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Fabrication of Snail Mucin-Silk Fibroin Hydrogel Scaffold for Guided Tissue Regeneration: A Zebrafish Fin Model Study

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ABSTRACT

Background: Scaffolds play a pivotal role in guiding tissue regeneration by providing a suitable environment for cell growth, migration, and differentiation. Among various materials, biopolymers such as silk fibroin and snail mucin have garnered attention due to their biocompatibility, natural origin, and potential to promote cellular activities. **Objectives:** In this study, we explore the fabrication of a novel Snail Mucin-Silk Fibroin hydrogel scaffold designed to enhance tissue regeneration. **Materials and Methods:** The hydrogel scaffold has been prepared by mixing 28% w/v gelatin, 21% w/v PVA in distilled water and 10% HPMC, and heated to 40ºC followed by addition of a snail mucin and silk fibroin at a lower temperature and freeze dried at 4ºC for 12 hr. **Results:** The characterization tests showed the scaffold's favourable biological properties and morphology, such as biocompatibility, hemocompatibility, ability to retain moisture, and promotion of cellular activities essential for tissue repair. Histopathological analysis showed that the snail mucin-silk fibroin hydrogel scaffold significantly enhanced fin regeneration compared to the control groups. **Conclusion:** Thus, the snail mucin hydrogel used in this study showed better soft tissue regeneration potential and hence be used for various applications in regenerative medicine.

Keywords: Hydrogel, Regeneration, Silk Fibroin, Snail Mucin, Zebra Fish.

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INTRODUCTION

Tissue regeneration and repair are crucial aspects of medical science, particularly in the field of regenerative medicine.[1] A significant challenge in tissue engineering is developing effective scaffolds that can promote the regeneration of damaged tissues while ensuring biocompatibility, mechanical stability, and adequate cell signalling. The tissue adhesives which are available currently have poor wet adhesion potential and/or biocompatibility.[2] Natural biomaterials have recently emerged as appealing possibilities for scaffold fabrication due to their unique features, such as biocompatibility, biodegradability, and the ability to enable cell adhesion and proliferation.[3] Among these, Silk Fibroin (SF) and Snail Mucin (SM) have garnered significant attention for their regenerative potential.

Snail mucin, a glycoprotein extracted from the slime of snails, has recently attracted interest for its beneficial properties in wound healing and tissue regeneration.[4] Snail mucin is rich in bioactive compounds such as glycosaminoglycans, proteoglycans, and antimicrobial peptides, which are known to promote cell migration, collagen synthesis, and tissue repair.[5] Additionally, snail mucin has anti-inflammatory properties, making it a valuable component for tissue regeneration applications.[6] When combined with other natural polymers like silk fibroin, snail mucin can potentially improve the performance of scaffolds for guided tissue regeneration.

Silk fibroin, a protein derived from the silkworm "Bombyx mori", is known for its excellent mechanical properties, biocompatibility, and versatility in tissue engineering applications.[7] Silk fibroin scaffolds have been studied in depth for numerous tissue engineering applications, including bone, skin, and nerve regeneration. Silk fibroin's ability to support cell growth and its controlled degradation profile makes it an ideal candidate for scaffold fabrication. However, while SF scaffolds have shown great promise, the incorporation of other natural biomaterials could potentially enhance their regenerative properties.

The development of composite scaffolds combining snail mucin and silk fibroin can result in materials that not only possess the mechanical strength of silk fibroin but also the bioactive properties

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of snail mucin. This combination could enhance scaffold performance by providing a more conducive microenvironment for cell attachment, migration, and differentiation. Guided Tissue Regeneration (GTR) is an approach that uses scaffolds to direct the growth of specific tissues and organs, and composite scaffolds could significantly improve the outcomes of this strategy.[8]

To assess the regenerative potential of these composite scaffolds, it is essential to use a reliable *in vivo* model. The zebrafish fin model has become a widely recognized model for studying tissue regeneration due to its remarkable regenerative abilities and the similarity of the processes to human tissue repair. Zebrafish fins can regenerate after amputation, making them an ideal model to investigate the efficacy of tissue-engineering scaffolds in promoting tissue growth and healing.[9] By utilizing the zebrafish fin model, this study aims to evaluate the regenerative potential of the Snail Mucin-Silk Fibroin (SM-SF) scaffold for guided tissue regeneration.

This study will explore the fabrication of Snail Mucin-Silk Fibroin composite scaffolds and their application in tissue regeneration, specifically focusing on their ability to promote regeneration in the zebrafish fin model. Through this investigation, we aim to provide insights into the synergistic effects of combining these two natural materials in scaffolds and their potential for advancing the field of guided tissue regeneration. These results have significant implications for understanding the adhesion process of snail-mucus hydrogels and developing next-generation bio-inspired adhesives.

MATERIALS AND METHODS

Preparation of snail mucin hydrogel scaffold

Snail mucin was collected from garden snails "*Cornu aspersum*" using a controlled milking technique, ensuring that the mucin is harvested under hygienic conditions to maintain its bioactive properties. Simultaneously silk fibroin was extracted from "*Bombyx mori"* silkworms and aqueous silk fibroin solution was prepared at a concentration of 5-10% w/v.

The scaffold has been prepared by mixing 28% w/v gelatin, 21% w/v PVA in distilled water and 10% HPMC and heated to 40ºC followed by addition of the extracted snail mucin and silk fibroin at a lower temperature. The mixture was homogenized to ensure a uniform distribution of snail mucin within the silk fibroin matrix. The resulting solution was then cast into moulds and allowed to air dry or freeze-dry at 4ºC for 12 hr to form scaffolds (Figure 1).

Scaffold Characterization

The structural, mechanical, and biological properties of the fabricated SM-SF scaffolds were assessed by a series of characterization techniques. The surface morphology of the scaffolds was observed using Scanning Electron Microscopy (SEM). This helps to assess the pore structure, surface roughness, and the uniformity of the composite materials. Fourier Transform Infrared (FTIR) spectroscopy analysis was done to identify the bond formation between the compound used for the scaffold. Blood compatibility testing was done to evaluate the toxicity of the scaffold against RBC cells.

Cell Culture

To assess the cytocompatibility of the SM-SF composite scaffolds, 3T3 fibroblast cell lines were seeded onto the scaffolds and incubated for 24 hr to identify the growth of the cells.

In vivo Evaluation Using the Zebrafish Fin Regeneration Model

The zebrafish fin regeneration model was used to evaluate the regenerative potential of the SM-SF composite scaffolds *in vivo*. Adult zebrafish (*Danio rerio*) were maintained in a controlled environment and was fed a regular diet on a 14-hr light/10-hr dark cycle. Zebrafish was anesthetized with tricaine methane sulfonate (MS-222) prior to the surgical procedure. A portion of the caudal fin was amputated to initiate regeneration. After fin amputation, the scaffold was placed at the wound site, and the fish was allowed to recover in a tank with a controlled temperature and water quality (Figure 2).

Histological Analysis

The zebrafish were euthanized at specific time points (7,14-, and 21-days post-implantation). The fin portion of the fishes were cut and fixed in formalin for histological analysis of the wound. The formalin fixed tissue was then processed and sectioned and stained with haematoxylin and eosin stains.

Ethical Considerations

All experiments involving zebrafish was conducted in accordance with ethical guidelines for animal research, with appropriate permissions obtained from the Institutional Animal Ethical Committee of Saveetha Dental College and Hospitals, Saveetha Institute of Medical and Technical Sciences (SIMATS).

Through this methodology, the regenerative potential of Snail Mucin-Silk Fibroin composite scaffolds was comprehensively assessed, providing insights into their application for guided tissue regeneration in both *in vitro* and *in vivo* models.

RESULTS

SEM Morphology

This fabricated membrane was examined for morphology (Figure 3). Porous and slightly rough surface was observed. Porous nature of the membrane helps in the supply of nutrients, blood and oxygen for the regeneration of the tissue. Flexible morphology helps folding and twisting of the membrane which can be placed anywhere in the oral region. Slightly rough surface which micron in nature helps in the attachment of cells and also to grow and multiply effectively. Thus, the porous and flexible surface which slight roughness helps in the better regeneration of the cells and tissues.

Functional group analysis by FTIR

A rapid and reasonably priced method for identifying chemicals is FTIR, or infrared spectroscopy. According to FTIR, the s-GAG shares a repeating sequence with the previously described s-GAG from snail bodies, which is →4)-α-GlcNAc (1 → 4)-α-IdoA2S (1 →). C-N vibrations, -C-O stretching, N-H bending vibration for secondary amines, C=C stretching of α, β-unsaturated ring, C-H stretching, O-H stretching, and N-H amide bending were the principal peaks that were seen at wave numbers of 1074.39, 1240.27, 1528.64, 1641.48, 2946.36, 3275.24, and 3421.83 cm-1, respectively (Figure 4).

Biocompatibility and biodegradability

Blood compatibility

Blood compatibility testing was done as per ASTM standard F7500. As per standard the samples showed the lysis percentage which is less than 2% (Figure 5). This proves that the fabricated membrane was compatible and it does not show any toxicity with RBC cells when incubated. Bleeding that results from accident, trauma, or surgical treatments can have serious consequences. Uncontrolled bleeding is the leading cause of death on battlefields and during traumatic events. Bio adhesive polymers with haemostatic properties can assist reduce blood loss and facilitate wound closure.

Adhesion performance

Under pathophysiological conditions, ideal bio adhesive materials promote bio integration and tissue regeneration. The hydrogel's high stickiness can prevent detachment from the target tissue, enabling bio integration and healing. The ability of fresh and dried SM-SF hydrogels to adhere to wet tissues was tested.

Confocal Laser Scanning Microscopical (CLSM) analysis of 3T3 (fibroblast cells) cultured over the snail mucin membrane

3T3 fibroblasts cell lines were cultured over the snail mucin membrane in order to evaluate the attachment growth and regeneration of the cells. It is observed the cells get attached effectively and the growth and multiplication of the cells are better after 24 hr incubation. Figure 6 shows the CLSM of surface and cross view of the cells attached over the membrane after 24 hr (Figure 6).

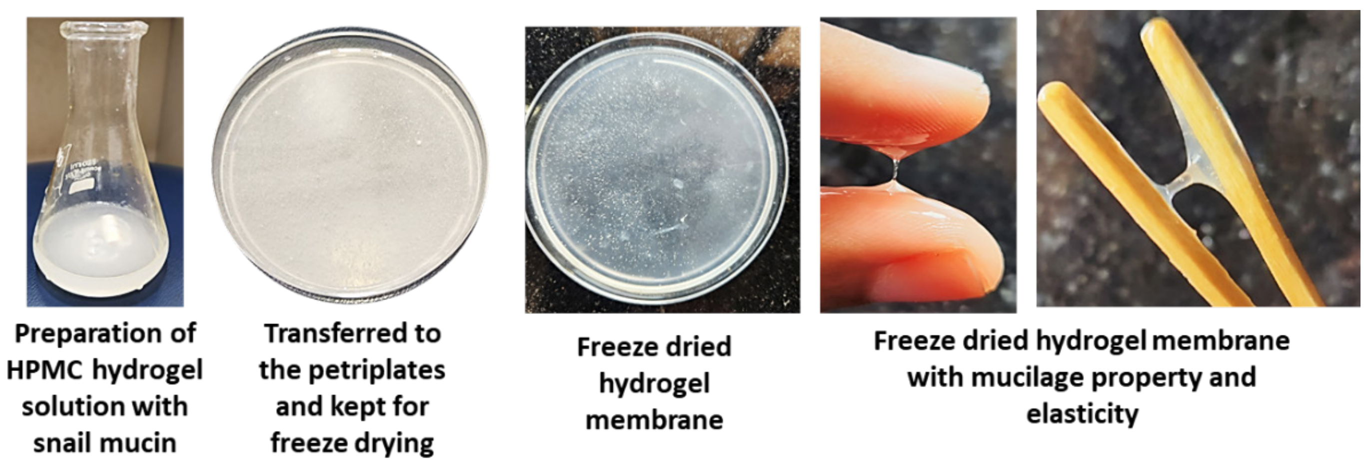

Figure 1: Preparation of snail mucin hydrogel scaffold.

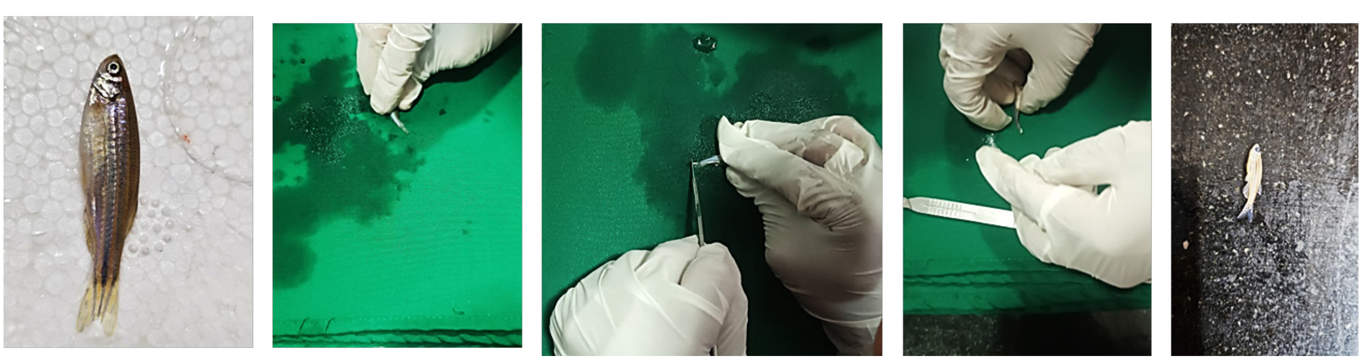

Figure 2: Process of fin regeneration procedure using hydrogel membrane.

Fin regeneration in Zebrafish-using snail mucin membrane

The idea of using snail mucin membrane in fin regeneration might focus on leveraging its beneficial properties for wound healing or stimulating tissue regeneration. Possible mechanisms by which snail mucin could be useful include Wound Healing. The mucin might act as a barrier that accelerates the healing process by promoting the formation of new cells and tissue. In zebrafish, it could be used in models where the regeneration of the fin is impeded or slowed down, to see if it enhances the rate of regeneration. Reduction of Inflammation: Snail mucin has anti-inflammatory properties that could potentially reduce scarring and tissue damage, both of which are important for successful regeneration. Stimulating Growth Factors: Certain components in snail mucin might help activate pathways that promote stem cell proliferation or the regeneration of specific tissues.

Snail mucin has already been incorporated into various cosmetic products for skin regeneration, particularly in products aimed at reducing scars, wrinkles, and other forms of skin damage. It has been shown to promote the production of collagen and elastin, both critical proteins for skin's structural integrity. As a result, snail mucin has been used in topical ointments, gels, and creams to accelerate wound healing and improve skin appearance after injury.

Histopathological analysis of zebrafish tissue which is treated with the snail mucin membrane

The H&E-stained sections revealed increase in the proliferation of the undifferentiated mesenchymal cells (blastema cells) in the dermis. There was also evidence of increase in the number of fibroblast cells, dense collagen bundles formation and increase in vascularity in the fin portion treated with the scaffold. The epidermis showed a bulge along the edge of the tail suggestive of regeneration of the fin portion of the zebrafish treated with the snail mucin scaffold (Figure 7).

DISCUSSION

Biological adhesives present appealing alternatives to sutures and staples.[10] The discovery and development of biomimetic tissue adhesives has received a lot of interest in recent years. Biobased extracts contain a variety of bioactive components and are commonly utilised in tissue engineering applications to improve the bioactivity and physical properties of biomaterials. Garden snail *Helix aspersa* has gained popularity among animal sources due to its antibacterial and regenerative extracts, which have the potential to regenerate tissues. In this study, bioactive *H. aspersa* (snail mucus) was injected into an HPMC matrix to create porosity scaffolds for tissue regeneration.

The study explores an innovative approach to tissue regeneration using a combination of snail mucin and silk fibroin in a hydrogel scaffold. This research highlights the promising role of snail mucin in promoting wound healing and tissue repair due to its mucopolysaccharide content and growth factor properties. Silk fibroin, known for its biocompatibility, strength, and biodegradability, is used here as a matrix to support the mucin. The use of zebrafish as a model organism is significant due to their regenerative abilities, particularly in fin regeneration. This study combines biological materials with cutting-edge tissue engineering techniques to enhance the regeneration of damaged tissues.

Hydrogel scaffolds have been extensively studied in tissue engineering due to their excellent water retention properties and ability to mimic the Extracellular Matrix (ECM). Studies by authors such as Lee *et al*., and Zhang *et al*., focused on the use of various biopolymers like collagen, alginate, and gelatin for scaffold fabrication, which supported cellular growth and tissue repair.[11,12] However, the combination of snail mucin with silk fibroin in hydrogel scaffolds is a novel concept in the

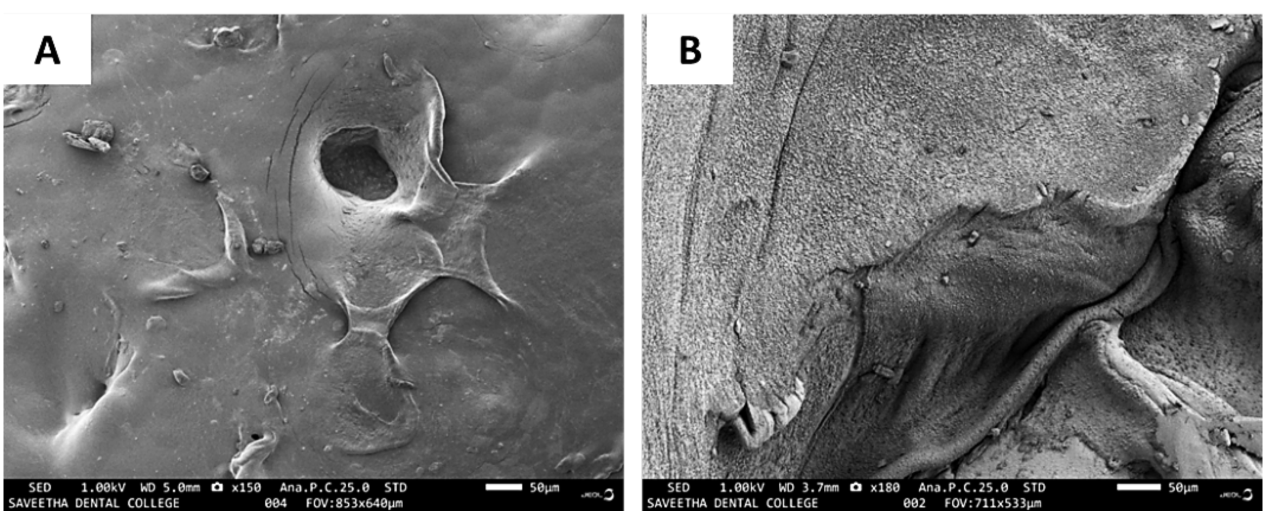

Figure 3: SEM morphology of hydrogel membrane.

current study. Unlike previous studies that have primarily explored collagen-based or synthetic polymer scaffolds, this study introduces a dual-component scaffold that leverages the regenerative potential of snail mucin, potentially enhancing tissue regeneration by providing bioactive molecules that promote cell adhesion and proliferation.

The inclusion of snail mucin in the scaffold for tissue regeneration is an innovative approach that sets this study apart from previous work. Snail mucin has been shown to possess properties such as anti-inflammatory effects, moisture retention, and wound-healing abilities.[13] While previous studies have mainly focused on snail mucin's use in cosmetic and dermatological applications, such as skin regeneration, its potential in tissue engineering is relatively underexplored.[14] The current study expands on this idea by integrating snail mucin with silk fibroin in a hydrogel matrix, aiming to improve not just skin regeneration but also other tissue types, as shown in the zebrafish fin model.

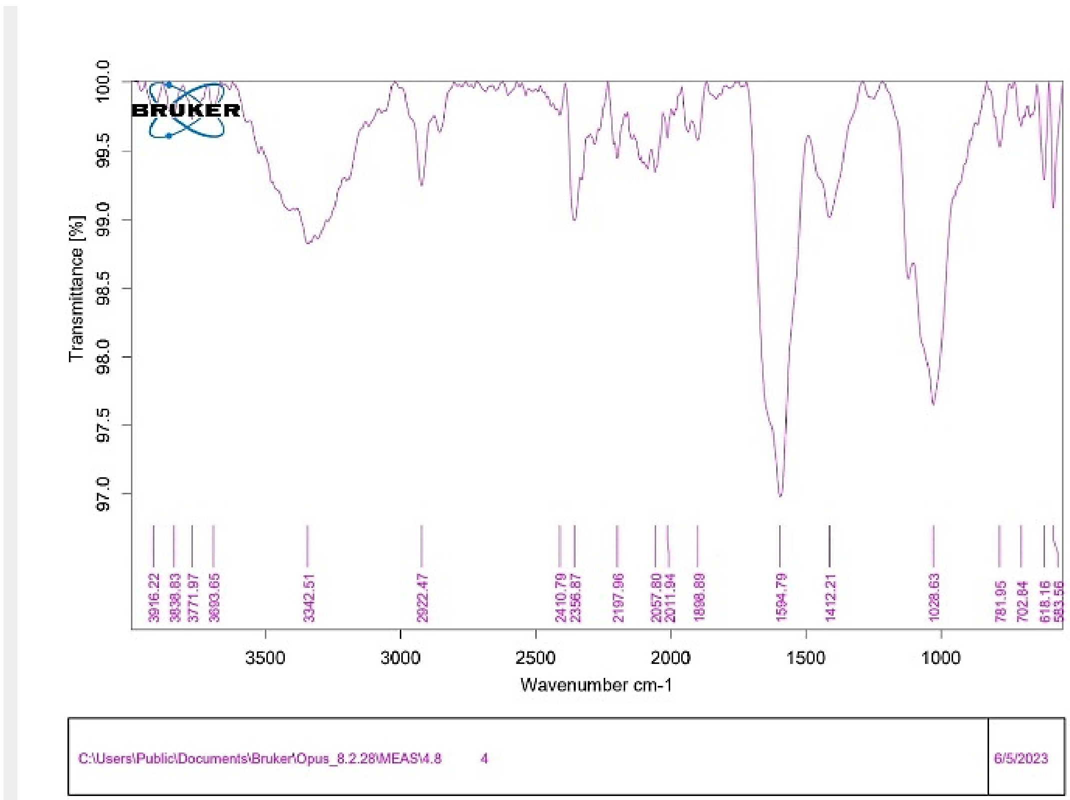

Figure 4: FT-IR spectrum of hydrogel membrane.

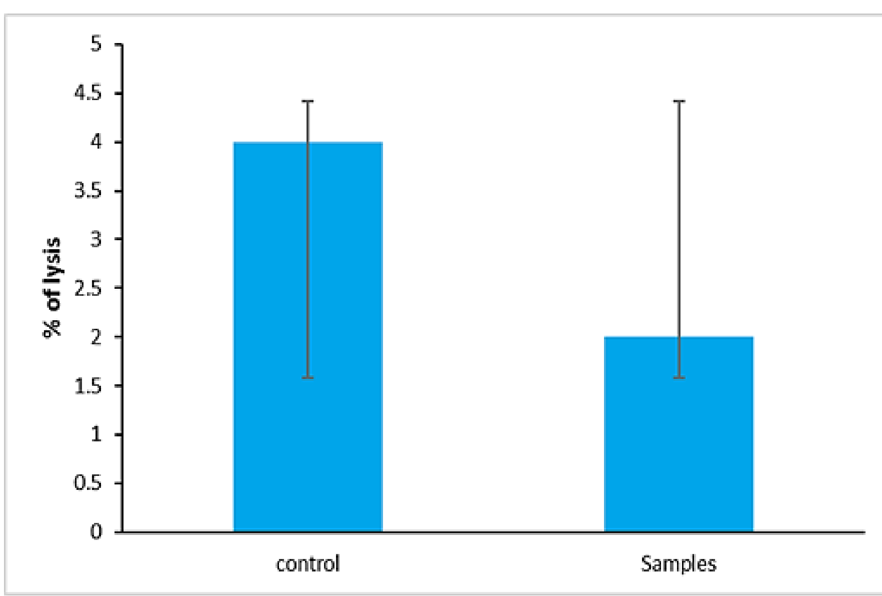

Figure 5: Hemocompatibility of hydrogel membrane.

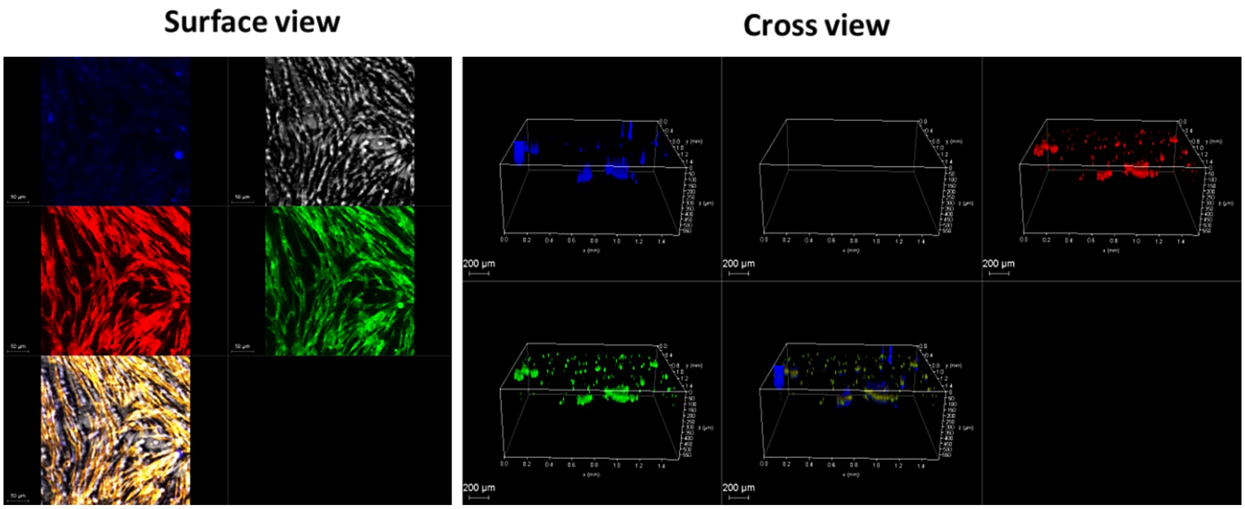

Figure 6: Fibroblast attachment and proliferation on the membrane and its CLSM image.

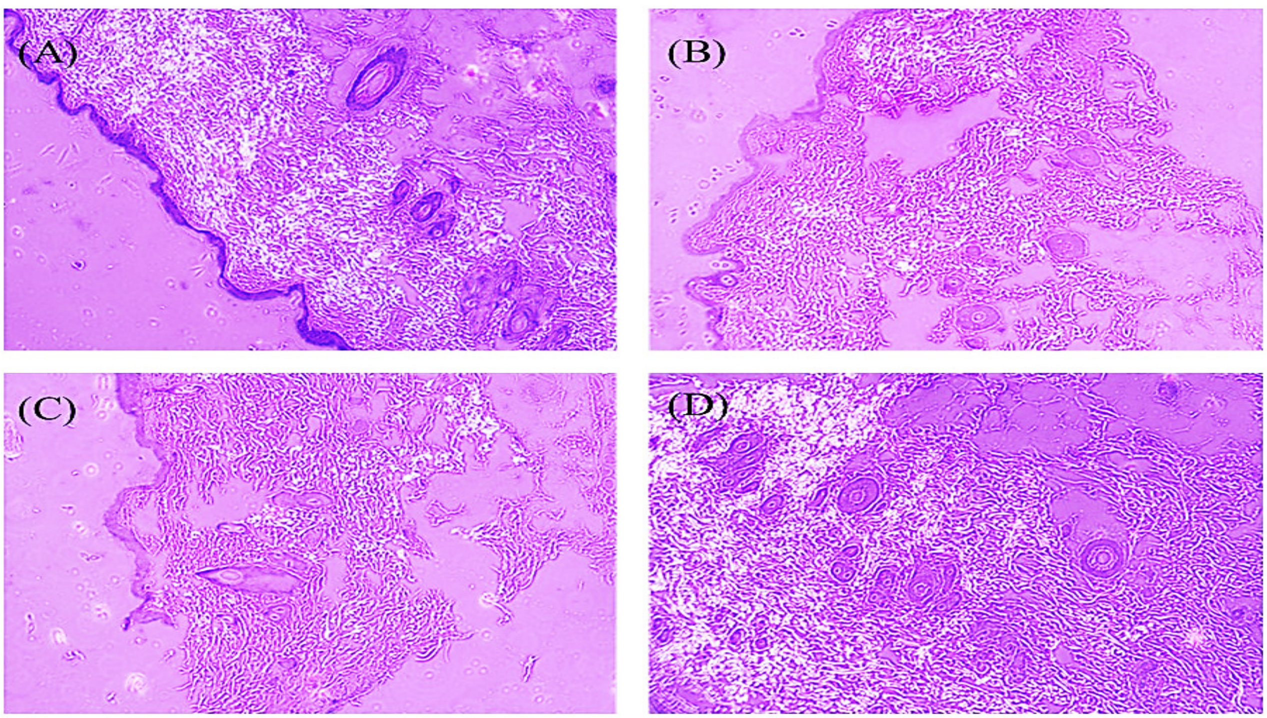

Figure 7: Histopathological analysis of the zebrafish tissue treated with the scaffold.

Silk fibroin has long been a focus in regenerative medicine due to its remarkable mechanical properties and biocompatibility. Previous studies, such as those by Li *et al.*, have explored its use in creating scaffolds for bone, cartilage, and nerve regeneration.[15] Silk fibroin's ability to mimic the natural ECM and support cellular behaviours has made it a preferred material in scaffold development. In the current study, silk fibroin serves as a structural component of the hydrogel scaffold, providing both mechanical support and a conducive environment for the regenerative properties of snail mucin. This combination has not been extensively studied before, making it an exciting advancement in the field.

The use of the zebrafish fin model is another critical aspect of this study. Zebrafish are a popular choice for regenerative studies due to their remarkable ability to regenerate various body parts, including fins, heart, and spinal cord.[16] Previous research has successfully employed zebrafish to study bone and tissue regeneration, with studies showing that the fin regeneration model can provide valuable insights into cellular processes such as proliferation, migration, and differentiation.[17] The current study builds on this by using zebrafish to test the effectiveness of the snail mucin-silk fibroin scaffold in promoting tissue regeneration, further validating its potential for human applications.

Recent advances in tissue engineering have focused on the development of multifunctional scaffolds that incorporate various bioactive materials to promote regeneration. For instance, Choi *et al*., investigated the role of growth factors and biomaterials in promoting tissue healing.[18] The combination of bioactive molecules, like snail mucin, and biomaterials, like silk fibroin, is in line with this trend, offering enhanced therapeutic outcomes. The current study adds to this growing body of literature by introducing a new scaffold material that not only supports tissue regeneration but also actively participates in the healing process through its bioactive components.

CONCLUSION

Thus, the bioactive extract loaded SM-SF scaffolds showed enhanced physical properties and *in vitro* bioactivity. The snail mucin-silk fibroin hydrogel used in this study showed better soft tissue regeneration potential and hence be used for various applications.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

SM-SF: Snail mucin-Silk fibroin; **GTR:** Guided Tissue Regeneration; **SEM:** Scanning Electron Microscopy; **FTIR:** Fourier-Transform Infrared Spectroscopy; **CFSM:** Confocal Scanning Microscopy; **HPMC:** Hydroxy Polymethyl Cellulose; **ASTM:** American Society for Testing and Materials; **ECM:** Extra Cellular Matrix.

SUMMARY

This study presents the development of a novel Snail Mucin-Silk Fibroin hydrogel scaffold for tissue regeneration. The scaffold was fabricated using a mixture of gelatin, PVA, and HPMC, followed by the incorporation of snail mucin and silk fibroin, then freeze-dried. Characterization tests confirmed its biocompatibility, hemocompatibility, moisture retention, and ability to promote cellular activities essential for tissue repair. Histopathological analysis demonstrated significantly enhanced fin regeneration compared to control groups. These findings highlight the scaffold's potential for soft tissue regeneration, making it a promising candidate for regenerative medicine applications.

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