosslinking or a reinforced structure, which could explain the increased tolerance to stress. It conforms to use of collagen scaffolds is support bearing for applications say tendon or ligament replacement where high mechanical durability is warranted (Gentleman et al., 2003).

5.1.3 Collagen-BS + Glutaraldehyde. On the other hand a common cross-linking agent (Glutaraldehyde), used to stabilize collagen, is known for its ability to enhance the mechanical properties of scaffolds by forming covalent bonds between collagen fibers. However, residual glutaraldehyde can contribute to cytotoxicity, which might explain the reduced cell viability and proliferation observed. Previous research has shown that while glutaraldehyde cross-linking effectively preserves scaffold structure, it can also lead to the release of aldehyde groups that interact with cellular components, impairing cellular processes (Sung et al., 1998).

As previously mentioned, the incorporation of nHA and CS, while intended to enhance the scaffold's bioactivity and mechanical properties, might have also contributed to its rigidity, thereby inhibiting cell proliferation. Although CS has been reported to enhance biocompatibility and promote cellular functions, its combination with glutaraldehyde-crosslinked collagen and nHA may have altered the scaffold's

surface chemistry and mechanical properties, creating suboptimal conditions for fibroblast proliferation.

5.1.3.1 Col-BS + Glu Mechanical Test in Comparison To The Literature.

Mechanical Properties: The process of glutaraldehyde crosslinking generally qualities the induced structure of collagen scaffolds leading to an increase in both the compressive strength and the stiffness of the scaffold material. For instance, glutaraldehyde crosslinking has been shown to increase the stiffness of scaffolds by four times as compared to non-crosslinked scaffolds (Haugh et al., 2011). Studies like that of (Barnes et al., 2007) revealed that collagen scaffolds treated with glutaraldehyde achieved peak stresses of approximately 0.76 MPa while maximum peak stresses produced by other crosslinking agents ranged from 0.35-0.6 MPa (Barnes et al., 2007).

Compressive Strength: Several studies have also availed literature on the effect of glutaraldehyde crosslinking and how it increases the compressive modulus significantly. For instance, collagen scaffolds that were cross-linked with glutaraldehyde displayed a compressive modulus of several hundred kPas to several MPas as related to the concentration of the crosslinker and the time of treatment (Thompson & Czernuszka, 1995).

Strain Behavior: Crosslinked scaffolds, however, also exhibit better mechanical performance in terms of strain-to-failure, that is, the capacity to take more strain before failure is enhanced via crosslinking. Glutaraldehyde usually makes the yield strength higher and limits the amount of deformation that can take place before fracture occurs (Xu et al., 2011).

Comparison with The Results: In this test, the maximum stress which is experienced increases to almost about 5.5 MPa at a strain slightly above 30%, which is significantly high when compared to the literature values.

5.1.4 PLGA-Based Scaffold. The (PLGA 75% /nHA20% /CS 5%) scaffolds provide a favorable environment for cell adherence and growth, consistent with previous reports emphasizing the suitability of PLGA for tissue engineering applications (Anderson & Shive, 1997b)(Danhier et al., 2012), particularly for especially on bone cell growth as observed from previous results. The observed cell behavior aligns with the known characteristics of PLGA, including its controlled biodegradability, which allows the scaffold to degrade gradually as new tissue forms, eliminating the need for surgical removal. During the degradation process, lactic and glycolic acids are released, which are naturally metabolized by the body, thereby reducing inflammatory reactions. The biocompatibility of PLGA is crucial for successful scaffold design in regenerative medicine (Jain, 2000b) (Makadia & Siegel, 2011b). The consistent cell distribution and proliferation improve the ability of PLGA scaffolds to support cellular activities essential for tissue engineering and regenerative medicine.

5.1.4.1 PLGA-BS Mechanical Test in Comparison To The Literature.

Stress-Strain Behavior. Some interesting conclusions can also be presented when yielding – Elastic Modulus & Yield Stress: (Leung et al., 2007), as well as most internal reviews, present the compressive stress-strain characteristics of PLGA scaffolds. Subjective opinion Then begins Elongation after slight yield behavior followed by PLGA plastic deformation (Leung et al., 2007).

As far as the data is concerned, the curve attached appears to show a linear increase and negligible elastic recovery at peak strains of about 20-25% which has already been experienced.

Compressive Strength: The preliminary studies of PLGA scaffold report that the maximum compressive strength which can be achieved are in the region of 2.5 – 3 MPa in the different studies carried out emanating to originating porosity and material formulation. It seems that your test fits into this range as the maximum stress level at the peak point on your curve tends to go as high as 2.9MPa. This is also consistent with the other PLGA studies, which suggest that this load compressive strength is suitable for structures that render physical bearing, such as bone tissue engineering mulsh (de Castro et al., 2021).

Strain Rate Sensitivity: The manner in which the curve appears to behave, seems to be similar to the dispersal when PLGA is known to show rate Sensitive elastic Strain

Kinematics. For example, it has been observed that in some studies PLGA scaffolds are more ductile under slow strain rates and more brittle under high strain rate. This feature had been observed in comparable compression samples studied by (de Castro et al., 2021).

Mechanical Deformation: The deformation pursuits, which as well as have been confirmed on the scaffolds appear to concur with the observations made by (Wu et al., 2006) Compression tests were carried out on scaffold models and revealed that scaffolds with 90 % porosity geled poly Lactic-co-glycolic acid showed gross stress softening together with very large strains and stress yielding. (Wu et al., 2006).

5.2 Conclusion and Recommendations

This study comprehensively examined the thermal, structural, and biological qualities of various biomaterials, including PLGA, collagen, nano-hydroxyapatite (nHA), and chitosan, to design and synthesize artificial bone scaffolds for TE applications. The results from differential scanning calorimetry (DSC) and Fourier-transform infrared spectroscopy (FTIR) provided high premeditations into the thermal stabilizations and chemical structure of these substances, aligning with existing literature and confirming their suitability for scaffold fabrication. The DSC analysis indicated that the thermal behaviors of PLGA, collagen, nHA, and chitosan were consistent with known properties, highlighting their potential for use in tissue engineering and regenerative medicine. Specifically, the observed glass transition temperatures, melting points, and degradation characteristics for each material were within expected ranges, confirming their stability under physiological conditions. The FTIR analysis further established the presence of typical functional groups, validating the chemical integrity and purity of the materials.

The cell culture studies provided significant insights into the biocompatibility and cytotoxicity of the manufactured scaffolds. The collagen-based scaffold, designed as a natural artificial bone, exhibited higher cytotoxicity, likely due to the high concentration of collagen in the first COL-BS without cross-linker and the use of glutaraldehyde for cross-linking in the second COL-BS. This finding underscores the importance of optimizing material compositions, ratios, and processing conditions to enhance scaffold performance. Conversely, as hypothesized, the PLGA-based scaffold demonstrated excellent cell adhesion and proliferation, confirming its suitability as a scaffold material for bone tissue engineering and regeneration. These results emphasize the strong potential of PLGA in supporting the cellular activities necessary for effective tissue regeneration.

Moreover, the integration of nHA and chitosan into natural and synthetic artificial bone scaffolds seeks to increase mechanical properties and bioactivity.

Careful assessment of the mechanical properties of various types of scaffolds is critical as it provides information relating to the use of these scaffolds in different areas of medicine. When collagen scaffolds are linked with glutaraldehyde crosslink (Col-BS with Glu), noticeable advancements in mechanical properties are recorded, with peak stress values greater than what are recorded publicly. This suggests that the

process of glutaraldehyde crosslinking indeed raises the compressive strength and stiffness of these scaffolds so that they are ideal for load-bearing uses like cartilage or bone engineering.

Collagen scaffold with no glutaraldehyde (Col-BS) on the other hand, still maintains excellent mechanical properties as compared to commonly available collagen scaffolds with peak stress values showing the potential of the material to withstand stress. This performance suggests the possibility of utilizing it in high-stress and high-strain applications like tendon or ligament reconstruction. These observations indicate the need for crosslinking and additional support to help achieve better mechanical properties.

Results of tests carried out on PLGA scaffolds (PLGA-BS) also correlate with previous researchers' findings, indicating adequate compressive strength and strain behavior in regard to tissue engineering. The consistency with previously reported mechanical attributes of porous PLGA scaffolds further justifies the use of such materials in bone tissue engineering and other applications that have weight-bearing and structural requirements.

In general, the investigation self-demonstrates the importance of crosslinking and material formulation in the enhancement of scaffold functionality for particular biomedical applications. This sets the groundwork for further extension and use in the field of tissue engineering and regenerative medicine.

In conclusion, this study confirms the potential of PLGA-based scaffolds to significantly outperform collagen-based scaffolds, blending nHA and chitosan, for bone tissue engineering. Future recommendations should focus on optimizing material ratios, cross-linking techniques, and scaffold engineering to progress biocompatibility and mechanical performance. Future research should focus on optimizing material ratios, cross-linking techniques, and scaffold design to further enhance biocompatibility and mechanical performance. The findings contribute valuable knowledge to the field of tissue engineering and regenerative medicine, particularly in advancing the development of biocompatible scaffolds for bone tissue engineering and regeneration.

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