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BASIC RESEARCH:

Development, Characterisation and Biocompatibility Analysis of a Collagen-Gelatin-Hydroxyapatite Scaffold for Guided Bone Regeneration

Desarrollo, caracterización y análisis de biocompatibilidad de un andamio de colágeno-hidroxiapatita para la regeneración ósea guiada

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ABSTRACT: Guided Bone Regeneration (GBR) is the choice of treatment for improving the horizontal and vertical bone volume through bone grafting. GBR membranes work on the principle of preventing epithelial migration into the defect space while maintaining the space for cell migration and differentiation at the defect site. Hydroxyapatite has been commonly used as a bone graft for infrabony defects. The study was conducted at the Department of Biomaterials at Saveetha Dental College. GBR membrane was prepared and its material characterization was done using Scanning Electron Microscope (SEM), Energy Dispersive X-ray (EDX) analysis, Fourier Transform Infrared Radiation (FTIR), and Confocal Analysis. The developed GBR membrane revealed SEM properties conducive to cell attachment. EDX and FTIR analysis showed the successful development of the collagen-gelatin-hydroxyapatite membrane. Cell culture and confocal analysis revealed excellent biocompatibility with a homogenous layer of viable cells. The developed composite GBR membrane is a biogenic membrane with relevant biomineralization potential that should be applied for GBR applications.

KEYWORDS: Biocollagen; Guided bone regeneration; Hydroxyapatite; Periodontitis; Quality of life.

RESUMEN: La regeneración ósea guiada (GBR) es el tratamiento de elección para mejorar el volumen óseo horizontal y vertical mediante injertos óseos. Las membranas GBR funcionan según el principio de prevenir la migración epitelial hacia el espacio del defecto. La hidroxiapatita se ha utilizado habitualmente como injerto óseo para defectos infraóseos. El estudio se realizó en el Departamento de Biomateriales del Saveetha Dental College. Se preparó un scaffold o andamio, el cual se caracterizó mediante microscopio electrónico de barrido (SEM), análisis de rayos X de dispersión de energía (EDAX), radiación infrarroja por transformada de Fourier (FTIR) y análisis confocal. El andamio desarrollado reveló propiedades propicias para la unión celular. Los análisis EDAX y FTIR mostraron el desarrollo exitoso de la membrana de colágeno-gelatina-hidroxiapatita. El cultivo celular y el análisis confocal revelaron una excelente biocompatibilidad con una capa homogénea de células viables. El andamio desarrollado es una membrana biogénica con un potencial de biomineralización relevante que puede utilizarse para aplicaciones GBR.

PALABRAS CLAVE: Biocolágeno; Regeneración ósea guiada; Hidroxiapatita; Periodontitis; Calidad de vida.

INTRODUCTION

Bone defects and tooth loss are some of the most common intraoral findings in patients with periodontal disease (1). The preference for fixed tooth replacements in the form of dental implants due to their improved function and esthetics (2-3) has led to the development of newer regenerative therapeutics. Guided Bone Regeneration (GBR) is the choice of treatment for improving horizontal and vertical bone volume through bone grafting (3). Block or particulate bone grafts obtained from autogenic, allogenic, or xenogenic sources are employed along with GBR membranes for the regeneration of the lost bone.

GBR membranes work on the principle of preventing epithelial migration into the defect space while maintaining the space for bone cell migration and differentiation at the defect site. Various generations of GBR membranes have been developed for the same purpose, with a focus on improving biocompatibility, tissue integration, and osteogenesis. With the non-resorbable membranes, though there was good space maintenance, the need for a second surgery with issues of membrane exposure and infection (4) has led to the development of resorbable membranes. Of the resorbable membranes, collagen-based membranes have been employed predominantly. Collagen plays a biomimicry role in the post-operative healing phase. It is the most abundant extracellular matrix protein in human connective tissue. The collagen membranes would thus biomimic and modulate cell behavior, differentiation, and maturation. However, collagen membranes are associated with a rapid rate of resorption, which may be aggravated in the presence of infections (5, 6, 7). Hence, maintaining a resorption rate of the membrane that is conducive to the regeneration of the osseous tissue is imperative. This search has led to the addition of osteogenic and osteoinductive agents to membranes.

Among the calcium phosphate ceramics, stable-phase hydroxyapatite has been commonly used as a bone graft for intrabony defects (8) Bone formation with ceramics is a multifactor process that is regulated by several aspects such as chemical composition, resorption and dissolution rates, physical structure (e.g., the geometry of the pores, porosity, as well as surface topography),

and implantation site (9). The chemical composition of a bioceramic material influences the rate of solubilization and resorption as well as its bioactivity (10-11). Hydroxyapatite with the release of high concentrations of calcium into microenvironments, results in the formation of a bioactive apatite layer responsible for osteoconductivity and osteoinductivity (12). Also, as hydroxyapatite is slowly resorbed, a hydroxyapatite-incorporated collagen membrane would have an improved and controlled rate of absorption that would be ideal for space maintenance and the prevention of epithelial migration. It would also have the added advantage of osteogenic induction due to the release of calcium ions.

In the current study, we have prepared a lyophilised collagen and hydroxyapatite based guided bone regeneration membrane. The membrane was further characterized with Scanning Electron Microscope (SEM), Energy Dispersive X-ray (EDX), Fourier Transform Infrared Radiation (FTIR), and Confocal analysis to study the biocompatibility and cell adhesion properties of the membrane.

MATERIALS AND METHODS

The study was conducted at the Department of Biomaterials at Saveetha Dental College. Approval was obtained from the Scientific Review Board of Saveetha Dental College for the conduction of the study, with approval number SRB/SDC/UG-1914/23/PERIO/052.

PREPARATION OF GBR MEMBRANE

All the chemicals and reagents of analytical grade were employed in the synthesis of the membrane. Fish origin collagen was procured from SRL Chemicals and gelatin was procured from MERCK USA. Hydroxyapatite crystals were synthesized for incorporation in the membrane. For the synthesis of hydroxyapatite crystals, calcium nitrate solution was added dropwise to diammonium hydrogen phosphate. The pH of this solution was maintained at 11 using ammonia. The resultant material was further washed with water and sintered at 400°C to obtain a powdered form of hydroxyapatite crystals. For membrane fabrication, a solution was prepared with an equal volume of collagen (10%) and gelatin (10%). To this mixture, 1% hydroxyapatite crystals were added. The solution was thoroughly mixed to obtain a homogenous composition. The mixture was poured on a petri dish to obtain a thin layer of material, which was lyophilized at 800°C to obtain a thin layer of the GBR membrane.

MATERIAL CHARACTERIZATION

The investigation of the novel composite Guided Bone Regeneration (GBR) membrane extended beyond its synthesis to encompass a thorough examination of its physical and chemical properties. One crucial aspect of this analysis was the exploration of its surface morphology, achieved through Scanning Electron Microscopy (SEM). To prepare the membrane for SEM imaging, it underwent a process of sputter coating with Platinum for 30 seconds. This coating enhances the membrane's conductivity, allowing for clearer visualization of its surface features. Subsequently, the membrane was subjected to observation using a Field Emission Scanning Electron Microscope (FE-SEM), specifically the JSM IT800 model manufactured by JEOL in Tokyo, Japan. Operating at an accelerating voltage of 1.5 kV and a resolution of 100 micrometers, the FE-SEM provided high-resolution images, enabling detailed examination of the membrane's topography.

In addition to SEM imaging, the sample underwent chemical characterization to elucidate its elemental composition. Energy Dispersive X-ray (EDX) analysis was employed for this purpose, utilizing instrumentation from Oxford Instruments. EDX analysis entails bombarding the sample with X-rays, causing the emission of characteristic

X-ray signals that are indicative of the elements present in the material. By analyzing these signals, the elemental composition of the composite GBR membrane could be determined, providing valuable insights into its chemical makeup.

Further investigation focused on the identification of specific chemical groups within the membrane structure. This was accomplished through Fourier Transform Infrared Spectroscopy (FTIR), a technique renowned for its capability to detect vibrational modes associated with functional groups in molecules. The membrane was subjected to FTIR analysis using a Bruker Alpha II instrument, which operates on the principle of measuring the absorption of infrared radiation by the sample. By analyzing the resulting spectra, the presence of characteristic vibrational modes corresponding to various chemical bonds present in the membrane could be confirmed.

By employing a combination of SEM, EDX analysis, and FTIR spectroscopy, a comprehensive understanding of the synthesized composite GBR membrane's physical and chemical properties was achieved. These analytical techniques provided valuable insights into its surface morphology, elemental composition, and molecular structure. Such detailed characterization is essential for assessing the membrane's suitability for applications in guided bone regeneration, as it offers insights into its structural integrity, biocompatibility, and potential performance in clinical settings.

BIOCOMPATIBILITY AND CELL ADHESION

The synthesized composite membrane was seeded with MG63 cells and analyzed under a confocal laser scanning microscope LEICA DMI8 to observe the viability, growth morphology, growth pattern and membrane depth penetrability using various dyes like methylene blue, rhodamine B, fluorescent acetate, and combination 3D staining. MG63 cell line was procured from National Centre for Cell Science, Pune, India.

The confocal laser scanning microscope facilitates high-resolution imaging, allowing for detailed observation of cellular behavior within the membrane structure. By utilizing different fluorescent dyes, distinct aspects of cell behavior and membrane interaction could be elucidated. Methylene blue, for instance, might highlight cell viability and distribution, while rhodamine B could provide insights into cell morphology and growth patterns. Fluorescent acetate might be utilized to assess metabolic activity within the seeded cells. Additionally, the combination of 3D staining techniques could offer a comprehensive understanding of cell distribution throughout the membrane's depth. By evaluating cell behavior and membrane interaction at a microscale level, this study contributes valuable information towards the development of advanced biomaterials for biomedical purposes. In the present study, the percentage of cell viability was assessed at various time periods (24, 48, 72, 90 and 120 hours).

STATISTICAL ANALYSIS

The data were collected, tabulated, and analyzed using IBM SPSS Statistics for Windows, Version 23.0 (Released 2015; IBM Corp., Armonk, NY, USA). A one-way ANOVA with a Tukey post hoc test was performed to assess the biocompatibility of the synthesized membrane in comparison to the collagen-gelatin membrane by assessing the percentage of cell viability at various time points, with a p value ≤ 0.05 set as statistically significant.

RESULTS

The developed GBR membrane, when observed under SEM (Figure 1), revealed a rough fibrillar structure with surface porosities. On closer analysis, the fibrous interconnected structures had a

fibrillar and lamellar stacking pattern that may be attributed to the presence of collagen and gelatin in the membrane. Observed porosities of various sizes appeared to be limited to the surface layer. Furthermore, a fine granular structure could be appreciated on the surface. This may be attributed to the presence of hydroxyapatite crystals on the surface of the membrane.

On chemical analysis of the developed GBR membrane using EDX analysis (Figure 2), it was observed that Carbon (C) and Oxygen (O) were the predominant compounds, with a weight percentage of 68.6 and 28.5, respectively. This may be attributed to the presence of collagen and gelatin. Calcium (Ca), Sodium (Na), and Phosphorous (P) appeared as trace compounds with 1.0, 1.1, and 0.6 weight percentages respectively. This may be accredited to the presence of hydroxyapatite crystals in the membrane.

FTIR analysis (Table 1, Figure 3), performed to assess the molecular constituents of the membrane revealed absorbance or peak bands corresponding to O-H stretching at 3291, amide I group and C-O bonding at 1643, amide II group, N-H stretching, C-N deformation at 1544, and bending and stretching of P-O ranging from 608 to 1084. The absorbance band for -OH stretching corresponds to the presence of collagen, gelatin and hydroxyapatite in the membrane. Similarly, the presence of amide groups, C-O bonding, N-H stretching, C-N deformation and P-O bonding all prove the presence of collagen and gelatin in the membrane.

To assess the biocompatibility, the developed GBR membrane was cultured with MG63 lineage cells. They were stained with methylene blue, rhodamine B, fluorescent acetate and combination stain (Figure 4). On methylene blue staining, the fibroblast cell organelles were well appreciable. The nucleus and cytoplasm were well stained. On rhodamine B staining active cells with well stained mitochondria were observed. The cells were elongated, spindle-shaped and arranged in a stacking pattern. Fluorescent acetate staining was performed to assess the cellular morphology. It revealed cells that were well developed and adherent to the membrane. The cells were arranged in a stacking pattern with a well appreciable cytoskeleton, nucleus, and filopodia. This proves that the membrane is biocompatible and is not cytotoxic to the cells. Different layers of the adhered cells were also appreciable. On combination 3D staining, different layers of stacked cells were appreciable. Also, the cells extending filopodia towards the adjacent cells could be appreciated. All the staining methods proved that the developed GBR membrane was biocompatible and allowed adherence of gingival fibroblast cells onto the surface. On comparing the synthesized GBR membrane with the collagen-gelatin membrane, no statistically significant difference was noted in the percentage of cell viability at various time points (Table 2, Figure 6), suggesting that the synthesized composite membrane is biocompatible. Optimum cell viability was observed with both the synthesized composite membrane and the control membrane.

On 3D depth analysis to study the cell penetrability through the membrane (Figure 5), it was seen that cells did not penetrate through the depth of the membrane. Cell adherence was limited only to the superficial layers of the membrane. This proves that the membrane would achieve its primary role of compartmentalizing tissues when used *in vitro*.

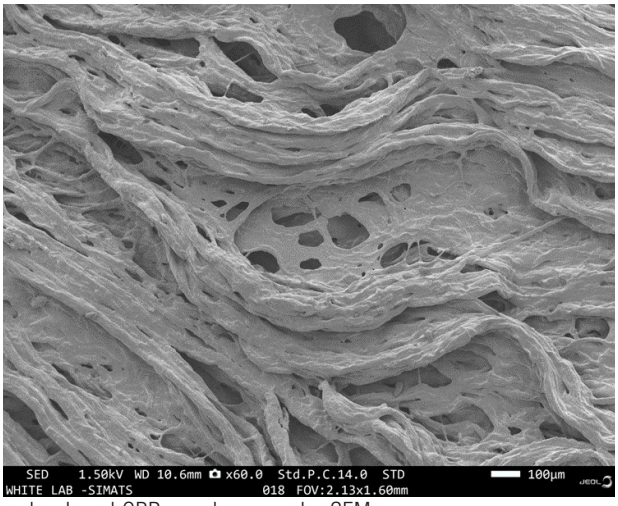

Figure 1. Surface morphology of the developed GBR membrane under SEM.

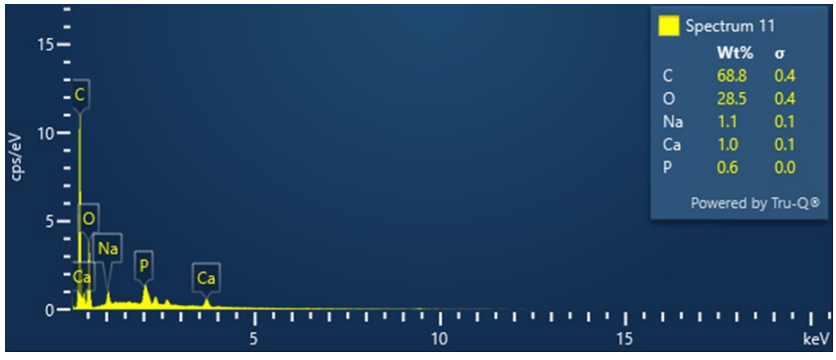

Figure 2. Chemical composition of the developed GBR membrane analysed with EDX.

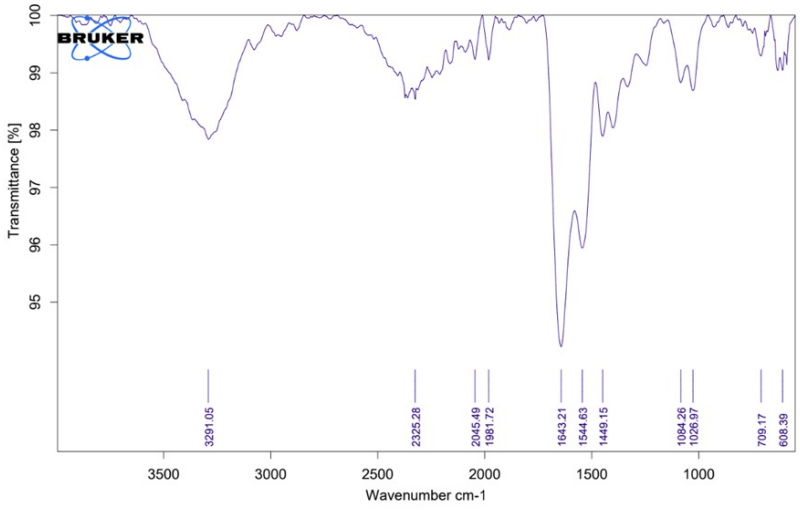

Figure 3. FTIR analysis of the developed GBR membrane.

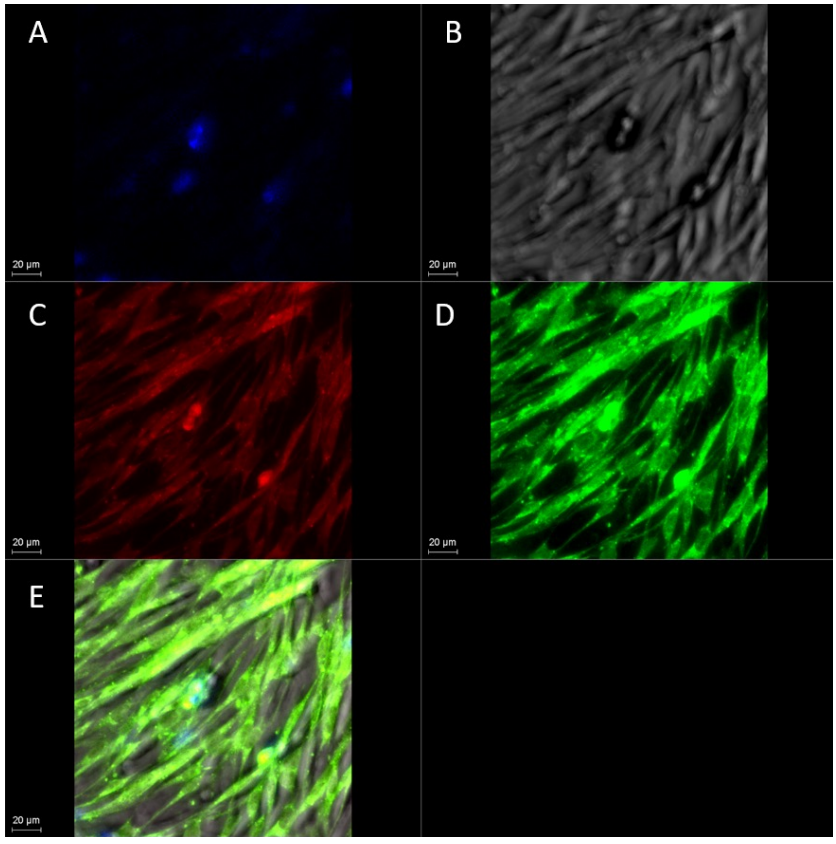

Figure 4. Cell culture staining and confocal analysis of the developed GBR membrane. A. Methylene blue staining, B. Plain, C. Rhodamine B staining, D. Fluorescent acetate staining, E. Combination staining.

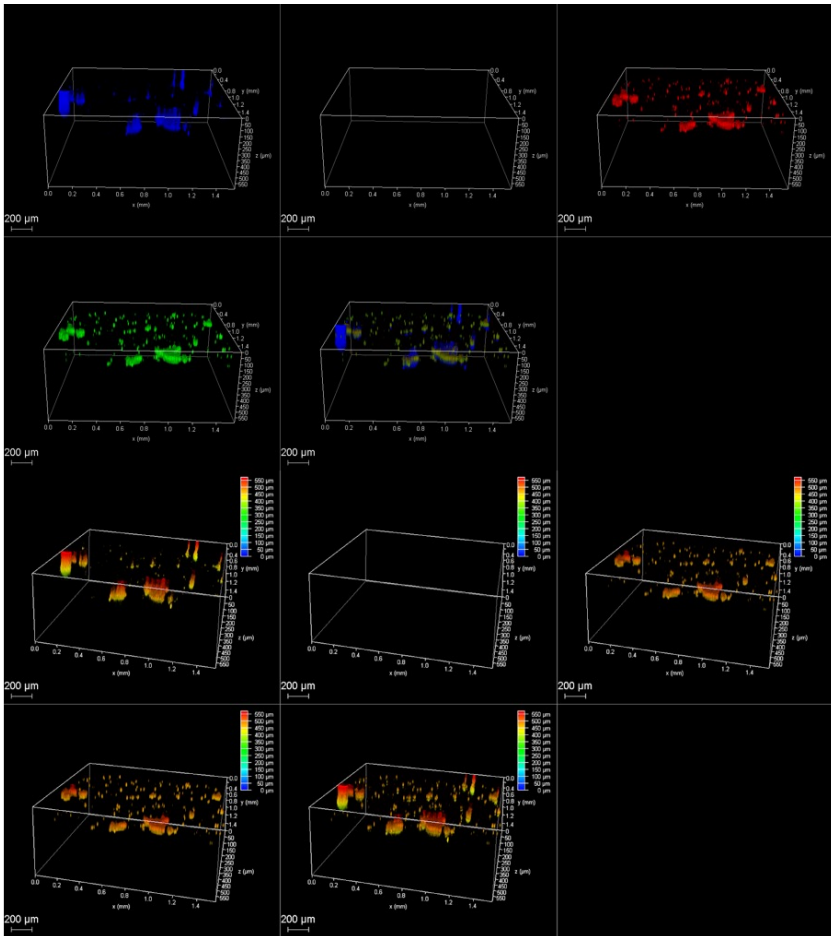

Figure 5. 3D cross-sectional depth analysis.

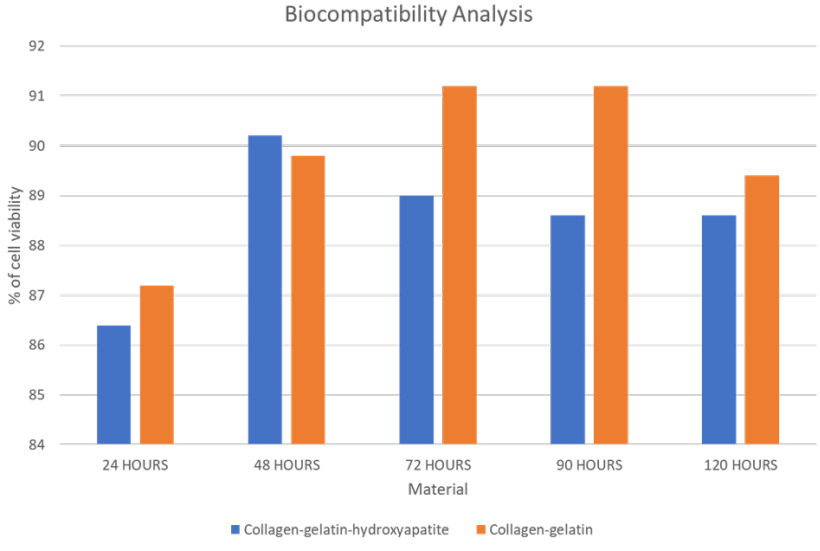

Figure 6. Comparative analysis of the biocompatibility of the developed collagen-gelatin-hydroxyapatite composite membrane with the control depicted as percentage of cell viability.

Table 1. FTIR analysis of the developed GBR membrane showing characteristic absorbance peak values of hydroxyapatite, collagen and gelatin.

3291	-OH stretching HYDROXYAPATITE, COLLAGEN, GELATIN
1643	amide I, C=O COL-GEL
1544	amide II, N-H stretching and C-N deformation
1449	C-N deformation
1084	bending and symmetric stretching vibrations of P-O
1026	
709	
608	

Table 2. Comparison of the biocompatibility of the developed collagen-gelatin-hydroxyapatite composite membrane with the control - Independent t test.

Time	Sample	N	Mean	Standard deviation	P value
24 hrs	Collagen and gelatin	5	86.40	1.140	0.327
	Collagen, gelatin and HA	5	87.20	1.924	0.327
48 hrs	Collagen and gelatin	5	90.20	1.924	0.378
	Collagen, gelatin and HA	5	89.80	2.775	0.378
72 hrs	Collagen and gelatin	5	89.0	1.581	0.189
	Collagen, gelatin and HA	5	91.2	2.864	0.189
90 hrs	Collagen and gelatin	5	88.60	3.362	0.674
	Collagen, gelatin and HA	5	91.20	2.387	0.674
120 hrs	Collagen and gelatin	5	88.60	2.320	0.617
	Collagen, gelatin and HA	5	89.40	3.209	0.617

DISCUSSION

Implants have become the mainstay of treatment for missing teeth due to their improved masticatory efficiency and enhanced esthetics. However, insufficient bone housing and bone defects following periodontal disease and trauma impair the placement of implants. With advances in tissue engineering applications, guided bone regeneration has emerged as a therapeutic solution to improve bone housing through augmentation (3, 13, 14). Various generations of GBR membranes have been developed and used over the years to optimize the regenerative outcomes (15). In our effort to achieve predictable regeneration, we developed a novel composite GBR membrane composed of collagen gelatin and hydroxyapatite.

On material characterization with SEM (Figure 1), the surface appeared as a rough fibrillar surface. The fibres had a lamellar pattern and minute porosities limited to the surface. Granular surface roughness was also noted. These features can be attributed to the presence of collagen-gelatin and hydroxyapatite in the membrane. EDX analysis (Figure 2) revealed C and O as the predominant components, which proves the presence of collagen-gelatin as the primary composition,

while trace amounts of calcium, phosphorus, and sodium prove the presence of hydroxyapatite crystals in the membrane. FTIR analysis (Table 1, Figure 3)also proved the composition of the membrane as collagen, gelatin, and hydroxyapatite through the presence of characteristic absorbance bands corresponding to OH stretching, C-N stretching, N-H stretching, P-O bending, and amide groups. The results of this study are in accordance to recent studies on composite collagen-based membranes that had similar surface and compositional features that were proven to promote cell adhesion to the membrane, membrane adhesion to bone, osteoblast differentiation, and bone deposition (15, 16).

Collagen is a ubiquitous protein of the mammalian extracellular matrix. It plays an important role in bone metabolism and wound healing. It has a biomimetic role wherein it promotes cell differentiation, cell migration and cell attachment while providing mechanical strength and flexibility (17, 18, 19). It's trimolecular highly organized structure resists mechanical stress while enabling cell growth (20). Gelatin, a derivative of collagen, is also highly biocompatible and hygroscopic with porosities that promote cell adhesion, differentiation, and proliferation (21). Collagen from marine sources was used for membrane fabrication in this study. As bovine and porcine-based xenografts have been associated with bovine spongiform encephalopathy and virion transfer, marine sources were used. Moreover, bovine and porcine sources are not being accepted by the patients due to religious constraints. Marine collagen is derived from marine waste like fish skin, fish bones, molluscs, and crustaceans, which are abundantly available. They are biocompatible, easily available at a lower cost, readily accepted by patients, and are not associated with any disease transfer. Studies have proven that marine collagen promotes osteoblastic activity and bone density (22).

The developed GBR membrane had hydroxyapatite incorporated into it to provide osteopromotive sites for the cells. Hydroxyapatite is a bioactive ceramic that binds directly to bone through the calcium apatite layer. It has been proven to have an osteopromotive and osteoinductive effect on bony defects (23). This could be attributed to the increased concentration of calcium and phosphorus in the defect microenvironment as they are released from the osteopromotive components of the membrane. The mildly acidic environment created due to the release of the ions has been proposed to cause a mild reactionary phenomenon that promotes new bone formation. It has the property of slow degradation that, when used in combination with collagen membrane, proves to be advantageous. As collagen membranes have an unpredictable degradation process, the addition of hydroxyapatite crystals in the membrane would modulate the resorption rate of the GBR membrane to that conducive to new bone formation. It would also enable the attachment of undifferentiated mesenchymal cells and their differentiation into osteoblasts, thus promoting bone deposition. Similar results were observed in recent studies that proved that the addition of hydroxyapatite to the GBR membranes improved new bone formation (24-25).

On cell culture studies with MG63 (Figure 4), it was noted that the developed GBR membrane was highly biocompatible and no cytotoxic behavior was observed in the cells. The cells were uniformly adhered to the membrane, with different layers of cells arranged in a stacking pattern. They also revealed a good proliferative rate with a well appreciated cytoskeleton. The cells were spindle-shaped and elongated. They had well-defined cell organelles that were visualized using different dyes. The proliferating cells also had extended filopodia, revealing cell interaction and migration. On comparing the synthesized membrane with the collagen-gelatin

membrane as a standard, no statistically significant difference was noted in the percentage of cell viability (Table 2, Figure 6). Optimum cell viability was noted with the synthesized membrane. Hence, it can be concluded that the developed GBR membrane had excellent biocompatibility and cell adhesion properties, which are crucial for the success of GBR procedures. The results of our study are in accordance with other studies that analyzed composite collagen membranes, which showed improved biomineralisation, biocompatibility, and cell proliferation (26, 27). Studies have also shown that the incorporation of hydroxyapatite into GBR membranes improves the mechanical strength and degradation rates of the membranes (28).

Another recent systematic review assessed the various barrier membranes developed with the addition of osteoinductive agents (29). The study stated that there was a shift in the composition of the developed membranes from using inert components to bioactive osteo-immunomodulatory components for improved regenerative outcomes. Calcium phosphate, hydroxyapatite, bioglass, polydopamine, and bisphosphanates have been explored as membrane additives to improve bone formation. Biomaterials and growth factors like chitosan, emdogain, and bone morphogenetic proteins have also been considered as promising candidates. Metal nanoparticles and antimicrobial drugs have also been considered to prevent infection-related failure of the regenerative site. Chu *et al*. developed and characterized an epigallocatechin-3-gallate cross-linked collagen membrane that was coated with nanohydroxyapatite membranes (30). They also conducted an animal study to explore its regenerative potential *in vivo*. They stated that the membrane showed improved mechanical properties with enhanced bone regenerative capacity and that the addition of nanohydroxyapatite improved the surface properties of the membrane while retaining the collagen backbone. Another study assessed polymer membranes that were sonicated with nanohydroxyapatite for guided tissue regeneration (31). They stated that the addition of hydroxyapatite slowed down membrane degradation while also improving the wettability of the membrane, which contributed to the good cellular activity of MG-63 cells leading to bone regeneration. Gavinho *et al*. developed zinc-containing bioactive glass-coated polycaprolactone membranes for guided bone regeneration and concluded that the addition of zinc did not improve the antimicrobial activity of the membrane (32). However, they stated that zinc promoted cell viability and alkaline phosphatase activity.

The developed biomineralised GBR membrane has good surface, chemical, biocompatible, and cell proliferative properties that are conducive to GBR applications. The collagen and gelatin would provide biomimetic cues, while the hydroxyapatite would provide osteoinductivity. The limitations of the study are that mechanical behaviour, degradation analysis, and cell line studies to assess the effect on osteoblast cells were not performed. We propose that the developed membrane has great potential for tissue engineering applications, which needs to be explored through further long-term *in vivo* studies.

CONCLUSION

In conclusion, the developed composite GBR membrane is a biogenic membrane with relevant biomineralisation potential that should be applied for GBR applications. This would greatly enhance the regeneration outcomes to progress towards predictability. Further *in vivo* studies need to be conducted to study its effect clinically.

AUTHOR CONTRIBUTION STATEMENT

Contributed to the acquisition, analysis, or interpretation of data for the work: P.S.
Helped in designing the methodology, drafting the work, revising it critically for important intellectual information, and final approval of the version to be published: P.A.
Helped in data collection and interpretation of the data: S.K.

CONFLICT OF INTEREST

The authors declare no conflict of interest in the study.

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