A STUDY OF THE EFFECT OF PREPARATION PARAMETERS ON THE MECHANICAL PROPERTIES OF FREEZE-DRIED GELATIN-ELASTIN-HYALURONATE SCAFFOLDS

MANSOUR QAMASH
Marquette University

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By

Mansour Qamash

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ABSTRACT

A STUDY OF THE EFFECT OF PREPARATION PARAMETERS ON THE MECHANICAL PROPERTIES OF FREEZE-DRIED GELATIN-ELASTIN-HYALURONATE SCAFFOLDS

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This thesis is dedicated to a detailed study of changes in the properties of Gelatin-Elastin-Hyaluronate (GEH) tissue engineering scaffold resulting from changes in preparation parameters. More specifically, utilizing a combination of foaming and freeze-drying techniques, this research investigates the effects of different parameters, including agitation speed, duration time, and chilling temperature on the scaffold's structural integrity, porosity, and mechanical properties. The methodology involves a carefully calibrated process in which the scaffold matrix is initially prepared by incorporating 8% gelatin, 2% elastin, and 0.5% hyaluronate (w/v) into a homogenous aqueous solution, followed by controlled agitation and subsequent freezing at designated temperatures. The freeze-drying stage solidifies the foam structure, creating a porous matrix essential for cell growth and nutrient delivery.

The findings reveal that porosity and mechanical properties, such as compressive Young's modulus, of scaffolds are significantly influenced by fabrication parameters, with higher agitation speeds and longer duration times leading to increased porosity and decreased modulus. Moreover, the degradation rates of the scaffolds processed at both −20 and −80°C were found to be comparable, indicating a similar level of preservation in physiological conditions. Morphological analyses, including laser microscopy and scanning electron microscopy (SEM), indicated optimal pore sizes (100–300 μm) that promote effective cell interaction and tissue regeneration, confirming the successful application of the freeze-drying and foaming methods in creating highly interconnected porous structures. Based on the findings, a decrease in chilling temperature correlates with a slight increase in pore size within the scaffold matrix. The methodical fabrication process developed in this study emphasizes the control of agitation speed and duration to modulate scaffold porosity, which is an essential characteristic for cellular infiltration and vascularization in tissue engineering. The research outcomes demonstrate that scaffold properties can be finely adjusted through the preparation process, offering the potential to match the structural needs of specific tissue engineering applications.

The thesis contributes significant advancements in scaffold design, providing a robust framework for the development of tissue scaffolds with controlled porosity and improved mechanical properties. By understanding and harnessing the effects of fabrication parameters, this research offers a pathway to design scaffolds that more accurately replicate the extracellular matrix, promoting enhanced tissue repair and regeneration.

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CHAPTER 1: INTRODUCTION

1.1. Background

Tissue engineering has emerged as a transformative field at the confluence of biology, materials science, and engineering, aiming to develop functional substitutes for damaged or diseased tissues. The concept was formalized in the late 20th century, though its roots trace back to earlier scientific inquiries into cell culture and regeneration. A pivotal milestone in its evolution was the development of the first artificial skin in the 1980s, which demonstrated the potential of engineered tissues in clinical applications [1]. Since then, the field has expanded to include a vast array of tissues, including bone, cartilage, vascular grafts, and more complex organs.

The progress in tissue engineering has been driven by technological advancements, notably in biomaterials, scaffold fabrication techniques (such as electrospinning, 3D printing, and freeze-drying), and bioreactor design [2]. These technologies have enabled the creation of scaffolds that not only support cell growth and differentiation but also closely replicate the physical, chemical, and mechanical environments of native tissues. Innovations such as the incorporation of bioactive molecules and dynamic mechanical stimuli in scaffold designs have further enhanced the functionality of engineered tissues, moving the field closer to the realization of fully integrated tissue replacements.

Scaffolds are central to the practice of tissue engineering, serving as the physical substrates that support the formation of new tissue. Their role is multifaceted, encompassing the provision of mechanical support, the facilitation of cell attachment and proliferation, and the delivery of biochemical cues necessary for tissue development [3]. By mimicking the extracellular matrix (ECM), scaffolds create an environment that is not only structurally beneficial to tissue formation but also biochemically helpful to cells [4].

The ECM in native tissues provides crucial signals that guide cell behavior, including differentiation, migration, and extracellular matrix deposition. Similarly, engineered scaffolds are designed to present cells with cues that influence their function and outcome. This is achieved through careful selection of scaffold materials and fabrication methods that result in desired physical properties (such as porosity and stiffness) and biochemical functionalities (such as the incorporation of growth factors or peptides) [4,5].

The effectiveness of a scaffold in regenerative medicine is largely determined by its ability to integrate with the host tissue, a process that requires the scaffold to be biocompatible, to possess mechanical properties that match the target tissue, and to degrade at a rate that matches the formation of new tissue [5]. Thus, the development of scaffolds involves a delicate balance between mimicking the natural ECM and tailoring the scaffold's properties to meet the specific needs of the engineered tissue.

As mentioned earlier, one of the pivotal aspects of the successful integration of engineered scaffolds into target tissues involves ensuring their mechanical compatibility, specifically through aligning the scaffold's stiffness and elasticity with the native tissue characteristics [6]. Mechanical properties are fundamental in determining how a scaffold interacts with the biological environment, influencing not only the physical support it provides but also how it guides cellular behavior and tissue development [7]. Stiffness, or the scaffold's resistance to deformation under applied force, must be carefully tuned to resemble that of the target tissue. A mismatch in stiffness can lead to inadequate mechanical support or can even cause undesirable cellular responses, affecting the regeneration process [8]. For example, scaffolds intended for bone tissue engineering require higher stiffness and strength compared to those used for soft tissue regeneration, such as skin, gingiva, or vascular tissues [9]. Elasticity, the ability of the scaffold to return to its original shape after deformation, is equally important, especially for soft tissues. The scaffold's elasticity affects its ability to accommodate changes in shape, which is crucial for maintaining normal physiological

functions [10]. Research has shown that the mechanical properties of scaffolds can influence cell adhesion, proliferation, migration, and differentiation—key processes in tissue regeneration [11]. Cells can sense the mechanical cues from their surrounding matrix, a phenomenon known as mechanotransduction, and respond in ways that affect the tissue healing process [12]. Therefore, achieving mechanical compatibility with native tissues is not just a matter of structural support but also of facilitating the appropriate cellular activities for successful tissue integration and regeneration.

The mechanical properties of scaffolds are influenced by several parameters, such as fabrication methods, material selection, and cross-linking, which play a crucial role in determining their applicability for specific tissue engineering purposes. For instance, the method of scaffold fabrication significantly impacts the scaffold's porosity, interconnectivity, and, consequently, its stiffness and elasticity [13]. Material selection is another critical factor affecting the mechanical properties of scaffolds. The choice of materials, ranging from natural polymers like gelatin, elastin, and hyaluronate to synthetic polymers such as polylactic acid (PLA) and polyglycolic acid (PGA), dictates the scaffold's biocompatibility, degradation rate, and mechanical behavior [9]. Combining different materials or incorporating composite structures can enhance scaffold performance by combining the advantageous properties of each component [14]. Cross-linking density in hydrogel-based scaffolds, achieved through chemical or physical methods, on the other hand, can modulate their mechanical stiffness and degradation behavior, allowing for the fine-tuning of scaffold properties to match those of the target tissue [15].

Optimization of these parameters—fabrication methods, material selection, and cross-linking density—requires a comprehensive understanding of their effects on the scaffold's mechanical and biological properties. Through systematic investigation and adjustment of these variables, it is possible to develop scaffolds that not only meet the mechanical requirements of the target tissue but also support the dynamic processes of cell adhesion, proliferation, migration, and

differentiation. This optimization effort is essential for advancing scaffold-based tissue engineering strategies, moving closer to the realization of scaffolds that effectively mimic the natural extracellular matrix and facilitate successful tissue regeneration.

Recently, gelatin, elastin, and hyaluronate have emerged as popular choices for use in the fabrication of scaffolds for soft tissue engineering applications, due to their unique properties that mimic the natural extracellular matrix (ECM) [16]. Gelatin, derived from collagen, offers excellent cell adhesion and proliferation properties, making it a staple in scaffold design for its biocompatibility and ease of processing [17]. Elastin, known for its elasticity, allows scaffolds to withstand mechanical stresses, enabling the engineered tissues to replicate the dynamic environments of native tissues [18]. Hyaluronate, a critical component of the ECM, enhances moisture retention, cell proliferation, and migration, essential attributes for tissue repair and regeneration [19].

Recently, Tayebi et al. [20], Rasoulianboroujeni et al. [21], and Dehghani et al. [22] integrated these materials, i.e., gelatin, elastin, and hyaluronate, to develop scaffolds that exhibit enhanced biological response, facilitating more effective tissue regeneration. Their methodology, which utilizes a combination of foaming and freeze-drying techniques, has led to the production of scaffolds with an optimized composition, demonstrating considerable promise particularly in dental applications. However, the fabrication process they employed involves several critical parameters. Given the direct impact of these parameters on the mechanical properties of the scaffolds, there is an acknowledged need for further investigation, particularly concerning chilling temperature, as well as agitation time and speed. This thesis is focused on studying the properties of these scaffolds by a detailed investigation into these specific preparation parameters. The project aims to study the effects of agitation speed, duration time, and chilling temperature on the scaffold, analyzing their individual and combined influences on structural integrity, porosity, and mechanical strength.

1.2. Research Objectives

This thesis is dedicated to advancing the field of tissue engineering through studies of the properties of Gelatin-Elastin-Hyaluronate (GEH) scaffolds. With a focus on the precise influence of specific preparation parameters—namely agitation speed, duration time, and chilling temperature—this study aims to investigate how these factors collectively impact the scaffold's structural integrity, degradation, porosity, and mechanical properties. The objective is to explore the complexities involved in scaffold fabrication, enhancing their functionality for soft tissue engineering applications. By assessing the individual and combined effects of these parameters on GEH scaffolds, this research seeks to refine the manufacturing process, thereby optimizing scaffold performance and tailoring its characteristics to better meet the demands of medical applications.

The investigation is structured around a series of detailed objectives, beginning with an exploration of how agitation speed affects the scaffold's mechanical properties, which the essential for ensuring tissue integration and regeneration. It extends to examining the role of duration time on the scaffold's stability and degradation rate, alongside the influence of chilling temperature on its internal structure and mechanical properties. These areas of focus are crucial for understanding the scaffold's behavior in a biological context and its potential for tissue engineering applications. By exploring the synergistic effects of these preparation parameters, the research intends to develop a more robust fabrication protocol that maximizes the bioactivity and mechanical performance of GEH scaffolds. Ultimately, this study is set to make significant contributions to regenerative medicine by enhancing the understanding of the effect preparation parameters on the scaffolds' properties. Through this comprehensive approach, the thesis endeavors to facilitate the development and use of freeze-dried scaffolds, thus promoting progress in regenerative medicine and advancing medical applications that can benefit from improved scaffold design.

1.3. Structure of the Thesis

This thesis is organized into five comprehensive chapters, each designed to systematically explore the properties of freeze-dried Gelatin-Elastin-Hyaluronate scaffolds, focusing on their fabrication parameters and their implications for tissue engineering and regenerative medicine. The structure is laid out as follows to provide a consistent flow from the introduction of the subject to the presentation of research findings and conclusions:

Following the current chapter, i.e., introduction, the Literature Review chapter contextualizes the research within the broader scientific dialogue. It presents a comprehensive review of the scaffold fabrication techniques, particularly focusing on freeze-drying and the utilization of gelatin, elastin, and hyaluronate. This examination of previous studies lays the basis for understanding the significance and potential impact of this thesis's contributions to the field.

In the Materials and Methods section, the thesis details the experimental approach and the specific methodologies employed in fabricating and analyzing the GEH scaffolds. This chapter is critical for understanding how the research was conducted and the rationale behind the chosen preparation parameters, including agitation speed, duration time, and chilling temperature, and their expected impact on the scaffolds' properties.

The Results chapter delves into the findings of the current thesis, showcasing the effects of the varied preparation parameters on the structural and mechanical properties of the GEH scaffolds. This section meticulously analyzes findings related to porosity, structural integrity, mechanical strength, and degradation.

Concluding the thesis, the Conclusion chapter presents the research findings, discussing their implications for regenerative medicine and tissue engineering. It evaluates how the study's objectives were met and reflects on the broader significance of GEH scaffolds. Additionally, it offers directions for future research.

CHAPTER 2: LITERATURE REVIEW

2.1. Introduction

This chapter investigates scaffold fabrication via freeze-drying in tissue engineering, emphasizing its role in producing porous structures ideal for tissue repair. We start with an overview of freeze-drying, noting its utility in scaffold creation. The focus then shifts to studies on gelatin, elastin, and hyaluronate scaffolds, highlighting their biocompatibility and effectiveness. We also examine the mechanical properties critical to scaffold design, analyzing how material choices and design impact mechanical stability. Additionally, we explore how preparation parameters, such as freeze-drying conditions and material concentrations, affect scaffold performance, including degradation and bioactivity. This review aims to summarize current research on scaffold development, pointing out significant advancements and future research directions to improve scaffold function in regenerative medicine.

2.2. Overview of Freeze-Drying as a Scaffold Fabrication Method

Freeze-drying, also known as lyophilization, is identified as a widely used method for the fabrication of scaffolds in tissue engineering [23,24]. This process involves the sublimation of water from a frozen sample under vacuum, preserving the structural integrity and porosity of the scaffold. The freeze-drying process is particularly suitable for the fabrication of gelatin-elastin-hyaluronate scaffolds due to the hydrophilic nature of these biomaterials [25]. While using this approach, it should be considered that the process' parameters, such as freezing rate and primary and secondary drying conditions, significantly influence the mechanical properties of the resulting scaffold.

As shown in Figure (2.1), this process begins with the freezing of the scaffold material, which is typically a solution or suspension of the biomaterials. Following the freezing stage, the primary drying phase begins, where the ice crystals formed are sublimated under vacuum. The rate of sublimation depends on the temperature and pressure conditions, as well as the thermal conductivity of the scaffold material. Higher temperatures increase the sublimation rate but could also lead to the collapse of the scaffold structure. Therefore, the primary drying conditions need to be carefully controlled to ensure efficient removal of ice while maintaining the structural integrity of the scaffold. After the primary drying phase, the secondary drying phase is carried out to remove the unfrozen water bound to the scaffold material. This stage is typically conducted at higher temperatures than the primary drying phase. The secondary drying conditions, particularly the final temperature, could affect the residual moisture content of the scaffold, which could influence its mechanical properties and degradation rate [23,24].

The freeze-drying process is a versatile and effective method for the fabrication of gelatin-elastin-hyaluronate scaffolds [25]. However, the process parameters need to be carefully optimized to achieve a balance between scaffold porosity and mechanical strength [26,27]. Future research should focus on understanding the relationships between process parameters and scaffold properties and developing strategies for the precise control of these parameters.

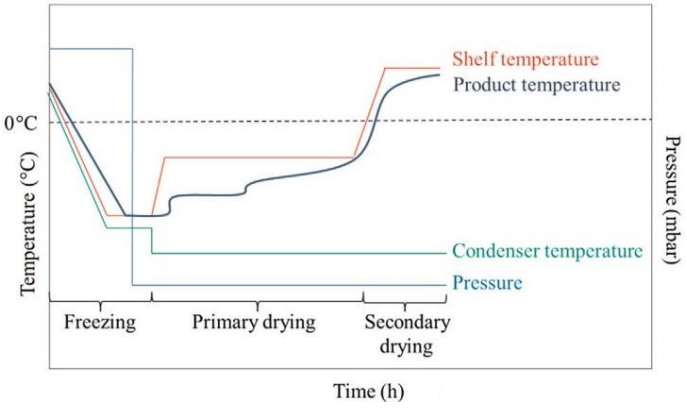


Figure 2.1: Temperature and pressure across freeze-drying stages [28]

2.3. Previous Studies on Gelatin, Elastin, and Hyaluronate-Based Scaffolds

Previous studies on gelatin, elastin, and hyaluronate-based scaffolds have significantly contributed to the development of biomaterials for tissue engineering and regenerative medicine. These biomaterials have been extensively explored for their ability to mimic the extracellular matrix, support cell attachment, proliferation, and differentiation, and promote tissue regeneration.

Gelatin, derived from collagen, is a vital biomaterial in tissue engineering due to its biocompatibility, biodegradability, and ability to mimic the extracellular matrix (ECM), facilitating cell adhesion, proliferation, and differentiation [29]. Its versatility allows for the fabrication of scaffolds through various methods, including electrospinning, 3D printing, and laser fabrication, to support the regeneration of diverse tissues [30]. Gelatin scaffolds are particularly favored for their enzymatic degradation capabilities, which are preserved even after chemical modifications, making them ideal for transient applications where scaffolds gradually degrade as new tissue forms [31]. These scaffolds have been extensively utilized across various applications, including bone, cartilage, skin, nerve, and vascular tissue engineering [32]. These scaffolds, especially when combined with other polymers, display enhanced physicochemical and biomechanical properties conducive to tissue regeneration [33]. Advanced fabrication techniques have enabled the development of scaffolds with controlled porosity and mechanical properties, facilitating the delivery of cells and bioactive molecules to the desired sites, thereby promoting effective tissue repair and regeneration [34,35].

Specifically, in dental tissue engineering, gelatin-based scaffolds have shown significant promise in supporting the regeneration of dental tissues, such as periodontal ligaments and alveolar bone [36]. The adaptability of gelatin scaffolds to various fabrication techniques allows for the creation of structures that closely mimic the natural environment of dental tissues, thereby enhancing cell-material interactions and promoting successful tissue integration and function restoration in dental applications [37,38]. Application of gelatin in the fabrication of scaffolds for

tissue engineering shows its significant potential across a variety of tissue types, including dental tissue engineering. The ability of gelatin-based scaffolds to support cell growth, differentiation, and the regeneration of functional tissues underscores their vital role in advancing the field of tissue engineering.

In another study about gelatin scaffolds, Rasoulianboroujeni et al. [21] developed dual porosity gelatin scaffolds with enhanced cell infiltration and proliferation by performing the foaming step. This research underscores the importance of scaffold architecture and porosity in promoting cellular activities critical for tissue engineering, such as nutrient transport and waste removal, while the use of a gelatin provided a conducive environment for tissue growth.

Elastin offers significant promise in tissue engineering due to its unique elastic properties and biocompatibility. It plays a crucial role in providing resilience and elasticity to tissues, making it an ideal component in the development of scaffolds that mimic the natural tissue environment. Through advanced fabrication techniques such as electrospinning and co-electrospinning with other polymers like collagen and poly(lactic-co-glycolic acid) (PLGA), elastin-based scaffolds have been engineered to support various tissue engineering applications, offering a balance between mechanical integrity and biological functionality [39,40].

Elastin's integration into scaffold materials has facilitated the development of structures that closely mimic the mechanical and biochemical cues of native tissues. For example, in cardiovascular tissue engineering, elastin enhances the mechanical properties and endothelialization of vascular grafts, promoting cell adhesion and proliferation essential for vascular repair [41,42]. Similarly, in dermal tissue engineering, elastin incorporation has been shown to improve scaffold elasticity, thereby supporting fibroblast infiltration and collagen deposition, crucial for skin regeneration [43,44].

In soft tissue engineering, scaffolds incorporating elastin or elastin-like peptides have been tailored to replicate the viscoelastic properties of soft tissues such as blood vessels, lung, and skin. These scaffolds not only support cellular attachment and proliferation but also enable dynamic mechanical stimulation, promoting tissue-specific cellular responses and matrix deposition. The development of elastin-based scaffolds that are mechanically robust and biologically active offers new opportunities for engineering soft tissues with functional properties akin to native tissues [45,46]. Incorporation of elastin into tissue engineering scaffolds represents a significant advancement in the field, providing materials that closely mimic the natural tissue environment. The unique combination of elastin's biocompatibility, elasticity, and ability to support cellular functions makes it an invaluable component for developing next-generation scaffolds for tissue regeneration.

Combining gelatin with elastin in scaffold fabrication merges the biodegradability and cell-supportive properties of gelatin with the elasticity and resilience of elastin, creating composite scaffolds that offer enhanced mechanical and biological functionalities for tissue engineering applications. These composite scaffolds are particularly advantageous in applications requiring both structural support and flexibility, such as in vascular and skin tissue engineering, where they facilitate a more dynamic interaction with the biological environment. By leveraging the strengths of both polymers, researchers aim to closely replicate the complex architecture and mechanical behavior of native tissues, promoting improved cellular response and tissue integration [39,40].

Research has shown that co-electrospun blends of gelatin and elastin not only support cell attachment and proliferation but also modulate cell behavior due to the bioactive cues presented by the composite material. The presence of elastin in these scaffolds enhances their elastic modulus and tensile strength, making them suitable for applications subjected to mechanical stress, such as in cardiovascular tissue engineering. Furthermore, the combination of gelatin and elastin has been found to encourage the deposition of extracellular matrix components by cells cultured on these

scaffolds, indicating their potential to support the formation of functional tissue constructs [41,42]. The interaction between gelatin and elastin in composite scaffolds represents a forward step in the design of biomaterials that more accurately mimic the natural extracellular matrix, supporting the regeneration of tissues with complex mechanical and biological requirements.

Hyaluronic acid (HA), a naturally occurring polysaccharide found within the extracellular matrix, has gained widespread attention in tissue engineering due to its inherent biocompatibility, hydrophilicity, and capacity to influence cell behavior. HA-based scaffolds, leveraging these properties, are designed to provide a conducive environment for cell attachment, proliferation, and differentiation, crucial for tissue regeneration. Advances in scaffold fabrication technologies, such as photocrosslinking, have enabled the development of HA hydrogels with tunable mechanical properties and degradation rates, providing the specific needs of various tissue engineering applications. These scaffolds have demonstrated promising results, promoting natural wound healing processes such as angiogenesis, and showing minimal inflammatory response when implanted, making them suitable for a broad spectrum of regenerative medicine applications, including soft tissue repair and vascular tissue engineering [47,48].

The versatility of HA scaffolds extends beyond their biocompatibility and hydrophilicity; their chemical structure allows for easy modification, enabling the incorporation of bioactive molecules to further guide tissue repair. This functionalization, combined with the scaffold's intrinsic properties, supports the design of biomimetic environments that closely replicate the native tissue's physical and biochemical cues. Recent developments in HA-based scaffolds have explored various forms, including hydrogels, sponges, and injectable solutions, highlighting their adaptability to different tissue engineering challenges. The ability to tailor scaffold properties to match specific tissue requirements underscores the potential of HA-based materials in advancing regenerative medicine and improving therapeutic outcomes [49].

The combination of hyaluronic acid (HA) and gelatin in scaffold fabrication merges the unique properties of both biomaterials, creating composite scaffolds that offer enhanced biocompatibility, mechanical strength, and bioactivity. This interaction improves scaffold performance in tissue engineering by providing a balanced environment that supports cell adhesion, proliferation, and differentiation while maintaining sufficient structural integrity and flexibility. These composite scaffolds are particularly beneficial in applications requiring robust yet biologically active matrices, such as in the engineering of soft tissues where both mechanical support and cellular interaction are critical [50].

By integrating gelatin, known for its ability to facilitate cell binding through its arginine-glycine-aspartic (RGD) sequences, with the hydrophilic and biodegradable nature of HA, researchers have been able to develop scaffolds that not only possess improved mechanical properties but also exhibit enhanced water retention and degradation characteristics suitable for tissue repair and regeneration. This combination has been found to encourage a more significant deposition of extracellular matrix components by cultured cells, thereby facilitating the formation of functional tissue constructs. The dual presence of HA and gelatin within the scaffold architecture also presents opportunities for further functionalization with growth factors and other bioactive molecules, amplifying their regenerative potential [51].

Leveraging the distinct yet complementary properties of HA, gelatin, and elastin, researchers have embarked on innovative scaffold designs in tissue engineering and regenerative medicine. This strategic combination of materials utilizes HA's biocompatibility and moisture retention, gelatin's mechanical strength and biodegradability, and elastin's elasticity, to create scaffolds that not only support enhanced cell attachment and proliferation but also promise comprehensive tissue repair across diverse applications. The integration of these biomaterials represents a significant advancement in fabricating more effective, biomimetic environments for facilitating tissue regeneration.

Tayebi et al. [20] explored the potential of 3D-printed membranes for guided tissue regeneration, utilizing the combination of gelatin, elastin and HA that take advantage of the mechanical robustness and biodegradability of gelatin and elastin, along with the biocompatibility and hydrophilicity of HA. The study highlights the ability of these composite scaffolds to support cell attachment and proliferation, underscoring their suitability for facilitating tissue repair and regeneration across various applications, including bone and dental reconstruction.

Furthermore, Dehghani et al. [22] applied a 3D-printed membrane composed of HA, gelatin, and elastin as an alternative to the amniotic membrane for ocular surface/conjunctival defect reconstruction. The study demonstrates the composite's ability to support ocular cell adherence and proliferation, indicating its potential as a versatile scaffold for ocular tissue engineering. The scaffold's biocompatibility and similarity to the natural ocular surface environment make it a promising option for reconstructive surgeries. It's noteworthy that this study, which was conducted in Dr. Tayebi's lab, also optimized the composition of gelatin, elastin, and HA, determining that a formulation of 8% gelatin, 2% elastin, and 0.5% HA (w/v) in an aqueous solution exhibited the best rheological properties. Consequently, this composition was selected based on the findings presented in this manuscript.

These studies collectively demonstrate the significant potential of gelatin, elastin, and HA, as well as their combination in scaffold design for diverse tissue engineering applications. The synergistic effect of these materials not only enhances the mechanical and biological properties of the scaffolds but also broadens their applicability across various fields of regenerative medicine, offering new avenues for the development of biomimetic and functional tissue-engineered constructs.

2.4. Exploration of Mechanical Properties in Scaffold Design and Tissue Engineering

The exploration of mechanical properties in scaffold design and tissue engineering is a critical aspect of biomaterial science [24,52], especially in the context of developing scaffolds like those composed of gelatin, elastin, and hyaluronate. These materials, when freeze-dried, offer unique structural and functional characteristics conducive to tissue engineering and regenerative medicine [53,54]. This section delves into the existing literature to understand how the mechanical properties of these scaffolds can be optimized through various preparation parameters.

Mechanical properties, including compressive strength, tensile strength, and elasticity, are paramount in scaffold design as they directly influence the scaffold's ability to support tissue formation and integration with the host tissue. The mechanical integrity of a scaffold ensures that it can withstand physiological forces without degrading or failing structurally before the new tissue is fully formed and functional. The ideal scaffold should mimic the mechanical properties of the tissue it aims to replace or support, facilitating cellular attachment, proliferation, and differentiation [24,52,55].

The freeze-drying technique, commonly employed in the preparation of these scaffolds, significantly affects their porosity, pore size distribution, and mechanical strength. The parameters of the freeze-drying process, such as cooling rate, final temperature, and duration of sublimation, need to be meticulously controlled to optimize the scaffold's structure and mechanical properties. Studies have demonstrated that altering freeze-drying parameters can lead to scaffolds with tailored porosity and mechanical strength, matching the requirements of specific tissue engineering applications [56].

The interplay between scaffold composition and mechanical properties is another area of intense research [57,58]. The ratio of gelatin, elastin, and hyaluronate can be varied to fine-tune the scaffold's mechanical characteristics. For instance, increasing the proportion of elastin in the

scaffold composition has been shown to enhance its elasticity, making it more suitable for engineering tissues that experience dynamic mechanical loads. Conversely, a higher concentration of gelatin has been found to improve compressive strength, beneficial for load-bearing applications like cartilage tissue engineering [24,52,57,58].

The mechanical properties of freeze-dried GEH scaffolds are critical determinants of their suitability for various tissue engineering applications. The preparation parameters, therefore, must be meticulously optimized to achieve a balance between mechanical robustness and biological functionality, aiming to replicate the target tissue's mechanical milieu closely. This delicate balance underscores the significance of understanding the mechanical properties of scaffolds within the broader context of tissue engineering, where the goal is to create biofunctional scaffolds capable of supporting successful tissue regeneration and integration with the host tissue.

2.5. Impact of Preparation Parameters on Scaffold Performance

Embarking on the journey to explain the impacts of preparation parameters on the performance of freeze-dried GEH scaffolds, a rigorous analysis of existing research reveals a complex landscape where these parameters serve as pivotal modulators of scaffold efficacy and functionality. The scaffolding process, a foundation in tissue engineering, necessitates a planning of variables to fabricate constructs that not only support cellular activities but also mimic the biomechanical and biochemical properties of native tissues.

Among these parameters, the freeze-drying process emerges as a critical determinant, influencing pore architecture, which in turn dictates nutrient flow, cellular infiltration, and ultimately, the integration of the scaffold with surrounding tissues [59]. Studies have shown that the lyophilization parameters, including the initial freezing temperature and the rate of temperature decrease, significantly affect the size and distribution of pores within the scaffold, with optimal

conditions leading to uniform pore sizes conducive to homogeneous cell seeding and proliferation [60].

Moreover, the concentration of the gelatin-elastin-hyaluronate mixture prior to freeze-drying has been highlighted for its role in determining the mechanical strength and elasticity of the final scaffold, with higher concentrations typically resulting in denser scaffolds that exhibit enhanced mechanical properties [61].

Cross-linking, another preparation parameter, greatly impacts scaffold performance by stabilizing the biomolecular structure, thereby affecting not only mechanical strength but also degradation rates—a critical factor in ensuring that the scaffold degrades at a rate consistent with tissue regeneration [62]. Chemical cross-linking methods utilizing agents like glutaraldehyde or genipin have been explored, with each agent offering distinct advantages in terms of biocompatibility and mechanical robustness yet also posing potential cytotoxicity challenges that necessitate careful consideration and balance [63].

The impact of scaffold hydration, a parameter often overlooked, is paramount, as it influences not only the immediate mechanical properties but also the scaffold's biological performance, including cell adhesion, proliferation, and differentiation. The hydrophilic nature of hyaluronate within the gelatin-elastin-hyaluronate composite plays a vital role in this context, enhancing water retention and thereby modulating the scaffold's viscoelastic properties better to mimic the natural extracellular matrix (ECM) [64]. Additionally, the pH and ionic strength of the solvent used in scaffold preparation have been found to affect the solubility, and consequently, the uniformity of the biomaterial distribution within the scaffold, which in turn influences its overall structural and mechanical integrity.

In synthesizing these insights, it becomes evident that the performance of gelatin-elastin-hyaluronate scaffolds is intrinsically tied to a complex interplay of preparation parameters. These

parameters not only define the physical and mechanical attributes of the scaffold but also its biological efficacy in supporting tissue regeneration. This complete understanding underscores the importance of a methodical and nuanced approach to scaffold design and fabrication, highlighting the need for ongoing research to further refine these parameters for the development of optimized scaffolds tailored to specific tissue engineering applications.

2.6. Conclusion

This chapter has explained the multifaceted nature of scaffold development within the field of tissue engineering, underscoring the critical role of fabrication parameters to fulfill specific biomedical objectives. It has showcased the imperative for a collaborative, interdisciplinary strategy that integrates the principles of materials science, biology, and engineering. This approach is essential for overcoming the complex challenges associated with designing scaffolds that are capable of mimicking the natural extracellular matrix and promoting effective tissue repair and regeneration. The insights derived from this review set the stage for future explorations, highlighting the need for innovative fabrication techniques and the development of new biomaterials aimed at addressing the current limitations in regenerative medicine. As research in this field advances, the vision of creating scaffolds that not only provide structural support but also actively facilitate the regeneration of damaged tissues moves closer to realization, offering promising prospects for the evolution of scaffold-based treatments. The forthcoming chapter will detail the experimental strategies and specific techniques utilized in this research to fabricate and evaluate the GEH scaffolds, followed by a presentation of the findings derived from this thesis.

CHAPTER 3: MATERIALS AND METHODS

3.1. Introduction

This chapter provides a detailed description of the experimental framework employed in the fabrication and characterization of GEH scaffolds. The methodologies outlined herein encompass the complete spectrum of the research process, from the initial preparation of materials to the comprehensive analysis of the fabricated scaffolds. Specifically, it defines the precise protocols followed in the scaffold fabrication process—including the agitation speeds, duration time, and chilling temperatures—and the subsequent post-processing techniques. Furthermore, this chapter elaborates on the various characterization methods applied to assess the structural integrity, porosity, mechanical strength, and degradation rate of the scaffolds. These methods include mechanical testing to assess elasticity, structural and morphological evaluation of the samples using laser microscopy and scanning electron microscopy (SEM) methods, and biochemical assays to determine the scaffolds' degradation characteristics. By precisely documenting the experimental approaches and techniques utilized, this chapter aims to provide a comprehensive and replicable protocol for the fabrication and optimization of GEH scaffolds, thereby contributing to the advancement of scaffold-based regenerative medicine.

3.2. Fabrication of the Scaffolds

The process of scaffold fabrication is a thorough one, requiring precise control over the materials and conditions used to ensure the creation of scaffolds that are conducive to tissue regeneration. This section introduces the materials employed in the fabrication of GEH scaffolds and outlines the protocol followed to produce them.

3.2.1. Materials

In the preparation of GEH scaffolds, the following materials were utilized, detailed in Table (3.1):

- Gelatin: Sourced from porcine skin, with a bloom strength of approximately 300, indicating its gelling power and suitability for creating a supportive matrix for cell growth.
- Elastin: Derived from bovine neck ligaments, providing the necessary elasticity to mimic the mechanical properties of natural tissues.
- Hyaluronic acid (HA): Obtained from a biotechnological process, ensuring high purity and consistency for supporting cell proliferation and migration.
- Deionized water: Used as the solvent for dissolving the scaffold materials, ensuring a pure and controlled environment for scaffold formation. Moreover, used for washing steps post-cross-linking to remove any unreacted substances and prepare the scaffolds for cell culture or storage.
- Hydrochloric acid (HCl): Employed to adjust the pH of the scaffold mixture, facilitating the proper incorporation of elastin.
- 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-Hydroxysuccinimide (NHS): Used for cross-linking the scaffold materials, enhancing their mechanical stability and longevity.
- Ethanol: Utilized for cross-linking, rinsing, and storage processes to ensure sterility and remove residual cross-linkers.
- Phosphate buffered saline (PBS): Used for washing steps post-cross-linking to remove any unreacted substances and prepare the scaffolds for cell culture or storage.

Table 3.1: List of the materials used in this thesis.

Material	Detail	Manufacturer	Country
Gelatin	Gelatin from porcine skin powder, gel strength ~300 g Bloom, Type A, BioReagent, for electrophoresis, suitable for cell culture	Sigma-Aldrich Life Science	USA
Elastin	Elastin From bovine neck ligament 95% purity, hydrolyzed and lyophilized.	EPC, Elastin Products Company, Inc.	USA
HA	Sodium hyaluronate, 95%, Thermo Scientific Chemicals	Thermo Fisher Scientific	China
HCl	Hydrochloric acid 6N 4L	The VWR Chemicals BDH	USA
EDC	$PC_8H_{18}ClN_3$ powder to crystalline powder to solid	Oakwood Products, Inc	China
NHS	$C_4H_5NO_3$ N-Hydroxysuccinimide, 98+%	Alfa Aesar Johnson Matthey Company	China
Ethanol	Ethanol, Alcohol Reagent, anhydrous, denatured, ACS, 94-96%,	Thermo Fisher Scientific, Chemicals	USA
PBS	PBS, Phosphate Buffered Saline, 10X Solution	Fisher BioReagents	USA

3.2.2. Protocol of the fabrication of the GEH scaffolds

We have chosen a specific composition consisting of 8% gelatin, 2% elastin, and 0.5% HA (w/v) in an aqueous solution, based on previously published findings, as the most suitable composition [20,22]. As schematically shown in Figure (3.1), the combination of foaming and freeze-drying methods was used in this thesis to fabricate the scaffolds [21]. As defined in Table (3.2), different agitation speeds (0, 500, and 1000 rpm), duration times (0, 5, and 15 min), and chilling temperatures (-20 and -80°C) were used to fabricate the scaffolds of 20 different groups of samples. The step-by-step procedure used in this study to fabricate the samples is as follows:

- Heat preparation: 120 ml of deionized water was first heated to 50°C, stirring continuously with a magnetic stirrer to ensure even temperature distribution.

- Gelatin incorporation: 9.6 gr of gelatin was gradually added to the heated water while maintaining the temperature at 50°C, ensuring the gelatin dissolves completely and the mixture becomes homogeneous.
- Hyaluronate addition: After the gelatin was fully integrated, 0.6 gr of hyaluronate was slowly added to the mixture, continuing to maintain the temperature at 50°C. Sufficient time was given to the solution to achieve complete homogeneity.
- pH adjustment for elastin: pH of the mixture was checked. To proceed with adding elastin, pH was adjusted to approximately 4 using HCl, adding it gradually if the initial pH is above 4.
- Elastin integration: 2.4 gr of elastin was slowly incorporated into the mixture, ensuring the temperature is steady at 50°C and the pH is around 4. The solution was stirred until became homogeneous.
- Agitation: The homogenous solution was agitated by a mechanical mixer (IKA, USA) at the specified speeds and duration times, based on Table (3.2), keeping the solution at 50°C to form a foam.
- Molding: The foam was transferred into molds for shaping.
- Freezing: The molds were placed in a freezer set to either −80°C (Fisherbrand Isotemp Ultra Low Temperature Freezer, USA) or −20°C (VWR Standard Series Refrigerator and Freezer Combo Units, USA) and left overnight to solidify.
- Freeze-drying: The frozen samples were subjected to freeze-drying at −55°C and a pressure of 1 Pa for 48 hours, removing all moisture without compromising the scaffold's structure he specified equipment used was (Labconco FreeZone 2.5 Liter Benchtop Freeze Dryer, USA).
- Cross-linking: The scaffolds were treated with a solution of 2 mg/ml EDC and 0.5 mg/ml NHS in 100% ethanol (v/v) at 4°C overnight to enhance their stability.
- Rinsing: The cross-linked scaffolds were rinsed in 100% ethanol to eliminate any residual cross-linker.

- PBS wash: The scaffolds were immersed in PBS once to remove ethanol and other soluble impurities.
- Water wash: The scaffolds were subsequently immersed in deionized water to further purify them.
- Secondary freezing: The washed scaffolds were transferred to a −20°C environment and left overnight to prepare for a second freeze-drying process.
- Final freeze-drying: The scaffolds were freeze dried for an additional 12 hours to ensure thorough drying.
- The finalized scaffolds were stored in a refrigerator at 4°C until they were ready for further use.

3.3. Characterization of the Samples

In the comprehensive evaluation of the fabricated GEH scaffolds, a multi-faceted measurement and analysis approach is meticulously designed to dissect the scaffold's essential properties, ensuring a complete understanding of its potential for dental applications.

3.3.1. Morphology, surface roughness, and internal microstructure

As shown in Figure (3.2), a Dino-lite digital microscope camera was employed to obtain low-magnification photos of the scaffolds after complete preparation. LEXT OLS4000 3D Laser Measuring Microscopy (Olympus, Japan) was used for imaging of the scaffolds. Moreover, the surface morphology and roughness were analyzed using the 3D Laser Measuring Microscopy. Surface roughness was assessed across five distinct regions of each sample, and the arithmetical mean height of the surface (Sa) was calculated for each area. It is important to note that the Sa value quantitatively measures the average deviation of the surface height from the mean plane across the entire measured surface area. This metric is crucial for evaluating and comparing the surface textures of various materials or manufacturing processes. The results are presented as the mean ± standard deviation (SD) for each sample.

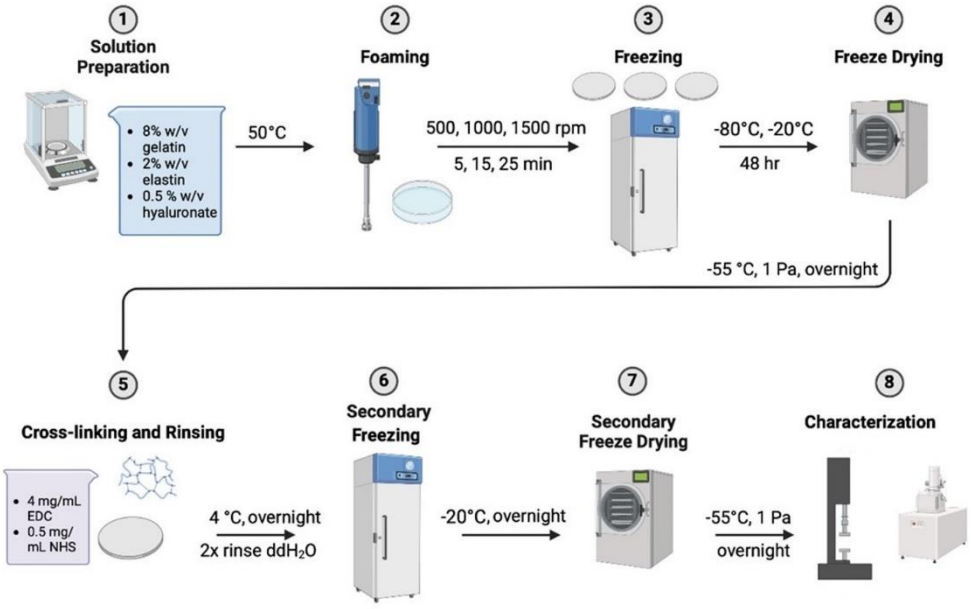


Figure 3.1: Schematic protocol for the fabrication of the scaffolds.

Figure 3.2: Investigation of the morphology and surface roughness of a typical sample by laser microscope.

Table 3.2: Sample specification.

No.	Name	Agitation (rpm)	Time (min)	Temp (°C)
1	$F_{0(Control)}$	0	0	−20
2	F_1	500	5	−20
3	F_2	1000	5	−20
4	F_3	1500	5	−20
5	F_4	500	15	−20
6	F_5	1000	15	−20
7	F_6	1500	15	−20
8	F_7	500	25	−20
9	F_8	1000	25	−20
10	F_9	1500	25	−20
11	F_{10}	500	5	−80
12	F_{11}	1000	5	−80
13	F_{12}	1500	5	−80
14	F_{13}	500	15	−80
15	F_{14}	1000	15	−80
16	F_{15}	1500	15	−80
17	F_{16}	500	25	−80
18	F_{17}	1000	25	−80
19	F_{18}	1500	25	−80
20	$F_{19(Control)}$	0	0	−80

The microstructure of the scaffolds was examined using a Scanning Electron Microscope (SEM, JEOL JSM-6510LV), operating at an acceleration voltage of 15 kV to ensure high-resolution imaging. This setup allowed for detailed observation of the scaffold's internal morphology and porosity, critical for evaluating its suitability for tissue engineering applications. Before imaging, samples were sputter-coated with gold to enhance conductivity and reduce charging, enabling the precise capture of the scaffold's microarchitecture. This SEM analysis provided essential insights into the interconnectivity between the pores, contributing significantly to the optimization of scaffold design for improved cellular interactions and tissue integration.

3.3.2. Porosity measurements

The porosity of the samples was measured using Archimedes' principle at room temperature. Ethanol was selected as the solvent as it infiltrates into the scaffolds' pores without swelling or shrinking the matrix. The porosity of the scaffold was calculated using the following equation [65]:

$$\text{Porosity } (\%) = (W_2 - W_1)/(W_2 - W_3) \times 100 \quad (3.1)$$

where W_1, W_2 and W_3 are the dry weight of the samples, weight of the scaffolds saturated with ethanol, and the weight of the scaffolds suspended in ethanol, respectively. Measurements were performed for three samples of each group.

3.3.3. Mechanical properties

To measure the compressive Young's modulus for each scaffold, a universal testing machine (UTM, AGS-X, Shimadzu) equipped with a 1 kN load cell was employed. Cylinders of ~ 6 mm height and ~ 3 mm radius were cut from each sample and used for the measurements. The experiments were conducted at the constant compression speed of 1 mm/min until failure. The strain was determined by dividing the change in length by the initial length of the samples. Modulus were obtained by calculating the slope of the stress-strain curve within the elastic range, measured

at the strain of 0.020. Measurements were performed in triplicate, averaged, and reported. Figure (3.3) shows a typical sample under the compression test used in this thesis.

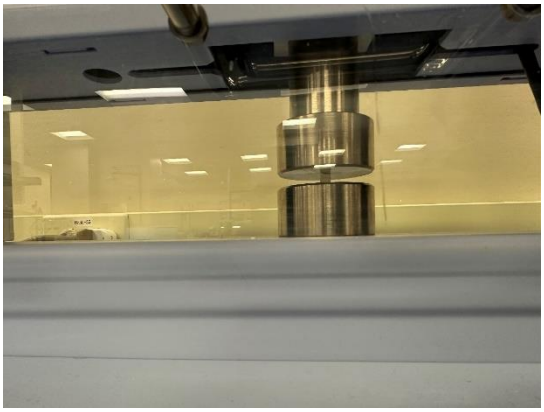


Figure 3.3: A typical sample under compression test.

3.3.4. Degradation

The degradation rate of the samples was evaluated by immersing them in phosphate-buffered saline (PBS) and storing them in a shaking incubator at 37°C. Initially, the weights of the samples were accurately determined. Subsequently, at predetermined time intervals (1, 2, 4, 7, 14, 21, and 28), samples were carefully removed and dried in an oven at 37°C for 24 hours, and then their weights were meticulously recorded. The degradation was quantified as the ratio of the sample's weight at each time point to its initial weight, illustrating the weight loss over time. Measurements were performed for three samples of each group.

3.3.5. Shrinkage monitoring

The shrinkage of the scaffolds after freeze-drying was evaluated to ensure the integrity and accuracy of the scaffold dimensions, which are critical for their intended application in tissue engineering. This evaluation involved measuring the scaffolds' diameter after freeze-drying using a digital caliper. These measurements were then compared to the original diameter of the mold used for scaffold fabrication, which was equal to 49.5 mm. The difference in measurements before and after freeze-drying provided a quantitative assessment of shrinkage, enabling the determination of

scaffold stability and structural fidelity. This step is crucial for verifying that the scaffolds meet the specific dimensional requirements necessary for successful integration and function within the target tissue environment.

3.4. Development of Mathematical Estimators for Scaffolds' Properties by Statistical Investigation of the Preparation Parameters

In order to investigate the effect of different preparation parameters on the properties of the scaffolds, a comprehensive statistical analysis was conducted. This process focused on varying agitation speeds and duration times across two distinct chilling temperatures. Four separate statistical analyses employing the Central Composite Design (CCD) and Response Surface Methodology (RSM) were conducted to explore the intricate effects of these parameters on the critical scaffold characteristics of mechanical properties and porosity [66].

For each chilling temperature, the study utilized data on the compressive modulus and porosity observed under different agitation speeds and duration times. These conditions were systematically examined using a three-level, two-factor fractional factorial CCD approach. This rigorous methodological framework was designed to examine the complex interactions between agitation speed and duration time and their collective impact on scaffold performance.

To derive precise mathematical estimators of the scaffold's behavior under these varied conditions, a second-order multiple regression analysis was employed on the CCD data. This analysis, facilitated by the least squares regression methodology, enabled the accurate description of the system's dynamics. The results of this analysis were presented as follows:

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n} \beta_{ii} X_i^2 + \sum_{i<j} \beta_{ij} X_i X_j \tag{3.1}$$

where Y represents the response variables (compressive modulus and porosity), X_i and X_j denote the independent variables (agitation speed, duration time, and chilling temperature), β_0 , β_i, β_{ii},

and β_{ij} signify the model constants, linear coefficients, quadratic coefficients, and interaction coefficients, respectively.

The employment of such an advanced statistical framework enabled a detailed exploration of how specific changes in agitation speed and duration time, adjusted for the chilling temperatures of -20°C and -80°C, influence the desired scaffold properties. This statistical method underscores the complexity of scaffold fabrication and highlights the necessity for precise control over fabrication parameters to achieve scaffolds with optimal physical and biological properties.

3.5. Conclusion

In conclusion, Chapter 3 thoroughly presents the comprehensive experimental framework adopted for the fabrication and characterization of GEH scaffolds, crucial for advancing scaffold-based regenerative medicine. The detailed protocols for scaffold preparation are pivotal in ensuring the scaffolds' suitability for tissue engineering applications. Through rigorous characterization methods, including mechanical testing, morphological analysis, and degradation studies, this chapter not only substantiates the potential of GEH scaffolds in dental and soft tissue engineering but also lays the groundwork for further investigation of the effect of fabrication parameters. This investigation is essential for tailoring the scaffolds' mechanical properties and structural integrity, ensuring their functional compatibility with native tissues. In the next chapter, the findings of this thesis will be presented.

CHAPTER 4: RESULTS

4.1. Introduction

This chapter presents the comprehensive findings obtained from the systematic experimental investigation conducted to evaluate the structural, mechanical, and degradation properties of GEH scaffolds. These scaffolds, fabricated under varying preparation parameters, including different agitation speeds, duration times, and chilling temperatures, were subjected to a series of detailed analyses to ascertain their suitability and effectiveness for tissue engineering applications.

The investigation was structured to provide insights into the morphology, surface roughness, and internal microstructure of the scaffolds, offering a foundational understanding of how these physical attributes contribute to the overall functionality of the scaffold. Porosity measurements were conducted to assess the scaffold's ability to facilitate cell infiltration and nutrient diffusion, critical factors for successful tissue regeneration. Mechanical properties, including compressive modulus, were evaluated to determine the scaffold's capacity to withstand physiological loads while promoting cellular activities. The degradation behavior of the scaffolds was analyzed to understand their biodegradability and compatibility with tissue healing processes. Additionally, shrinkage monitoring was performed to ensure the dimensional stability of the scaffolds post-fabrication, a vital aspect for their practical application in regenerative medicine.

Through studying the preparation parameters, this chapter aims to highlight the significant influence of fabrication conditions on the performance of GEH scaffolds. The results derived from this comprehensive study are intended to contribute to the ongoing efforts in the field of tissue engineering to develop scaffolds that closely mimic the natural extracellular matrix, promoting effective tissue repair and regeneration.

4.2. Morphology, Surface Roughness, and Internal Microstructure

Figure (4.1) shows the laser microscope images obtained from the control samples fabricated at the chilling temperatures of −20 and −80°C. The laser microscope images obtained from the typical samples fabricated at chilling temperatures of −20 (F_1 to F_9) and −80°C (F_{10} to F_{18}) are respectively shown in figures (4.2) and (4.3). The SEM images obtained from the control samples are shown in figure (4.4) without the agitation speed and time changes. Figures (4.5) and (4.6) show the SEM images of the typical samples fabricated at chilling temperatures of −20 (F_1 to F_9) and −80°C (F_{10} to F_{18}), respectively. These images show that the pores of the fabricated bone scaffolds possess appropriate interconnectivity and cohesion. The interconnection and overlap of pores are among the most crucial characteristics of any tissue engineering scaffold. Indeed, the presence of interconnected pores within scaffolds is essential to allow cells to grow and infiltrate throughout the scaffold. Additionally, these porosities play a significant role in nutrient delivery to cells, waste removal, and angiogenesis. The high volume of open porosities observed within the scaffolds fabricated in this study is directly attributed to the innovative application of the freeze-drying and foaming methods during their construction. These processes uniquely contribute to the development of a highly porous structure. The freeze-drying method effectively sublimates the solvent, leaving behind an extensive network of interconnected pores, while the foaming technique introduces gas bubbles that expand and create additional porosity within the scaffold matrix. This strategic combination ensures the formation of a scaffold with enhanced porosity and interconnectivity, essential for facilitating cell infiltration, nutrient diffusion, and tissue integration, thereby significantly improving the scaffold's performance in tissue engineering applications.

Figures (4.7) show the pore size distribution for different scaffolds fabricated in this project. To evaluate the pore sizes within the scaffolds, a precise and systematic approach was employed using a laser microscope. For each scaffold sample, five distinct regions were scrutinized to account for variability within the scaffold structure. Within each of these regions, five individual pores were

selected, and their diameters were calculated, totaling twenty-five pore measurements per sample. This method ensured a comprehensive assessment of the pore sizes across the entire scaffold. Our findings indicate that the diameters of the majority of pores present in the scaffolds fall within the range of 100 – 300 μm, a dimension considered highly beneficial for tissue engineering purposes. Pore sizes within this range are optimal as they allow for effective cell interaction, migration, and nutrient exchange, crucial for tissue regeneration.

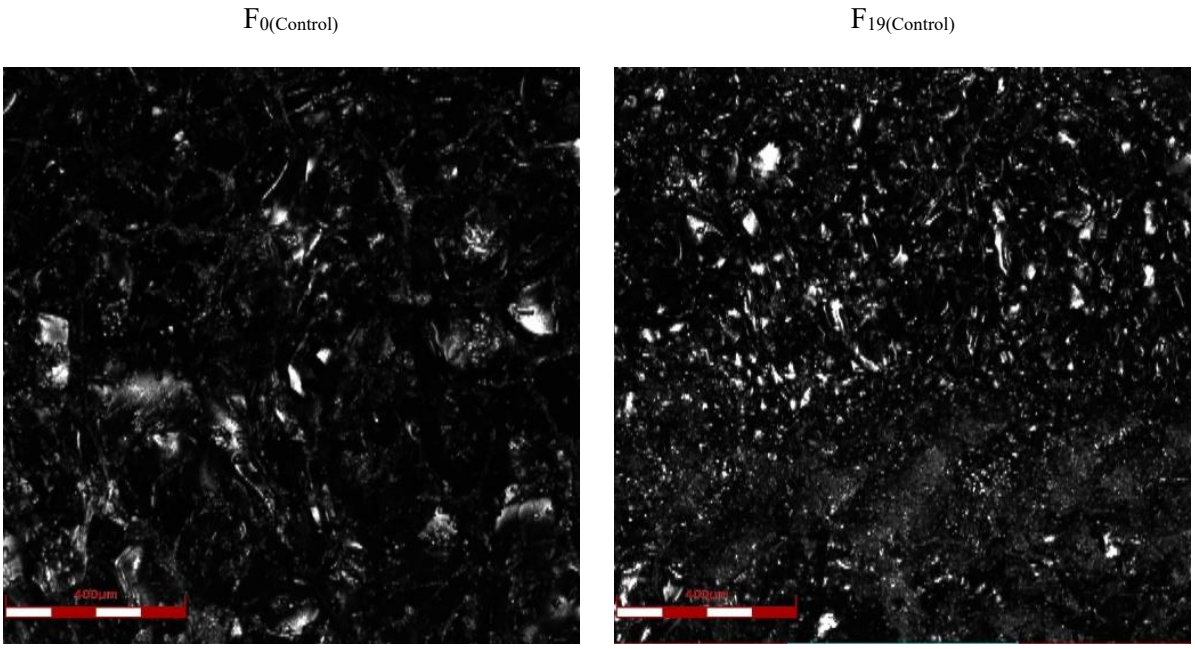


Figure 4.1: Laser microscope images of the control samples fabricated at −20°C ($F_{0(Control)}$) and −80°C ($F_{19(Control)}$). Scale bars are 400 μm.

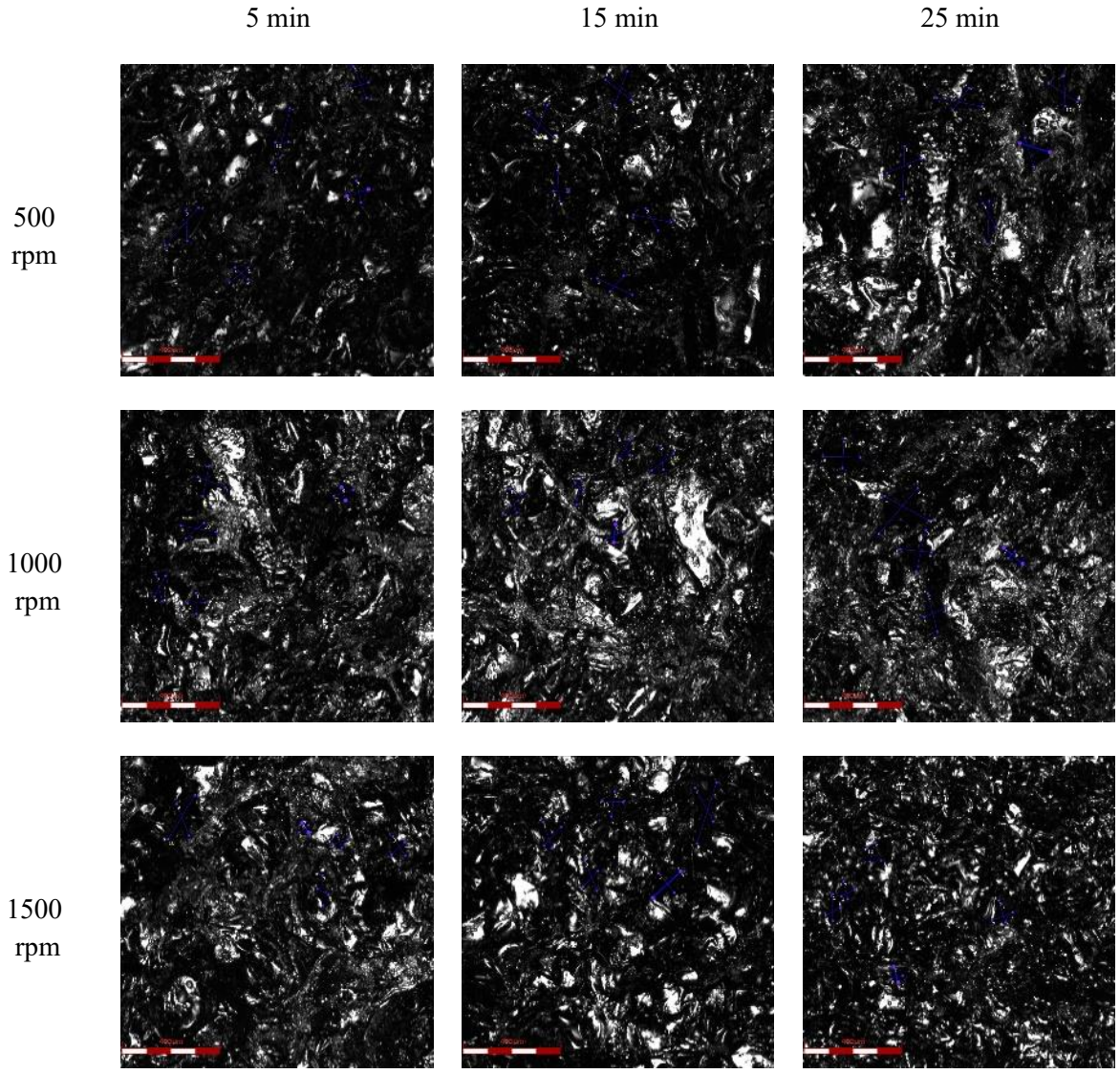


Figure 4.2: Laser microscope images of the typical samples fabricated at the chilling temperature of −20°C. Scale bars are 400 μm.

Pore sizes below this optimal range can severely restrict cell activities, potentially leading to the demise of cells adhered to the scaffold. Conversely, excessively large pores, surpassing 500 μm, reduce the surface area essential for cell attachment and compromise the scaffold's mechanical strength by increasing the void volume.

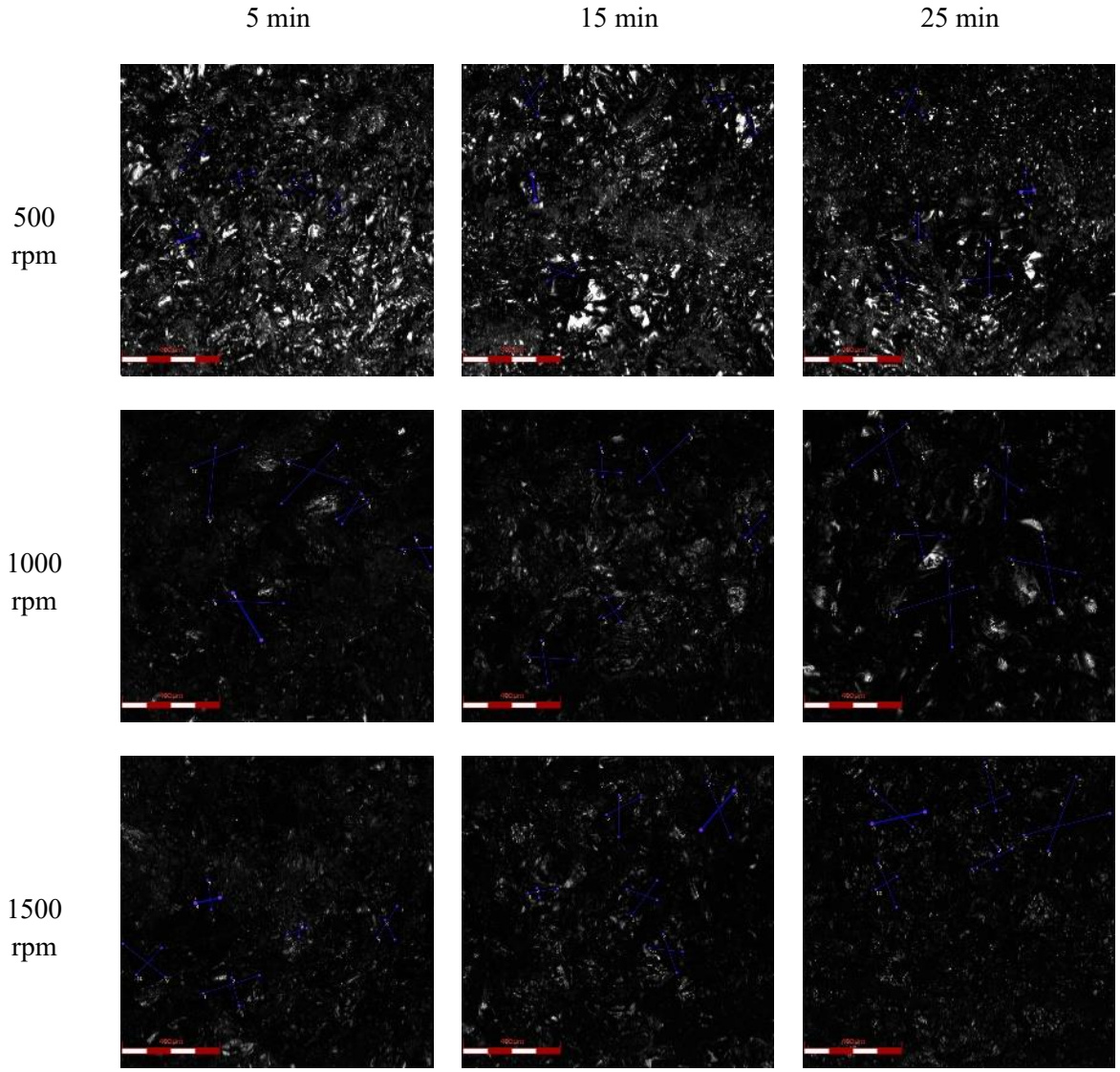


Figure 4.3: Laser microscope images of the typical samples fabricated at the chilling temperature of −80°C. Scale bars are 400 μm.

Perez and Mestres [67] highlight that the ideal pore size for scaffolds should be between 100 and 400 μm to support efficient cell-scaffold interactions, particularly under static seeding conditions. Pores exceeding 500 μm in size impede effective cell engagement with the scaffold, as cells may simply pass through without establishing attachment. Research has outlined specific pore size ranges that are considered optimal for the ingrowth and function of various cell and tissue

types, including ranges of 70–120 μm for chondrocyte ingrowth [68], 40–150 μm for fibroblast attachment [69], and 200–350 μm for facilitating osteoconduction [70].

To evaluate the effect of fabrication parameters on the pore size of the scaffolds, a series of one-way ANOVA tests were performed on agitation speed, agitation time, and chilling temperature. The results from these tests provide a statistical insight into the influence of each factor on the mean diameter of the pores created within the scaffolds.

For agitation speed, the ANOVA test yielded a p-value of ~ 0.84. Similarly, for agitation time, a p-value of ~ 0.91 was observed. Both p-values are substantially higher than the conventional alpha level of 0.05, which is commonly used as a benchmark for statistical significance. This indicates that within the scope of this study and at the 95% confidence level, neither agitation speed nor agitation time has a statistically significant effect on the mean diameter of the pores. Therefore, variations in these parameters do not contribute to meaningful differences in pore size that could impact the scaffold's performance in tissue engineering applications.

On the other hand, chilling temperature produced a p-value of ~ 0.06. While this value is still above the standard threshold of 0.05 for statistical significance, it is marginally close and suggests a potential trend where temperature might influence the mean pore diameter. This near-significant p-value points to a possible effect of chilling temperature on pore size, emphasizing further investigation.

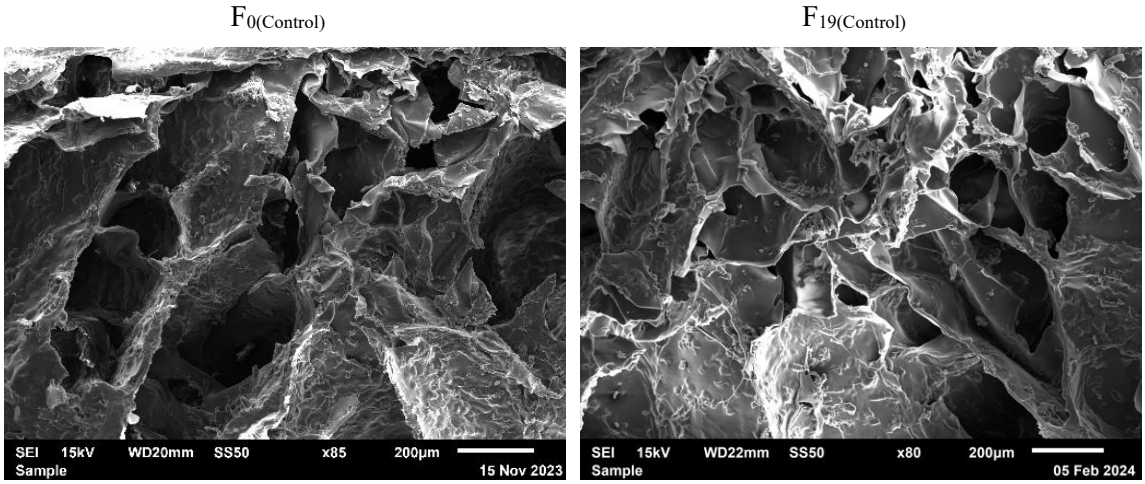


Figure 4.4: SEM images of the control samples fabricated at −20°C ($F_{0(Control)}$) and −80°C ($F_{19(Control)}$).

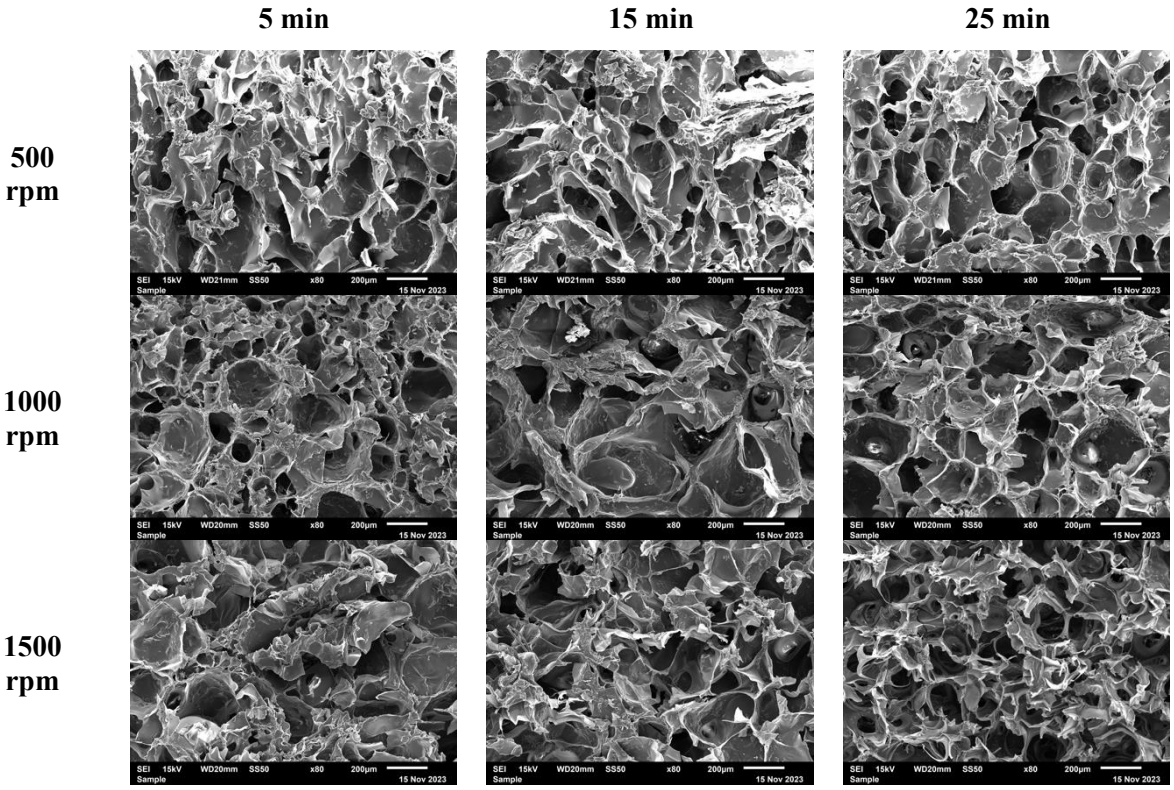


Figure 4.5: SEM images of the typical samples fabricated at the chilling temperature of −20°C.

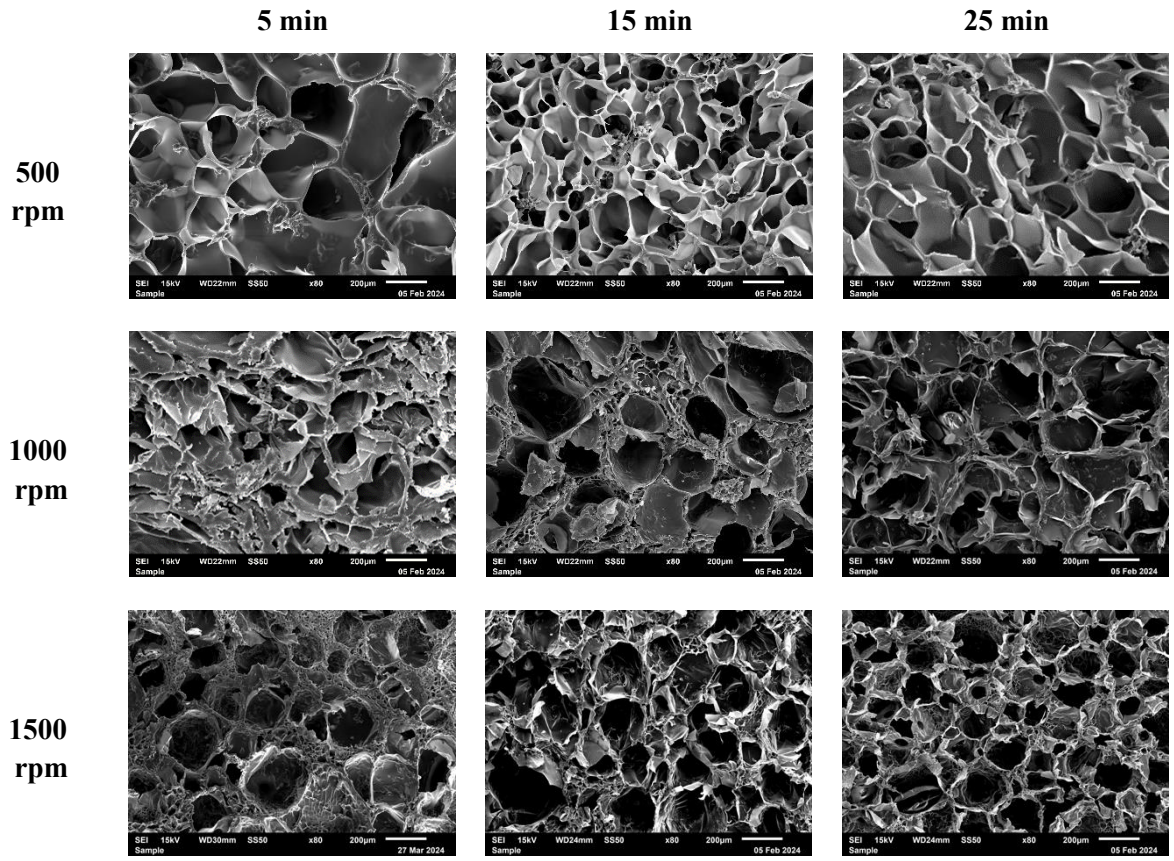


Figure 4.6: SEM images of the typical samples fabricated at the chilling temperature of −80°C.

The statistical analysis indicates that chilling temperature may have an impact on the pore size of the scaffolds, with a p-value marginally above the traditional threshold for significance. Complementing these findings, a careful examination of the SEM images, specifically figures (4.5) and (4.6), provides qualitative validation of the statistical data. These images suggest that a decrease in chilling temperature correlates with a slight increase in pore size within the scaffold matrix. This morphological trend observed in the SEM analysis resonates with the experimental outcomes reported by O'Brien et al. [26]. Their research, which meticulously explored the effect of varying freezing rates on pore sizes, found that a slower freezing process, typically associated with decreased temperatures, leads to larger pore diameters. However, as shown in figure (4.8), they noted that a very rapid freezing approach, such as quenching at −40°C, unexpectedly yielded larger pores—a finding that aligns with our observations where samples quenched at −80°C exhibited an

increase in pore size. This seeming paradox could be attributed to the complex interplay between freezing dynamics and ice crystal formation, where extremely rapid freezing might lead to less uniform ice crystal formation, and thus, larger pore spaces upon sublimation.

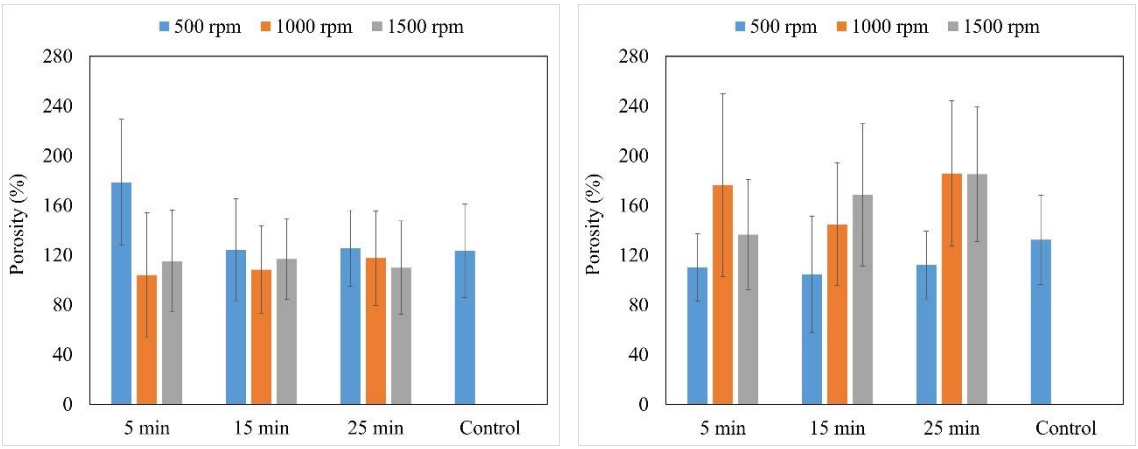


Figure 4.7: Pore size of the scaffolds at different agitation speeds and duration times fabricated at chilling temperatures of (a) −20 and (b) −80 °C.

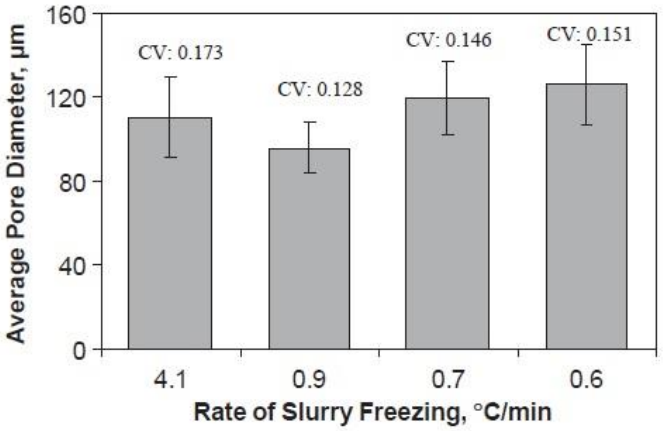
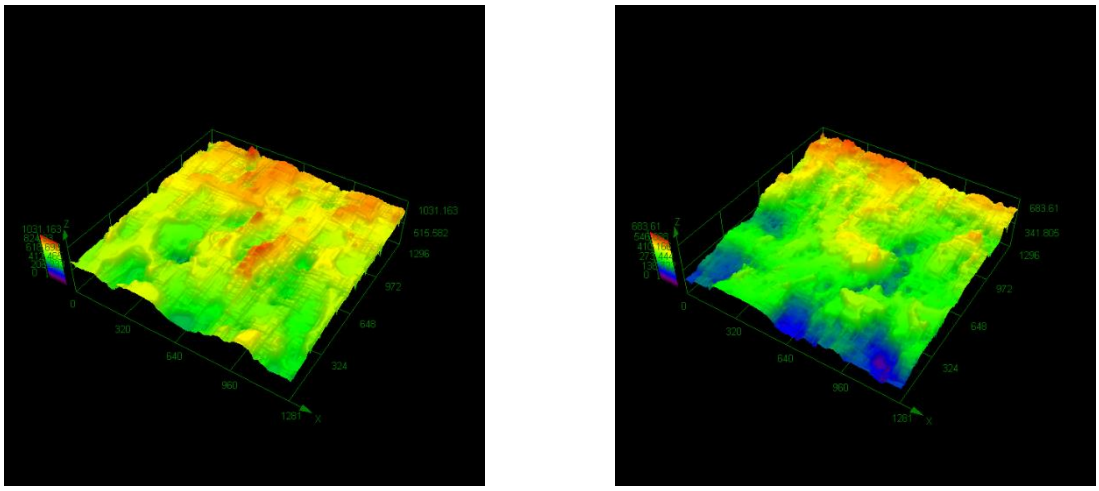


Figure 4.8: Average pore size for scaffolds fabricated with the quenching (4.1 °C/min) and different cooling rates of 0.9 °C, 0.7 °C, and 0.6 °C/min obtained from [26] with permission.

Figure (4.9) illustrates the surface roughness of control samples fabricated under chilling temperatures of −20°C and −80°C, with mean roughness values for samples $F_{0(Control)}$ and $F_{19(Control)}$ recorded at 36.54 μm and 21.29 μm, respectively. The surface roughness for the experimental groups, represented in samples F_1 to F_{18}, is typically shown in figures (4.10) and (4.11). Our observations indicated that the mean surface roughness for samples prepared at −20°C spanned from 31.31 μm to 49.29 μm. For those fabricated at the lower temperature of −80°C, roughness values ranged more broadly from 17.65 μm to 51.02 μm. This variance in surface roughness across different fabrication conditions underscores the impact of chilling temperature on the scaffold's textural characteristics. These findings contribute to our understanding of how fabrication parameters influence scaffold surface properties, which in turn can affect cell adhesion, proliferation, and overall tissue integration.

It should be noted that the effectiveness of laser microscopes in measuring surface roughness is compromised when assessing porous materials like foam. These materials feature uneven surfaces that can significantly distort measurements. Laser microscopes operate by scanning surfaces with a laser and capturing the reflected light to create a detailed three-dimensional profile of the surface. This method is highly precise, capturing fine surface details, which makes it particularly suitable for applications in materials science and manufacturing. However, given the inherent limitations when applied to porous structures, the results obtained from such samples may not accurately represent the true surface characteristics. Caution is advised in interpreting these results, acknowledging the potential discrepancies introduced by the complex topography of porous materials.

$F_{0(Control)}$ $F_{19(Control)}$



36.54 ± 8.21 µm 21.29 ± 2.00 µm

Figure 4.9: Surface roughness of the typical control samples fabricated at −20°C (F0(Control)) and −80°C (F19(Control)).

4.3. Porosity Measurements

The porosity of tissue engineering scaffolds is an important parameter that directly impacts their performance in biological applications. Figure (4.12) shows the value of the porosity of the scaffolds at different agitation speeds and duration times fabricated at chilling temperatures of −20 and −80°C. By examining the porosity measurements presented in this figure, we observe the interplay between agitation speed, time, and chilling temperature during the freeze-drying process on the resultant porosity of gelatin-elastin-hyaluronate scaffolds. From the analysis of the results, it is clear that scaffolds fabricated at both −20°C and −80°C generally exhibit increased porosity with greater agitation speeds and extended agitation times. This pattern indicates that increased agitation during preparation may enhance the scaffold's porosity. Specifically, the act of agitating may lead to the formation of a more open matrix, suggesting a potential refinement of the foam structure as agitation progresses, possibly due to the enhanced distribution of air pockets. The trend of higher porosity with increased agitation speed, particularly at 1500 rpm, may be attributed to the greater energy input, resulting in foam structures with better air incorporation and thus, a higher porosity.

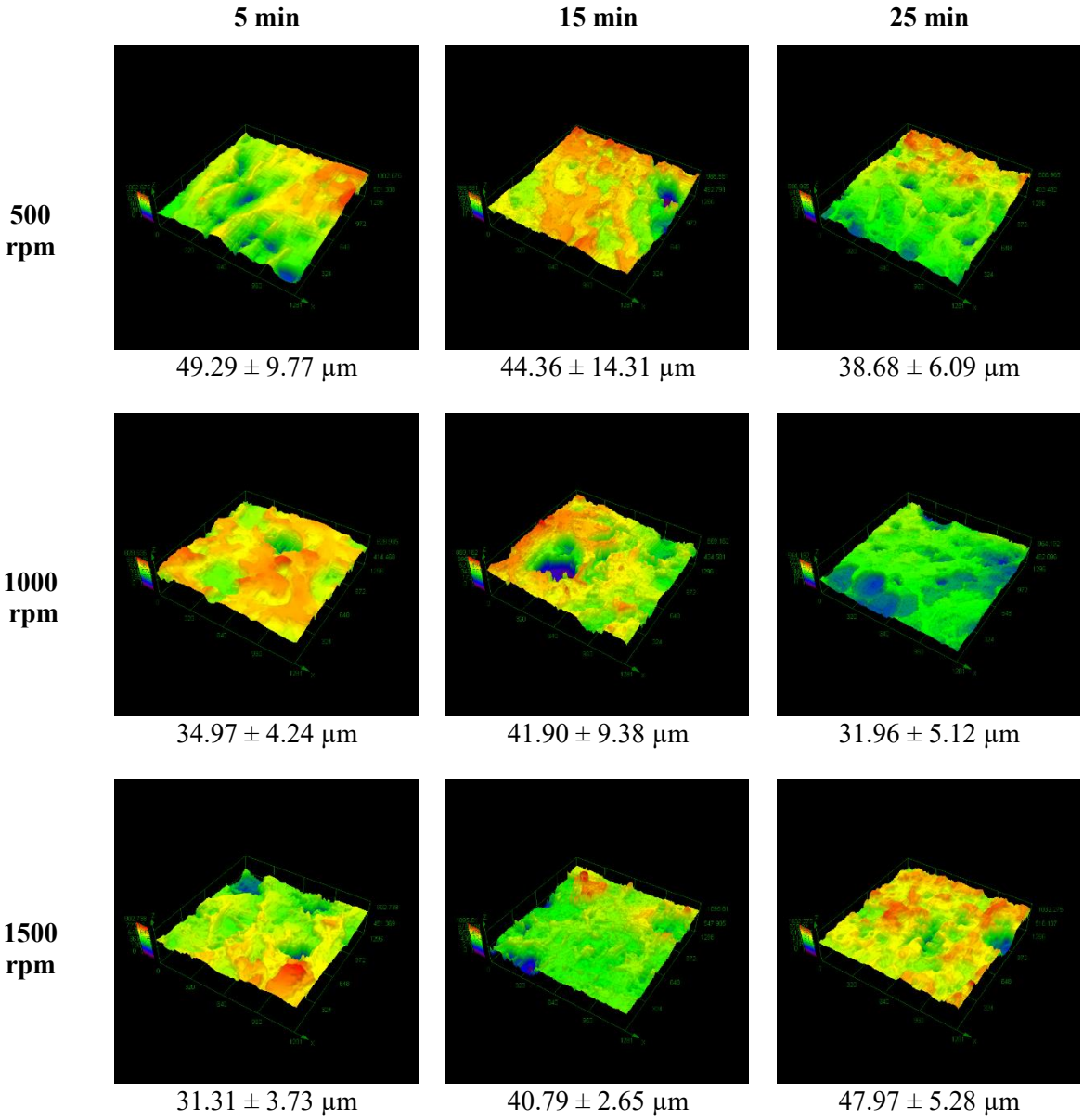


Figure 4.10: Surface roughness of the typical samples fabricated at the chilling temperature of −20°C.

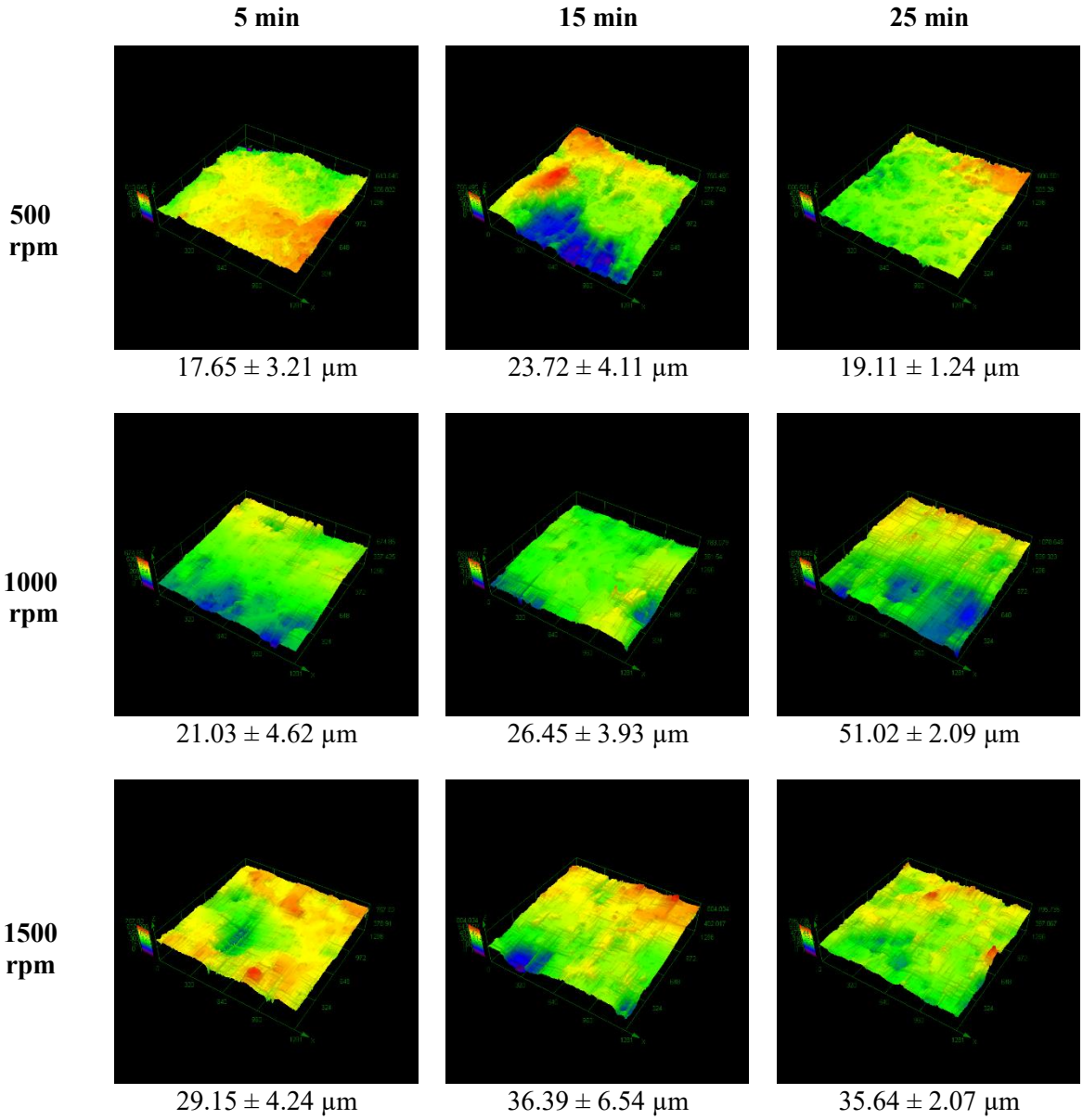


Figure 4.11: Surface roughness of the typical samples fabricated at the chilling temperature of −80°C.

When assessing the impact of chilling temperature, scaffolds processed at the lower temperature of −80°C exhibit a slightly increased porosity across all agitation parameters in comparison to the samples fabricated at −20°C. This observation supports the theory that lower freeze-drying temperatures contribute to the formation of larger ice crystals, which, upon sublimation, translate into more significant porosity within the scaffold.

Upon qualitative examination of the porosity trends from the scaffold data at −20°C and −80°C, it seemed that agitation speed plays a more pronounced role in influencing porosity, as demonstrated by significant changes across varied speeds. However, the impact of agitation time should not be overlooked, as there is a discernible increase in porosity corresponding to longer agitation periods. This observation underscores the importance of both factors in the scaffold's structural configuration, with speed seemingly exerting a stronger influence within the experimental scope. The consistency in these trends, regardless of the chilling temperatures, underscores a steadfast correlation between the chosen agitation parameters and the scaffold's porosity. This insight is crucial for tailoring scaffold architecture to meet the specific demands of biological applications in tissue engineering.

Overall, the analysis of these findings underscores the vital role that fabrication parameters play in determining the porosity of GEH scaffolds. Achieving optimal porosity is imperative to support cellular activities and mechanical stability, both of which are essential for the scaffold's function within a biological setting. The data emphasizes the necessity of refining preparation parameters to fine-tune the scaffold's porosity to meet the specific demands of tissue regeneration applications.

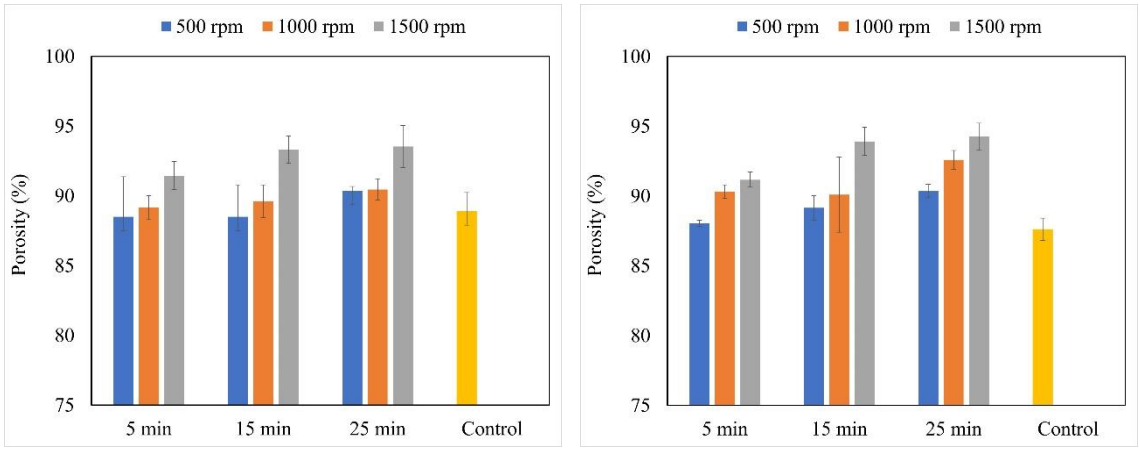


Figure 4.12: The value of the porosity of the scaffolds at different agitation speeds and duration times fabricated at chilling temperatures of (a) −20 and (b) −80 °C.

4.4. Mechanical Properties

The mechanical properties of tissue-engineered scaffolds are one of the most important factors that affect their performance in vivo. To ascertain the compression resistance of GEH scaffolds, compression tests were performed on the fabricated scaffolds. Figure (4.13) shows the results obtained from the compression test of a typical sample. Moreover, figure (4.14) demonstrates the value of the compressive Young's modulus of the scaffolds under different fabrication conditions.

Our findings showed that the mechanical properties of the samples were fundamentally influenced by the processing parameters, notably the agitation speed and duration during the pre-freeze mixing phase. The results suggest a complex interplay between these parameters and the resultant mechanical properties, highlighting a trend that suggests an increase in the agitation speed results in scaffolds with lower Young's modulus, pointing towards a less stiff structure. Moreover, prolonged agitation times, particularly at the highest speed of 1500 rpm, displayed a decrease in modulus, potentially implicating a degradation of structural integrity due to excessive porosity.

From the findings, generally, increasing the agitation speed and duration leads to an increase in porosity values, subsequently causing a decrease in compressive modulus.

These insights into the mechanical behavior of scaffolds are critical as they directly impact the design and application of tissue-engineered products. These results are important, not only to mimic the native mechanical properties of tissues but also to ensure that the scaffolds can withstand the physiological loads they will encounter post-implantation. In fact, this mechanical characterization study of GEH scaffolds provides a vital link between scaffold design parameters and their functional performance. It paves the way for the systematic optimization of tissue-engineered scaffolds, ensuring their mechanical robustness and, by extension, their success in clinical applications.

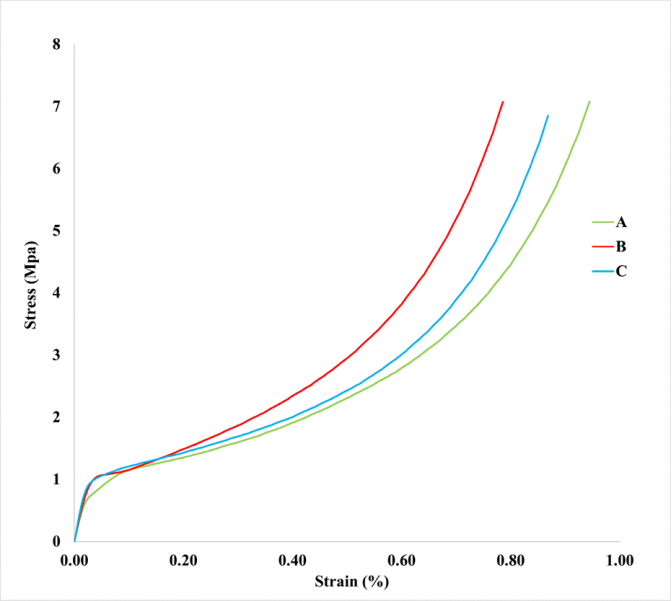
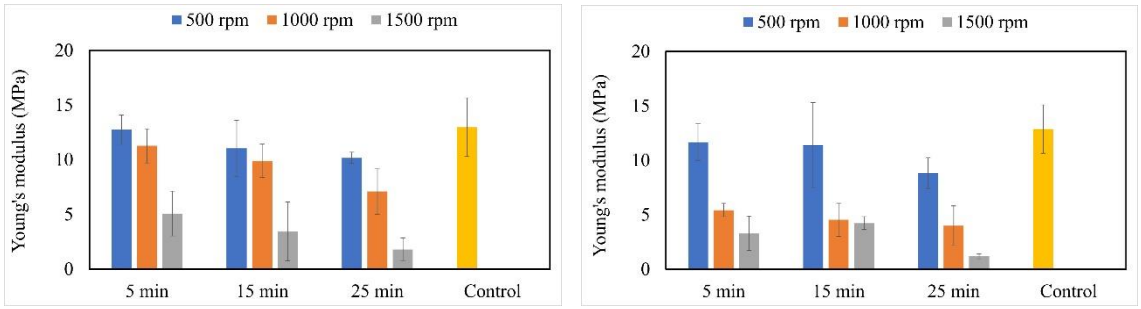


Figure 4.13: Three typical results (three repetitions) obtained from the compression test of sample F10.



(a) (b)

Figure 4.14: The value of the compressive Young's modulus of the scaffolds at different agitation speeds and duration times fabricated at chilling temperatures of (a) −20 and (b) −80 °C.

4.5. Degradation

In the investigation of scaffold degradation, samples from 18 experimental groups (F_1 to F_{18}) along with two control groups (F_0 and F_{19}) prepared under varying conditions of agitation, time, and temperature were analyzed to quantify their degradation over time. Tests were performed on three samples from each group, and the degradation was assessed at specific intervals: Day 0, Day 1, Day 2, Day 4, Day 7, and Day 14.

Figures (4.15) to (4.17) show the results obtained for the degradation analysis of the samples. The initial weight of each sample at Day 0 served as a reference for subsequent measurements. The relative weight of the samples at each time point was normalized by dividing the weight observed on a given day by the initial weight. This normalization process facilitated a comparative analysis of degradation rates across different groups.

Average weight percentages and standard deviations were calculated for each group at the specified time intervals to quantify the degradation behavior. The analysis showed that, generally, groups processed at −80°C (F_{10} to F_{18}) exhibited a slower degradation rate than those processed at −20°C (F_1 to F_9), underscoring the protective effect of lower temperatures against scaffold degradation.

This comprehensive degradation study provides valuable insights into the stability and longevity of scaffolds under various conditions, highlighting the importance of carefully selecting processing parameters to optimize scaffold performance for biomedical applications.

4.6. Shrinkage Monitoring

Maintaining the dimensional integrity of scaffolds following the freeze-drying process is crucial for their practical application in tissue engineering. However, in freeze-dried scaffolds, some shrinkage happens because of the capillary forces exerted on the scaffold's porous structure during the sublimation of ice. Indeed, as the ice sublimates into vapor, the porous structure previously supported by ice crystals collapses to a degree, leading to a reduction in overall dimensions. To evaluate this aspect, the shrinkage of the scaffolds was carefully measured against the original mold diameter of 49.5 mm. These post-processing measurements provided a quantitative assessment of the scaffold's ability to retain its intended dimensions, a critical factor for ensuring proper fit and function within the target tissue environment.

The assessment of scaffold shrinkage revealed that the samples exhibited a range of dimensional changes, as depicted in Tables (4.1) and (4.2). These values suggest that the scaffolds are relatively stable, with the majority experiencing only moderate shrinkage, which is within the acceptable limits for tissue engineering scaffolds. The findings indicate that while there is an expected degree of shrinkage due to the freeze-drying process, the scaffolds largely retain their dimensional properties with a minimum shrinkage of 5.9 % and a maximum of 18 %. This stability is essential for ensuring that the scaffolds meet the specific dimensional requirements necessary for successful integration and functionality within the biological setting. The collected data and subsequent analysis underscore the success of the freeze-drying protocol in maintaining scaffold integrity. The controlled shrinkage observed points to the efficacy of the chosen fabrication methods and conditions, reinforcing the scaffolds' suitability for tissue engineering purposes.

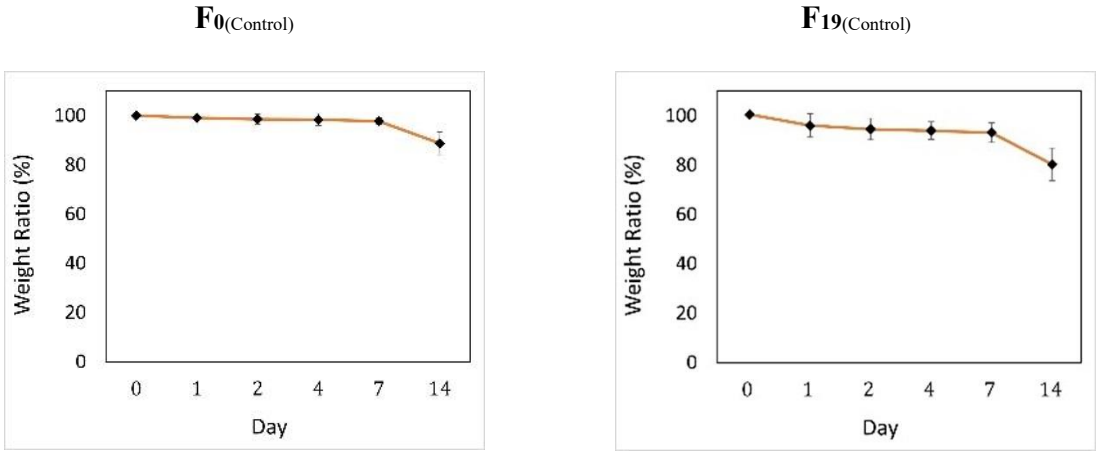


Figure 4.15: Degradation investigation for the control samples fabricated at −20°C ($F_{0(Control)}$) and −80°C ($F_{19(Control)}$).

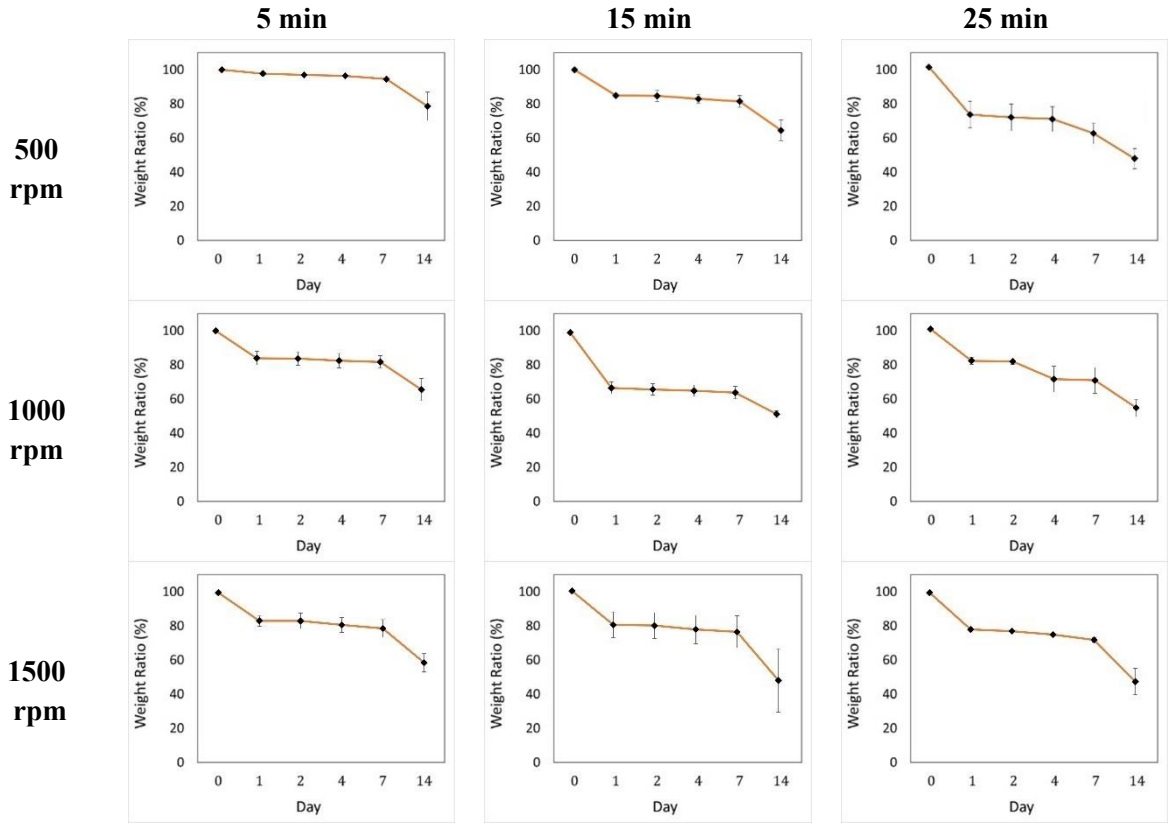


Figure 4.16: Degradation investigation for the samples fabricated at the chilling temperature of −20°C.

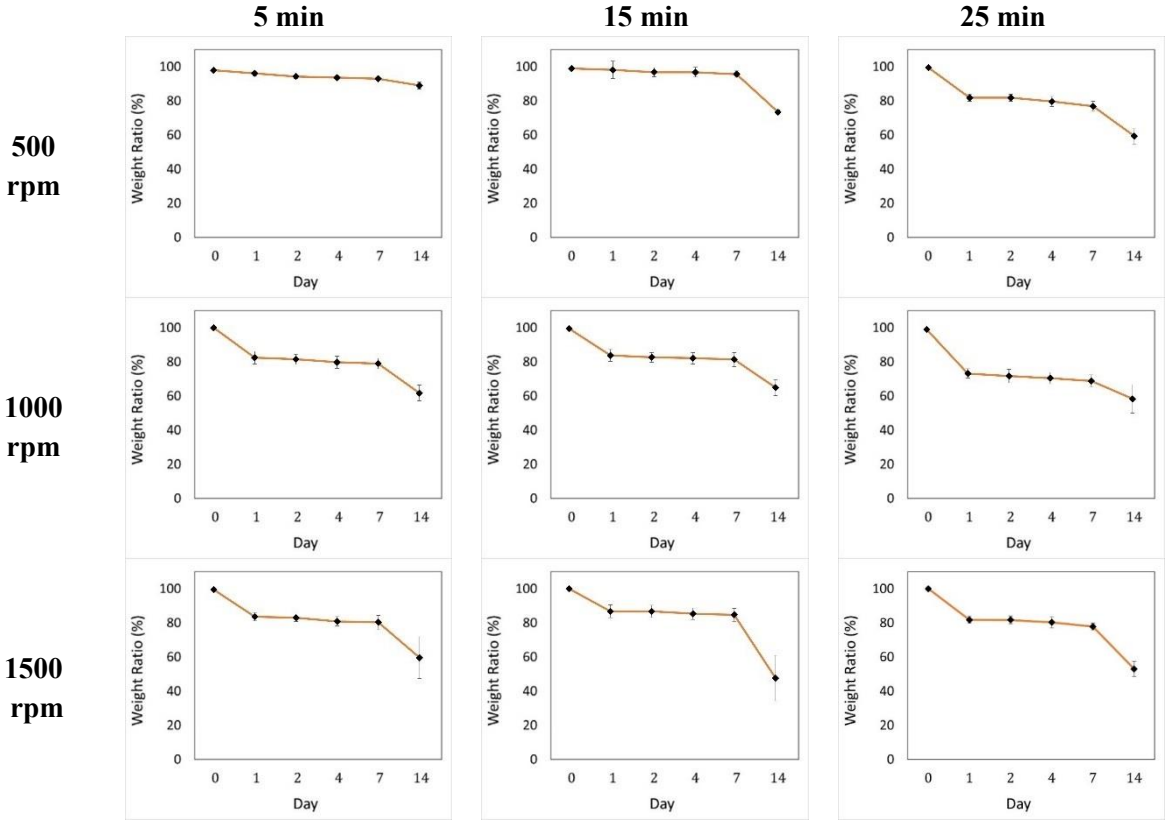


Figure 4.17: Degradation investigation for the samples fabricated at the chilling temperature of −80°C.

Table 4.1: The shrinkage values for the samples fabricated at −20 °C.

	$F_{0(Control)}$	F_1	F_2	F_3	F_4	F_5	F_6	F_7	F_8	F_9
FD 1	42.1	42.5	41	40.6	41.7	43.9	41.8	42.3	41.5	43.3
FD 2	41.8	41.5	41.5	41.5	42	44	42.2	41.7	42.7	43.1
DC 1	14.9	14.1	17.2	18.0	15.8	11.3	15.6	14.5	16.2	12.5
DC 2	15.5	16.2	16.2	16.2	15.2	11.1	14.7	15.8	13.7	12.9

FD 1 and FD 2 are the Final Diameter of samples 1 and 2 in *mm*, respectively. DC 1 and DC 2 are Diameter Change of samples 1 and 2 in %, respectively.

Table 4.2: The shrinkage values for the samples fabricated at −80 °C.

	F_{10}	F_{11}	F_{12}	F_{13}	F_{14}	F_{15}	F_{16}	F_{17}	F_{18}	$F_{19(Control)}$
FD 1	41.9	41.5	42.3	42.6	46.6	43.2	41.9	43.7	43.6	41.7
FD 2	41.5	41.2	41.9	42	45.5	43.5	42.2	43.5	43.2	41.6
DC 1	15.4	16.2	14.5	13.9	5.9	12.7	15.4	11.7	11.9	15.8
DC 2	16.2	16.8	15.4	15.2	8.1	12.1	14.7	12.1	12.7	16.0

FD 1 and FD 2 are the Final Diameters of samples 1 and 2 in *mm*, respectively. DC 1 and DC 2 are the Diameter Changes of samples 1 and 2 in %, respectively.

4.7. Mathematical Estimators for Scaffolds' Properties

In the realm of tissue engineering, the development of scaffolds with precise mechanical properties and porosity is a nuanced process that necessitates intricate planning and validation. Utilizing the robust capabilities of statistical analysis software like MATLAB and R, we sought to extrapolate empirical relationships that could accurately predict these critical parameters based on our experimental data.

Our data set included measurements of Young's modulus and porosity for scaffolds processed at chilling temperatures of −20 and −80°C. By employing regression analysis, we extracted estimators that described the relationship between our independent variables—time and speed of agitation—and our dependent variables, namely, the modulus and porosity at the two chilling temperatures.

The estimators were structured to capture the nonlinearities and interactions between the factors, providing a refined model for prediction. For instance, the estimators for modulus at −20 and −80°C revealed that, while both agitation speed and time influence the modulus, the speed has a more pronounced effect at both temperatures. This is crucial for scaffold development, as it emphasizes the need to optimize the mechanical mixing input during fabrication to achieve the desired mechanical strength. Similarly, the porosity estimators highlighted the nuanced effect of both variables on the porosity of the scaffolds. Our models suggest a fine balance is required

between agitation speed and time to ensure the scaffold possesses the necessary pore structure to support cell ingrowth and nutrient transport while maintaining its structural integrity.

While the scope of this thesis did not encompass a full optimization study, the obtained statistical estimators provide a gateway for predictive modeling in scaffold fabrication. They serve as a decision-making tool for selecting processing parameters that would yield a scaffold within the desired property range. These estimators are stepping stones towards a more extensive optimization framework that can accommodate the dynamic and multifaceted nature of scaffold development.

As mentioned before, a general formulation for our analysis could be considered as a linear model:

$$Property = \beta_0 + \beta_1 \mathrm{T} + \beta_2 \mathrm{V} + \beta_3 \mathrm{T}^2 + \beta_4 \mathrm{V}^2 + \beta_5 \mathrm{TV} + \epsilon \quad (4.1)$$

where property is either modulus or porosity at the specified chilling temperatures, T is duration of agitation, V is time of agitation, and β_0, β_1, β_2, β_3, β_4, and β_5 are coefficients which should be obtained from regression fitting. ϵ represents the error term accounting for randomness in the data.

Figure (4.18) shows the results obtained from the statistical model. Moreover, Table (4.3) reports the values obtained for coefficients calculated through regression analysis. Examination of these coefficients reveals that not all exert a statistically significant influence on the dependent variables, identified by p-values greater than 0.05. Based on these findings, and after discarding coefficients that lack statistical significance, we have refined our models to yield the following empirical estimators for modulus and porosity across the two chilling temperatures:

$$E_{-20} = 9.47972 - 1.66895\mathrm{T} - 3.9414\mathrm{V} - 2.03813\mathrm{V}^2,\ R^2{=}0.99 \quad (4.2)$$

$$E_{-80} = 5.3079 - 1.0655\mathrm{T} - 3.8576\mathrm{V} + 2.1152\mathrm{V}^2,\ \mathrm{R}^2{=}0.98 \quad (4.3)$$

$$\emptyset_{-20} = 89.67964 + 0.86910\mathrm{T} + 1.82147\mathrm{V},\ \mathrm{R}^2{=}0.95 \quad (4.4)$$

$$\emptyset_{-80} = 90.96153 + 1.27855\mathrm{T} + 1.9665\mathrm{V},\ \mathrm{R}^2{=}0.93 \quad (4.5)$$

where E_{-20}, E_{-80}, $\emptyset_{-20}$, and $\emptyset_{-80}$ shows modulus at −20°C, modulus at −80°C, porosity at −20°C, and porosity at −80°C, respectively.

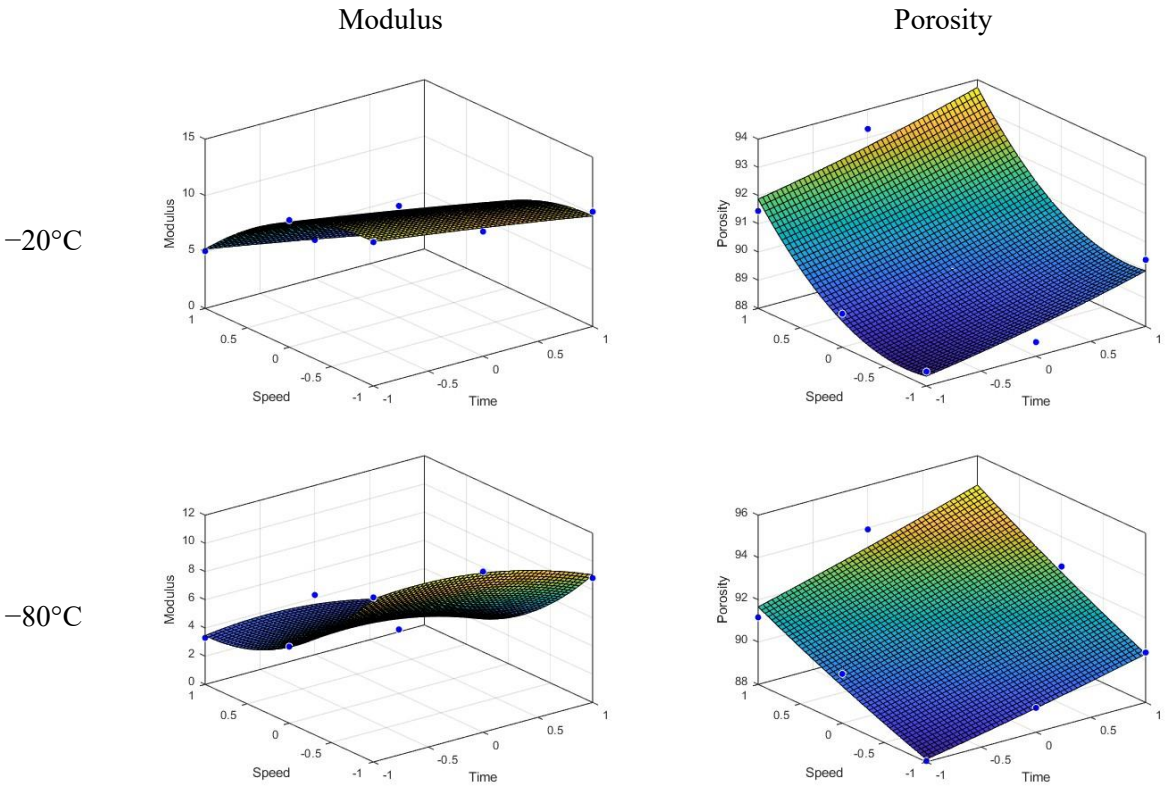


Figure 4.18: Curve fitting results for scaffold modulus and porosity at chilling temperatures of −20 and −80°C.

Table 4.3: Regression coefficients and P-values (in parentheses) for the prediction of scaffold modulus and porosity at different chilling temperatures. Values defined by * are not significant (P-value < 0.05).

	β_0	β_1	β_2	β_3	β_4	β_5
E_{-20}	9.47972 (0.000183)	-1.66895 (0.005192)	-3.94140 (0.000416)	-0.09458 (0.82530)*	-2.03813 (0.013917)	-0.17377 (0.576049)*
E_{-80}	5.3079 (0.00305)	-1.0655 (0.04791)	-3.8576 (0.00133)	-0.9869 (0.18174)*	2.1152 (0.03400)	0.1813 (0.68320)*
$\emptyset_{-20}$	89.67964 (3.16e-07)	0.86910 (0.04304)	1.82147 (0.00578)	0.11133 (0.81865)*	1.19023 (0.07544)*	0.05743 (0.86687)*
$\emptyset_{-80}$	90.96153 (7.92e-07)	1.27855 (0.0365)	1.96650 (0.0115)	0.06855 (0.9181)*	0.14360 (0.8300)*	0.19497 (0.6836)*

4.8. Conclusion

This investigation highlights the importance of fabrication parameters to the overall performance of tissue engineering scaffolds. Through meticulous experimentation, it was revealed that variables such as agitation speed and time, as well as chilling temperature crucially dictate the scaffold's pore structure, porosity, mechanical properties, and degradation behavior, which are fundamental to their effectiveness in tissue regeneration. The outcomes from this study emphasize the necessity of considering proper fabrication processes to tailor scaffold properties toward specific tissue engineering needs. Ultimately, these findings contribute significantly to our understanding of scaffold design, offering a foundation for future research aimed at advancing regenerative medicine technologies. The subsequent chapter will delineate the conclusions drawn from this study and propose directions for future research, thereby contributing to the progression of scaffold-based therapeutic approaches.

CHAPTER 5: CONCLUSIONS

5.1. Introduction

This thesis explored the effects of fabrication parameters—agitation speed, duration time, and chilling temperature—on the physical and mechanical properties of gelatin-elastin-hyaluronate (GEH) scaffolds. The results enhance our understanding of scaffold design and contribute to advancing tissue engineering. This concluding chapter summarizes the key findings and identifies potential areas for future research in scaffold-based therapies.

5.2. Conclusions

Gelatin-elastin-hyaluronate (GEH) scaffolds have shown a significant development in scaffold-based approaches for soft tissue engineering. The hypothesis of this research was that the physical and mechanical properties of these scaffolds could be significantly influenced by the conditions under which they are fabricated. This investigation focused on how factors such as agitation speed, duration time, and chilling temperature affect the properties of these scaffolds. Through an array of experimental designs, this study confirmed the hypothesis that these parameters are crucial in determining scaffold performance.

The findings from this work reveal that agitation speed and time do not have a significant effect on the size of the pores. However, chilling temperature slightly effects the pore size of the scaffolds, with quenching at lower temperatures favoring increased pore size. This enhancement in scaffold structure is important for facilitating cell infiltration, nutrient diffusion, and, ultimately, the regeneration of soft tissues. Meanwhile, scaffolds fabricated at both −20°C and −80°C exhibit increased porosity with greater agitation speeds and extended agitation times. Our results showed that higher agitation speed and duration time lead to lower Young's modulus, because of the increased porosity.

According to the results, samples with higher values of porosity degrade faster in both chilling temperatures. The degradation behavior of the samples assessed in this study points to their stability over time. This attribute is indicative of the scaffolds' ability to provide a temporary matrix for cell proliferation and differentiation, underscoring their potential for integration into the host tissue and subsequent replacement by natural tissue.

This research provides a foundation for the design and development of scaffolds with enhanced physical and mechanical properties, suitable for soft tissue engineering applications. The systematic approach adopted in this thesis, which carefully balances various fabrication parameters, paves the way for the creation of scaffolds that closely mimic the natural extracellular matrix, promoting effective tissue repair and regeneration. As the field of tissue engineering continues to evolve, the insights gained from this study will undoubtedly contribute to the ongoing efforts to develop more sophisticated and effective scaffold-based therapies. The research underscores the complexity of scaffold fabrication and highlights the critical need for precision in the design process to achieve the desired outcomes.

In conclusion, this research provides a basis for the development of scaffolds with improved physical and mechanical properties for soft tissue engineering. The systematic approach used in this thesis balances various fabrication parameters to develop scaffolds that simulate the natural extracellular matrix, facilitating effective tissue repair and regeneration.

5.3. Suggestions for Future Research

The research presented in this thesis provides a substantial foundation for the development of scaffolds in soft tissue engineering. However, the exploration into gelatin-elastin-hyaluronate (GEH) scaffolds also opens up several avenues for future investigation that could further enhance the efficacy and application of scaffold-based regenerative therapies. The following suggestions are proposed to extend the current understanding and application of tissue engineering scaffolds:

- *Incorporation of bioactive molecules:* Future studies should explore the integration of bioactive molecules, such as growth factors or peptides, into the GEH scaffolds. Investigating the effects of these molecules on cellular behavior, such as migration, proliferation, and differentiation, could provide insights into enhancing tissue regeneration. Additionally, examining the release kinetics of these molecules from the scaffolds would be crucial for ensuring their sustained bioactivity and effectiveness in promoting tissue repair.
- *Advanced fabrication techniques:* The adoption of novel fabrication techniques, such as 3D bioprinting, could offer greater precision in scaffold design and architecture. Future research could explore the use of these advanced techniques to fabricate scaffolds with complex, tissue-specific architectures that more closely mimic the natural extracellular matrix. This approach could improve the integration of scaffolds into target tissues and enhance their functional performance.
- *Mechanical property optimization:* While this thesis has highlighted the impact of fabrication parameters on the mechanical properties of scaffolds, there is a need for further optimization. Future work should aim to fine-tune these properties to match those of the target tissue more closely. This could involve exploring a wider range of fabrication parameters or combining different materials to achieve the desired mechanical characteristics.
- *In-vivo studies:* To validate the findings from in vitro experiments, future research should include in vivo studies assessing the performance of optimized scaffolds in animal models. Such studies would provide valuable information on the scaffolds' biocompatibility, degradation behavior, and efficacy in promoting tissue regeneration within a living organism. This step is critical for translating scaffold technologies from the laboratory to clinical applications.
- *Longitudinal studies on scaffold degradation and tissue integration:* Further investigation into the long-term behavior of scaffolds in a biological environment is necessary. Longitudinal

studies examining scaffold degradation, tissue integration, and regeneration over extended periods would offer insights into the lasting impact of scaffold-based therapies on tissue repair processes.

- *Customization for specific tissue types:* The potential for customizing scaffolds for the regeneration of specific tissue types represents an exciting area for future research. Tailoring scaffold properties, such as porosity, mechanical strength, and bioactivity, to meet the unique requirements of different tissues could significantly enhance the versatility and effectiveness of scaffold-based tissue engineering strategies.

By addressing these suggestions for future research, the field of tissue engineering can continue to advance toward the development of more sophisticated and effective scaffold-based therapies. The ultimate goal is to realize the full potential of regenerative medicine in restoring, maintaining, or improving tissue function and health in patients worldwide.

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