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Development and Characterization of a Magnesium Membrane Loaded with Hyaluronic Acid, Tricalcium Phosphate, and Quercetin for Bone Healing Applications

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Abstract

This study aims to develop and characterize a hyaluronic acid/Tricalcium Phosphate (TCP)/quercetin-doped magnesium membrane for Guided Bone Regeneration (GBR), targeting applications in periodontal and other biomedical fields. The goal is to create a biocompatible, hydrophilic membrane with enhanced properties suitable for promoting bone regeneration. A polymeric solution containing TCP, PVA, hyaluronic acid, and quercetin-doped magnesium nanoparticles was electrospun to create nanofibrous membranes. These membranes were analyzed using FTIR for chemical interactions, XRD for nanoparticle distribution, SEM for morphology, and water contact angle measurements for hydrophilicity. Cell viability was assessed with an MTT assay using Dental Pulp Stem Cells, and bone formation potential was evaluated using MG63 and osteoclast cells with Alizarin Red staining. The fabricated membranes demonstrated significant hydrophilicity, which is critical for GBR applications. SEM analysis revealed a nanofibrous structure with appropriate pore size, facilitating cell attachment and growth. The FTIR confirmed the expected chemical bonding, while XRD verified the incorporation of magnesium-doped nanoparticles. MTT assays showed high cell viability, indicating good biocompatibility. Furthermore, the bone formation assay confirmed the membrane's potential to support osteogenesis. These findings suggest that the hyaluronic acid/TCP/quercetin-doped magnesium membranes developed in this study exhibit favorable properties for use in guided bone regeneration, offering promising potential for addressing the limitations of current periodontal treatments and improving patient outcomes.

Keywords: *Bone Healing, Hyaluronic Acid, Quercetin, Tricalcium Phosphate.*

Introduction

The alveolar bone and periodontal ligament are both affected by the common chronic inflammatory disease known as periodontitis. It is primarily caused by bacterial infections from dental plaque buildup. This leads to the formation of deep pockets between gums and teeth, which can result in tooth loss, gum recession, and systemic health issues if untreated. Regular dental hygiene and professional care are crucial for prevention and treatment [1, 2].

Periodontitis has four primary subtypes: necrotizing, chronic, aggressive, and periodontitis associated with systemic diseases [1]. It is associated with increased risks of systemic illnesses such as cardiovascular problems and preterm low birth weight babies. Treatment options include systemic antibiotics, although these have limitations such as inadequate antibiotic concentration at the

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periodontal pocket site and rapid declines in plasma antibiotic levels. The previous research has described hollow cellulose acetate fibers loaded with tetracycline hydrochloride, effectively reducing pathogenic subgingival bacteria in the periodontal pocket.

However, current delivery systems have drawbacks like the need for mechanical attachment, removal of non-biodegradable systems, poor penetration into deeper periodontal pockets, and low patient compliance [3]. In order to repair periodontal tissues along with bone loss caused by periodontitis, guided bone regeneration (GBR) and guided tissue regeneration (GTR) are common treatments. A new generation of guided bone regenerating membranes, known as barrier membranes among soft tissue or periodontal defects, has gained popularity. These membranes prevent soft tissue ingrowth and allow slow-growing cells of bone to infiltrate the defect site.Despite numerous publications on these membranes, comprehensive reviews on their role in periodontal regeneration are scarce [4].

GBR is a prevalent surgical therapy for the additional alveolar bone to anchor dental implants in partially and fully edentulous patients. Barrier membranes are essential for preventing non-osteogenic tissue invasion into the bone cavity, which is critical for the success of GBR. These membranes are classified as non-resorbable or resorbable, with resorbable membranes not requiring a second surgery for removal [5].

The GBR approach has limitations, including needing a robust barrier membrane and creating a new cavity during surgery. The lack of soft tissue is also a limiting factor. A temporary scaffold is needed to act as an adhesion substrate of implanted cells & to give physical support for the creation of new organs in tissue engineering, which presents a viable alternative treatment for organ damage or loss. The scaffold should be highly porous, mechanically strong, biocompatible, biodegradable, and flexible to promote cell adhesion, encourage cell growth, and preserve differentiated cell activity. An exploration of guided bone regeneration using a hyaluronic acid/tricalcium phosphate/quercetin-doped magnesium membrane is presented here. The goal is to assess their potential in improving periodontal regeneration and addressing current treatment limitations.

Materials and Methods

Materials

Polyvinyl alcohol (PVA), Tricalcium Phosphate (TCP), hyaluronic acid, and quercetin-doped magnesium nanoparticles were obtained from reputable suppliers.

Fabrication of Membrane

PVA 10% by volume, hyaluronic acid 1%, B-TCP 0.5% w/v, and five milligrams of quercetin-doped magnesium (Q-MgO) at 37°C were combined to create a polymer solution. An electric charge of around 10 kV was applied to the solution after it was magnetically swirled for a day. The solution was injected at a rate of 0.9 ml/hour, and fibers were collected on an aluminum plate positioned 10 cm from the needle. The fabricated fibers were analyzed further [6].

Characteristics Analyzed

FTIR system with Attenuated Total Reflectance (ATR): After adding nanoparticles, functional groups were characterized, and chemical interactions were determined by Fourier Transform Infrared Spectroscopy (FTIR). Bruker ATR-FTIR spectroscopic examination was conducted using ATR infrared spectrometry (model) with a 400–4000 cm-1 range. The FTIR spectra validated the scaffolds' anticipated functional groups.

SEM Evaluation: The morphological characteristics of the created scaffolds were examined using the JSM-IT800 NANO scanning electron microscopy an electromagnetic emissions electron microscope

(MES) with a built-in JEOL energy dispersive X-ray spectrometer (EDS) [7].

After freeze-drying, the SEM and EDS were used to analyze membrane porosity and fiber diameter. Platinum was sputter-coated onto the samples at room temperature. The SEM pictures were analyzed using ImageJ software to determine the average fiber diameter and porosity. Micrographs were taken at a magnification of 2.50kX.

Contact Angle

The hydrophilicity of the scaffolds was determined by measuring water contact angles using goniometer software. After being divided into 1 cm by 1 cm squares, the scaffolds were positioned on the examination plate. A gentle drop of 50 µL distilled water was applied to the samples. Images were captured within two seconds of water droplet contact to measure the contact angles. Three measurements were taken on each scaffold at different locations [8].

Human Dental Pulp Stem Cell Culture

Following informed permission & ethical approval provided by the ethics committee, dental pulp stem cell lines (DPSCs) were extracted out of molars. DMEM, or Dulbecco's Modified Eagle supplemented with 10% FBS and 1% Penicillin/Streptomycin was used to cultivate the cells. A 48-well plate was seeded with 10,000 cells in order to examine the vitality and compatibility of the cells.

MTT Assay

A 1 mg/ml membrane of PVA/B-TCP/quercetin-doped magnesium nanoparticles was prepared. The sample was dissolved in 10% FBS and 1% Penicillin/Streptomycin in DMEM F12 medium. After 24 hours of immersion, the media were removed, and the cells were treated to assess compatibility. The cultivated cells were allowed to develop the formazan dye for four hours at 37°C after being incubated for twenty-four hours. After that, 10 µliter/100 mL of the MTT solution (5 mg/mL standard) was applied to the cells. After that, 200 µL of DMSO was added to the medium and left to sit for ten minutes. After the reaction product was transferred into a 96-well ELISA plate, the absorption intensity at 570 nm was determined using a plate reader for ELISA [9].

X-ray Diffraction (XRD analysis): The GBR membrane's magnesium nanoparticle content was verified by XRD analysis. Radiation from Cu K ($\lambda = 1.5406$ Å) was used with the D8 diffractometer platform for the analysis.

Bone Formation Assay

For 14 days, the MG63 osteoclast cultures were cultivated in a differentiation medium that contained magnesium oxide nanoparticles, ten millimoles of β-glycerophosphate, 0.05 millimoles of ascorbic acid, and DMEM F12. Alizarin red staining was used to identify calcium deposits. Cells were treated using 2% alizarin red solutions for 10 minutes after two weeks, and they were then twice rinsed with 1X PBS. Each well received 200 µL of DMSO for quantitative analysis, which was then incubated for an hour. The concentration of alizarin was measured with a spectrophotometer set at 405 nm. This assay helps assess the scaffolds' potential to promote cell differentiation into osteoblasts [10].

Statistical Analysis

Every value is shown as the average ± standard deviation of the mean, or SEM, derived from a minimum of three separate tests. Multiple comparisons were carried out using Scheffe's approach, and one-way ANOVA was used to assess significant differences. At $p < 0.05$, statistical significance was established.

Results

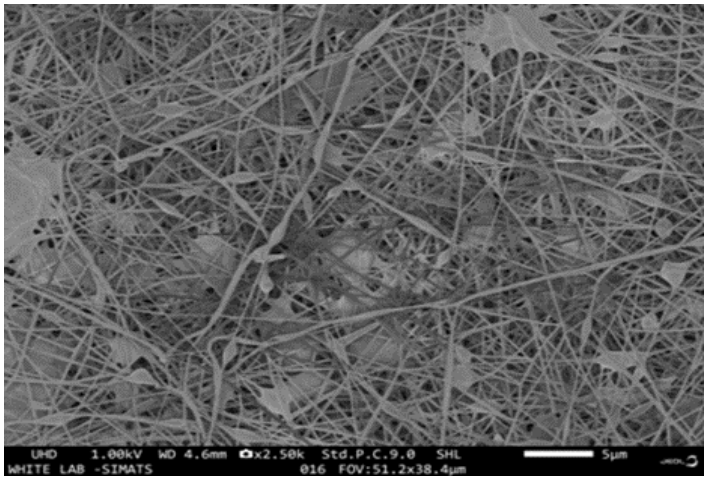

Figure 1. SEM picture of PVA nanofibers at 10% solid loading (control group)

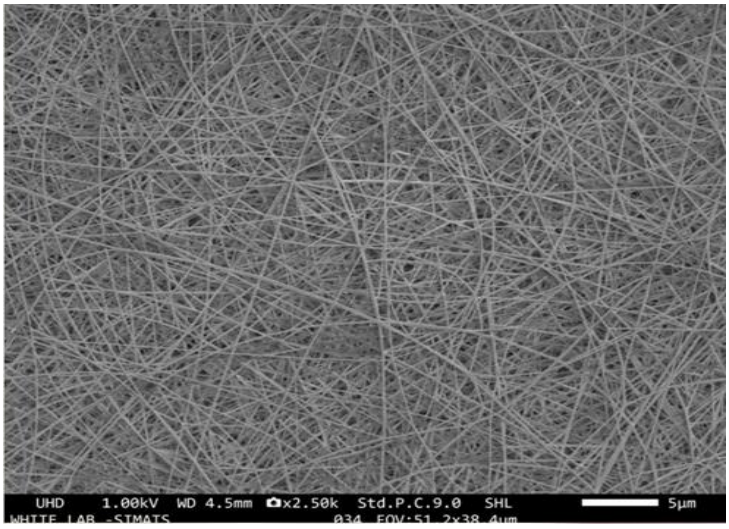

Figure 2. SEM picture of magnesium-incorporated nanoparticles in PVA fibers

The SEM image featuring exclusively PVA nanofibers displays increased porosity compared to the SEM image in Figure 2. This observation reinforces specific attributes such as its hydrophilicity, water permeability, and thermal and chemical resistance, which are advantageous for guided bone regeneration. Cell migration is facilitated only at a specific pore size. The diameter of the fibers in both Figure 1 and Figure 2 was determined to be five µm.

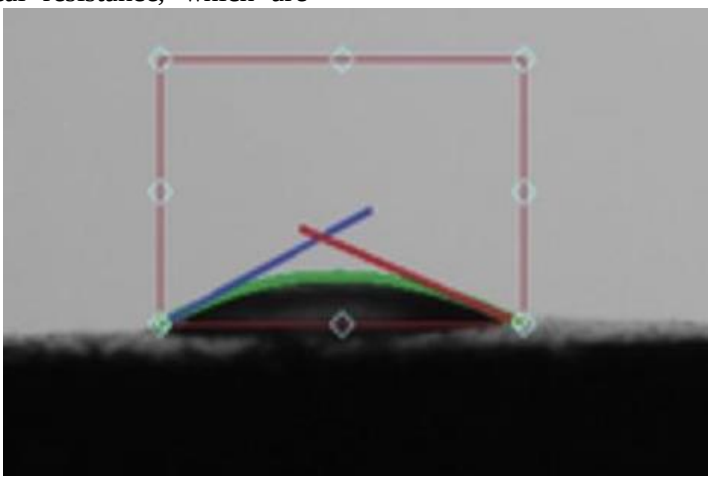

Figure 3. The contact angle of the scaffold

According to the information provided in Figure 3, the contact angle of PVA is 30°, while the average contact angle of magnesium nanoparticles is 24.57°. Incorporating Hyaluronic Acid, magnesium nanoparticles, and B-TCP in the scaffold has enhanced its hydrophilicity, endowing it with favorable hydrophilic properties and rendering it highly absorbable.

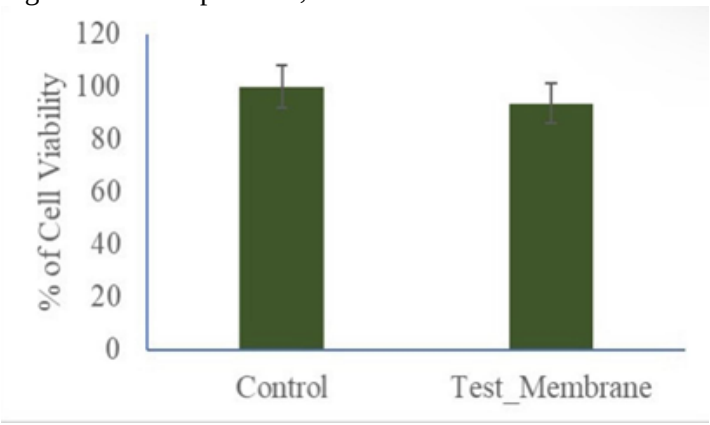

Figure 4. Comparison between cell viability of control group membrane containing only PVA and test group membrane containing magnesium nanoparticles.

Cell viability and compatibility are assessed using the MTT assay, a standard method for determining cell viability. If the compatibility of the synthesized material with the cells exceeds 80% (figure 4), it suggests that the material could be suitable for biological applications. Generally, a compatibility level of over 75% is considered favorable, indicating that the material is well-tolerated by the cells and has the potential for use in biological systems.

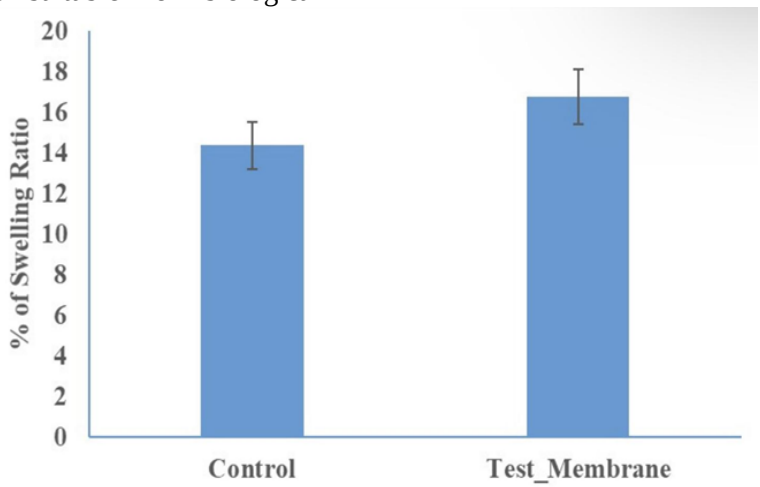

Figure 5. Comparison of the swelling ratio between control (PVA) and test membrane (magnesium membrane)

A swelling test result of less than 30% is considered ideal. In this range, the scaffold remains intact and ensures greater hydration, allowing for better nutrient flow due to increased porosity (figure 5). If too much swelling occurs, the scaffold loses its mechanical properties, while too minor swelling does not allow for proper material flow.

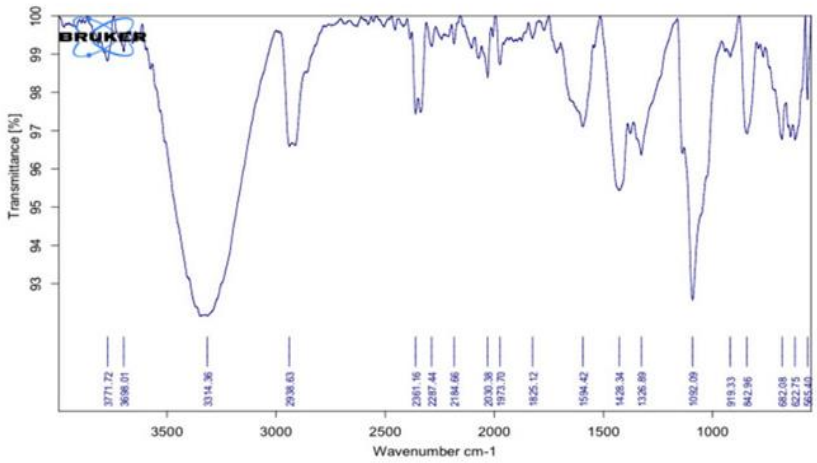

Figure 6. Depicts the FTIR spectra of PVA incorporated with magnesium membrane

The FTIR analysis has been utilized to evaluate the various functional groups present in the membrane. The prominent peaks of PVA were noted at 3314, 2938, 1428, 1326, and 1092 cm^{-1}, which are associated with the O-H stretching vibration of the hydroxyl group, CH2 asymmetric group, and C=O carbonyl stretch group. Upon the inclusion of magnesium nanoparticles, the significant peaks of MgO were identified at 842, 682, and 565 cm−1, indicating the presence of Mg-O-Mg bonds (figure 6).

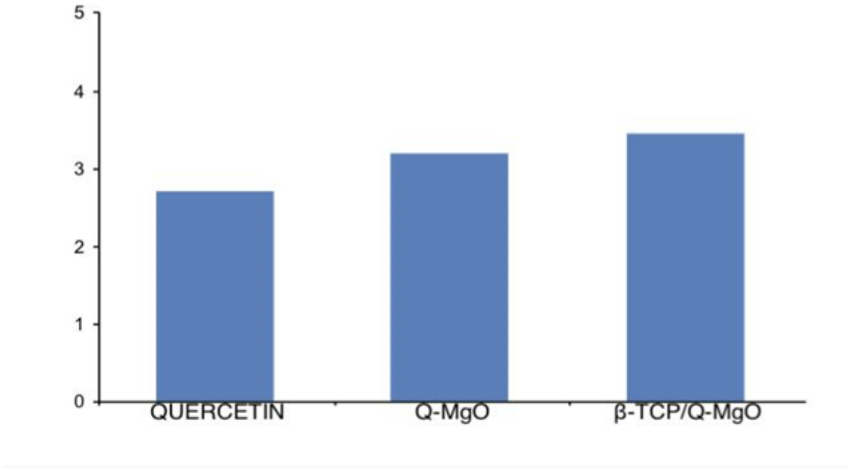

Figure 7. Depicts Alizarin red stain test

Since this anthraquinone derivative reacts more precisely and chelates with calcium cations, alizarin red S is the gold standard for detecting and measuring mineralization. Phase-contrast microscopy is used in this process to assess the staining of the cells after they have been fixed with formalin and stained with alizarin red S. Alizarin red S assay exhibits moderate sensitivity, making detecting early differentiation or minute but substantial variations in mineralization and osteoblast behavior challenging (figure 7). A colorimetric assay for quantitative analysis can remove and measure the dye. However, this method is not without limitations. Quercetin-doped magnesium oxide incorporated with B-TCP has a higher staining capacity.

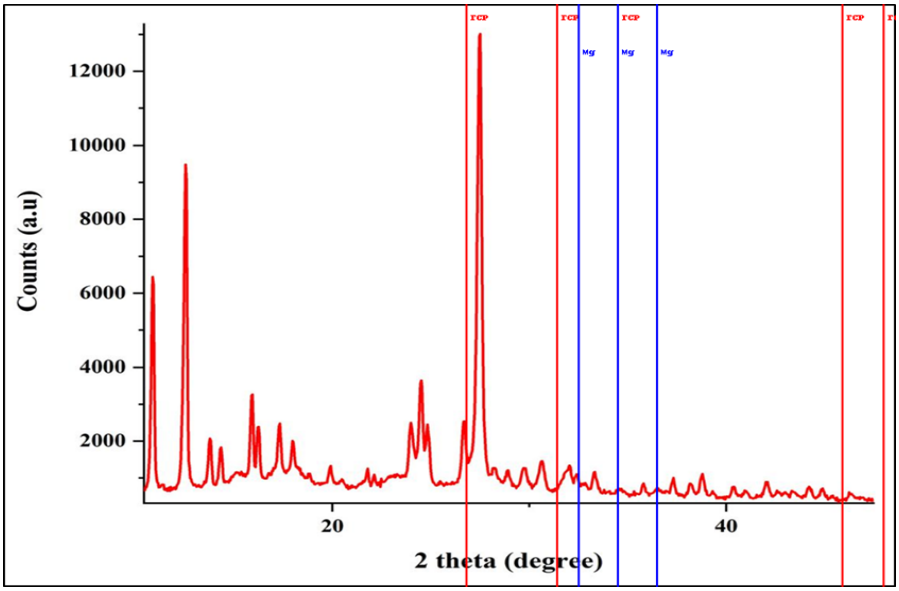

Figure 8. XRD analysis of the magnesium membrane loaded with hyaluronic acid, tricalcium phosphate (TCP), and quercetin

XRD

The XRD analysis of the magnesium membrane loaded with hyaluronic acid, tricalcium phosphate (TCP), and quercetin reveals (Fig 8) several distinct peaks that correspond to known reference patterns for TCP and magnesium. The experimental data shows prominent peaks around 25.9°, 31.0°, 34.4°, 47.0°, and 49.3°, which align well with the characteristic diffraction angles of TCP. Additionally, peaks observed at approximately 32.2°, 34.4°, and 36.6° match the standard positions for magnesium. The alignment of these peaks with reference patterns indicates the successful incorporation of TCP and magnesium into the membrane structure. The presence of these materials is crucial for the membrane's intended function in bone healing applications, as TCP is known for its osteoconductive properties, while magnesium contributes to bone regeneration. No significant peaks for quercetin were identified, which may suggest that it is either amorphous or present in a phase that is not easily detectable by XRD. Overall, the XRD pattern confirms the incorporation of key components necessary for the membrane's bioactive properties.

Discussion

The potential of electrically spun hyaluronic acid membranes in biomedical and tissue engineering applications is highlighted by this study, which offers insightful information on the production and characterization of these membranes. The remarkable biocompatibility and regenerative qualities of electrically spun synthetic hyaluronic acid membranes are drawing increasing interest. The successful fabrication and comprehensive characterization of these membranes are critical for their application in the biomedical field.

The successful fabrication of electrospun hyaluronic acid membranes represents a significant achievement. Due to its well-known biocompatibility and capacity for regeneration, hyaluronic acid is regarded as a promising substance for tissue engineering. The consistent production of these membranes suggests a scalable method suitable for large-scale applications, aligning with the literature that highlights the importance of reproducibility in biomedical materials fabrication [11].

The scanning electron microscope (SEM) examination, combined with energy-dispersive X-ray spectroscopy (EDS), revealed important details about the membranes' structure and

elemental composition. The microstructural characterization provided detailed information about fiber diameter, distribution, and overall architecture, which are crucial for ensuring the membrane's functionality in biomedical applications. EDS offered elemental mapping and quantification, contributing to quality control and ensuring that the membranes meet the necessary specifications for medical use. Studies have similarly emphasized the importance of detailed structural analysis in developing effective biomedical materials [12].

The porosity of the membrane is an important factor, especially for tissue engineering and drug delivery applications. Porosity test results confirmed the membrane's suitability by providing essential data on its permeability and potential for nutrient transport. High porosity is crucial for facilitating cell infiltration and nutrient exchange, necessary for effective tissue regeneration [13].

Stem cell recruitment is a fundamental aspect of regenerative medicine. The findings indicate that the electrospun hyaluronic acid membrane effectively recruits stem cells, a promising result for tissue regeneration applications. The cell attachment assay further demonstrated the membrane's favorable surface characteristics, supporting cell adhesion—an essential factor in cell-based therapies [14].

The cell viability test is a crucial indicator of the membrane's biocompatibility. The results showed that the membrane supports cell adhesion and growth without exhibiting cytotoxic effects, underscoring its safety for biomedical applications. Additionally, the membrane's potential for bone formation, as indicated by the bone-forming assay, suggests its applicability in bone tissue engineering. These findings correspond with existing studies that emphasize the importance of biocompatibility and osteogenic potential in tissue engineering scaffolds [15].

The reproducibility of electrospun membranes for large-scale biomedical applications has been emphasized, along with the importance of detailed structural analysis to ensure material efficacy. Key factors such as porosity, stem cell recruitment, and cell adhesion are critical for successful tissue regeneration [16]. The need for biocompatibility and osteogenic potential in scaffolds has also been validated. While the results are promising, concerns have been raised about the scalability and long-term stability of electrospun membranes, with challenges in maintaining consistent quality during large-scale production. [17].

Concerns have been raised regarding the long-term biodegradability and mechanical stability of electrospun hyaluronic acid membranes in dynamic physiological environments. Additionally, there is potential for batch-to-batch variability in production [18]. To address these issues, studies have focused on improving the mechanical properties of the membranes, particularly through chemical cross-linking techniques, which have demonstrated enhanced stability. Incorporating nanomaterials has also been shown to improve mechanical properties without compromising biocompatibility [19, 20].

Research has further explored integrating bioactive molecules to enhance the regenerative capabilities of these membranes [21]. Long-term studies have confirmed the gradual and complete degradation of electrospun hyaluronic acid membranes in physiological conditions, supporting their potential as temporary scaffolds in tissue engineering [22]. Artificial substitutes like calcium carbonate, PRF, and nano-hydroxyapatite have shown their clinical efficacy through mineralization in numerous applications [23-25]. Additionally, the use of these membranes in drug delivery systems has proven effective for sustained-release applications.

This study highlights the significant potential of electrospun hyaluronic acid membranes in various biomedical and tissue

engineering applications. The successful fabrication, coupled with detailed structural characterization, demonstrates the ability of these membranes to provide an optimal environment for bone formation. Their porous structure allows for nutrient exchange and cell migration, while their biocompatibility encourages osteoblast attachment and differentiation, ultimately leading to new bone matrix formation. Future research should focus on overcoming challenges related to large-scale production and improving long-term stability, which are necessary steps to fully realize their clinical potential in regenerative medicine. Additionally, understanding the underlying molecular mechanisms governing bone formation, such as the role of the membrane's degradation rate in guiding tissue remodeling, will be critical for optimizing their use in clinical settings.

Conclusion

In conclusion, this study establishes a solid foundation for the use of electrically spun hyaluronic acid membranes in biomedical and tissue engineering applications. These membranes' successful fabrication, structural characterization, porosity, stem cell recruitment, cell attachment, cell viability, and bone-forming capabilities make them versatile candidates for various applications in regenerative medicine, drug delivery, and tissue engineering. Further research can build upon these results to explore specific clinical applications and optimize the membranes' properties for various therapeutic uses.

Acknowledgement

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Conflict of Interest

Nil.

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