

In-vitro Evaluation of 3D Bioprinted Alginate Membranes for Periodontal Regeneration: Mechanical Properties, Biocompatibility and Drug Release Analysis

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ABSTRACT

Introduction: Alginate-based hydrogel membranes are commonly used in periodontal applications due to their biocompatibility. Rosuvastatin (RSV), a lipid lowering drug is known for inducing osteoblastic differentiation by several studies.

Aim: To assess the physical properties {using Scanning Electron Microscope (SEM), Fourier Transform Infrared (FTIR), surface roughness, and contact angle} and drug release behaviour (via Franz Diffusion Method) of alginate-based 3D bioprinted membranes, with and without 1.2% RSV.

Materials and Methods: This in-vitro study was conducted in Department of Periodontology, Sri Ramachandra Dental College and Hospital, Chennai, Tamil Nadu, India from September 2025 to November 2025. Two bioinks, including one with 1.2% RSV and other premade bioink CELLINK BONE®, were 3D printed

into 10 mm × 10 mm × 1 mm membranes. Each bioink was incorporated with RSV calcium (18 mg - CELLINK BONE® and 12 mg - 6% Alginate). Membranes were printed in rectilinear and honeycomb patterns and crosslinked using 0.5% Calcium chloride. Physical properties, haemocompatibility and drug release were then evaluated.

Results: SEM and FTIR analysis validated the formation and typical morphology of the 6% alginate membranes. The membranes were hydrophilic and haemocompatible, while the one with RSV had sustained drug release. However, the CELLINK BONE® membrane loaded with RSV was not structurally stable as it disintegrated after processing.

Conclusion: The results indicate that both membranes are promising for their use in Guided Tissue Regeneration (GTR), serving as scaffolds as well as local drug delivery systems.

Keywords: 3D bioprinting, Alginate, Guided tissue regeneration, Membranes, Rosuvastatin

INTRODUCTION

Periodontitis is a multifactorial inflammatory disease that leads to periodontal destruction resulting in tooth mobility and tooth loss [1]. The disease affects over 10% of the worldwide population, creating oral health complications affecting patient's quality of life and systemic well-being [2,3]. GTR serves as one of the promising treatment for periodontal regeneration [4]. GTR involves placement of a membrane that acts as a physical barrier and prevents epithelial migration ensuring space for the periodontal ligament, cementum and bone to form [5,6].

Ideal GTR membranes requisites include biocompatibility, mechanical occlusion, biodegradability and sufficient porosity to promote angiogenesis. Although this is achieved with 1st generation non-resorbable membranes, it requires a second surgery for removal following healing. Resorbable membranes avoid the need for removal but usually do not possess adequate mechanical strength to support proper soft tissue healing [7].

To address these challenges, different hydrogel membranes with Tricalcium Phosphate (TCP)-alginate (ALG) reinforcement have been developed. TCP is an efficient bone substitute with essential biocompatibility, stability, low density, and wear resistance [6]. The bone remodelling properties of TCP are well matched due to its micromorphology, interconnecting pore structure, and resorbability, which enhances its osteoconductivity [7]. Alginate (ALG) is a biocompatible, non-toxic, naturally derived polysaccharide from seaweed. It can be easily customised based on specific application needs by adjusting its mechanical strength, degradation rate, and porosity, thus it can be developed as injectable hydrogels, enabling minimally invasive delivery methods for tissue engineering or wound healing in anatomically complex sites [8].

Recent advancements include the '3D Bioprinting', an innovative approach for manufacturing regenerative membranes. It is uniquely defined by the high-resolution, spatial control that allows depositing bioinks in a layer-by-layer manner, thereby enabling the fabrication of complex, multi-layer architectures with biomimetic functionality. This facilitates customising scaffolds of desired shape, size, texture and patterns. When cells and biomaterials are incorporated, they are collectively termed as bioinks. Most common sources include agarose, alginate, collagen and hyaluronic acid. These materials replicate the extracellular matrix of the target tissue, thereby supporting cell proliferation, differentiation and controlled biodegradation [9].

CELLINK BONE® is a commercially available Bioink that consists of Sodium Alginate, TCP, and Cellulose fibrils. This is preoptimised and ready to print, and can also be incorporated with various drugs at suitable concentrations [10].

Statins are widely used lipid-lowering agents which also exhibit a range of pleiotropic effects such as cholesterol-independent anti-inflammatory activity [11], modulation of vascular response [12], microvascular reperfusion [13], and antimicrobial effect [14] and intrinsic antioxidant properties [12]. Statin promotes bone formation by upregulating BMP-2 expression in bone cells, thereby enhancing osteoblastic differentiation while inhibiting osteoclastic activity [15-17]. RSV, a synthetic sulfur containing hydrophilic statin that it is actively transported into osteoblastic cells via solute carrier transporters. It is shown to stimulate BMP 2 gene expression, secretion as well as increases alkaline phosphatase activity indicating enhanced osteogenic differentiation [15].

Although alginate has been extensively investigated as a bioink for tissue engineering, alginate-based 3D bioprinted membranes and

commercially available bioink CELLINK BONE® as scaffolds for periodontal regeneration remain unexplored. Therefore, the present study evaluated the physical properties and drug release of alginate-based 3D bioprinted membranes with and without the addition of 1.2% RSV.

MATERIALS AND METHODS

The present study is an in-vitro study conducted in the Department of Periodontology, Sri Ramachandra dental college and hospital, Chennai, Tamil Nadu, India from September 2025 to November 2025 (3 Months). The study was approved by Institutional Ethics Committee, Sri Ramachandra institute of higher education (CSP/25/AUG/165/391). As this was an exploratory In-vitro laboratory study evaluating the physicochemical properties and drug release characteristics of experimental bioinks, a formal sample size calculation was not performed. The number of specimens for each test was determined based on standard laboratory practice.

Material Preparation

Preparation of 6% Alginate Bioink [18]: The 0.6 g of alginate powder (Raw form obtained from Seakol Biopharm Pvt., Ltd., Chennai), was dissolved in 10 mL of distilled water using an electromagnetic stirrer. The sodium alginate was added in small increments in the distilled water while being stirred in the electromagnetic stirrer. The stirring was completed when the consistency of the mixture reached gelation stage. The Bioink was stored at 4°C till usage.

CELLINK BONE®: It is commercially available Pre-formulated Bioink patented by CELLINK BONE® Bioprinting and UPM-Kymmene Corporation. It was commercially bought from CELLINK BONE® e-commerce website (<https://www.cellink.com/product/cellink-bone/?n=1>). It is available in 3 mL cartridges. CELLINK BONE® is still under research and not indicated for human use yet. The bioink was stored at 20°C till usage. Its composition is mentioned in [Table/Fig-1].

Chemical name	CAS# *	EC No.	EC Class
Nanofibrillated cellulose	9004-34-6	None	Not classified as hazardous
Sodium alginate	9005-38-3	None	Not classified as hazardous
Tricalcium Phosphate (TCP)	7758-87-4	231-840-8	Not classified as hazardous
Proprietary buffer solution	None	None	Not classified as hazardous

[Table/Fig-1]: Composition of CELLINK BONE®.

*CAS# - Chemical abstracts service registry number

Drug incorporation [19]: The RSV Calcium was obtained in raw form (Amorphous powder form sourced from Middlemist Pharmaceuticals Pvt., Ltd., Chennai). In CELLINK BONE® bioink, according to manufacturer's instructions, the drug had to be dissolved in a water miscible solvent and mixed with the bioink. A limit of 150 µL of Drug (RSV) could be dissolved in the bioink. Therefore, 18 mg of RSV Calcium was dissolved in 150 µL of 5% propylene glycol solvent to obtain the 1.2% concentration of RSV Calcium. For 6% Alginate Bioink, 120 mg of RSV in amorphous powder form was dispersed in 10mL solvent to obtain 1.2% concentration of drug in the bioink [Table/Fig-2,3] [15-17].



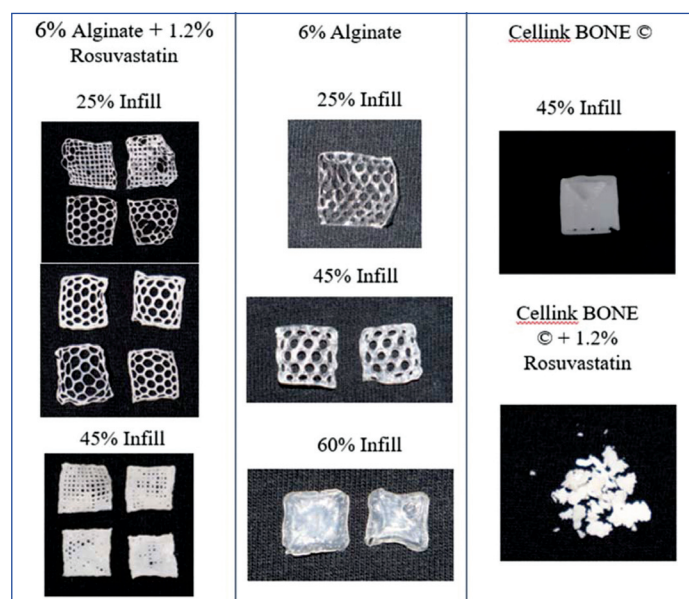
[Table/Fig-2]: Incorporation of 1.2% Rosuvastatin (RSV) in CELLINK BONE® Bioink.

3D bioprinting process [20]: Bioprinting was carried out using BioX bioprinter (CELLINK BONE®) in extrusion printing mode. Four



[Table/Fig-3]: Incorporation of 1.2% Rosuvastatin (RSV) in 6% Alginate.

Gauge size Nozzle was used for printing. The printing settings were determined by trial and error method and the setting in which a defined structure was obtained was deemed to be the final setting that bioink. For CELLINK BONE® with and without 1.2% RSV incorporation the printing settings were: Extrusion pressure - 40 to 45 psi, Print bed temperature -20°C, Nozzle temperature -20°C, Layer height - 0.5mm - 2 Layers 10mm x 10mm x 1mm in dimension. For 6% alginate bioink, with and without 1.2% RSV, the bioprinter was set at Extrusion pressure -70 psi, Print bed temperature -25°C, Nozzle temperature -37°C Layer height - 0.1mm - 1 layer, 20 mm x 20 mm x 1 mm in dimension. The membranes were printed in rectilinear and honeycomb patterns with different infill percentages (25%, 45%, 60%) [Table/Fig-4], which were preset in the bioprinter [Table/Fig-5,6].

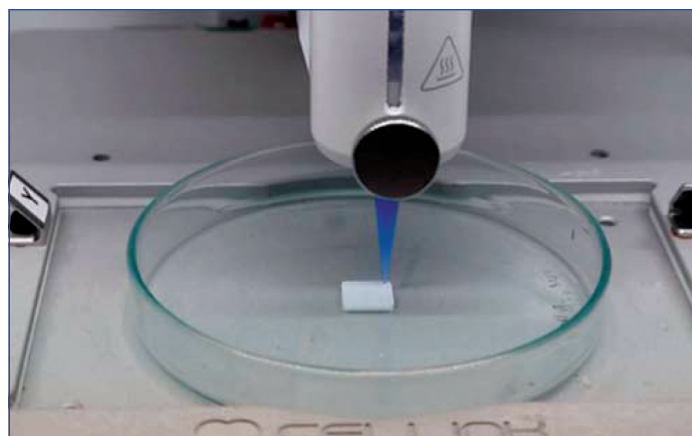


[Table/Fig-4]: Membranes after processing.



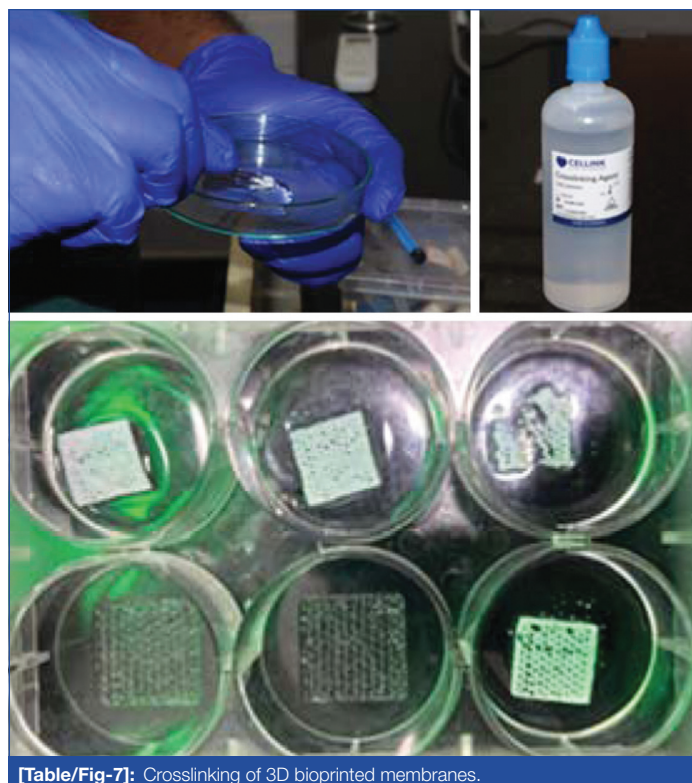
[Table/Fig-5]: BioX bioprinter (Cellink ©).

Post printing processing of membranes: All the membranes were crosslinked using 0.5% Calcium chloride for five minutes to obtain a rigid yet foldable membrane. After crosslinking, the membranes were heat dried at 60°C in a hot air drying chamber



[Table/Fig-6]: 3D bioprinting of the membrane.

aseptically. The membranes were stored at 10°C till further analysis [Table/Fig-7].



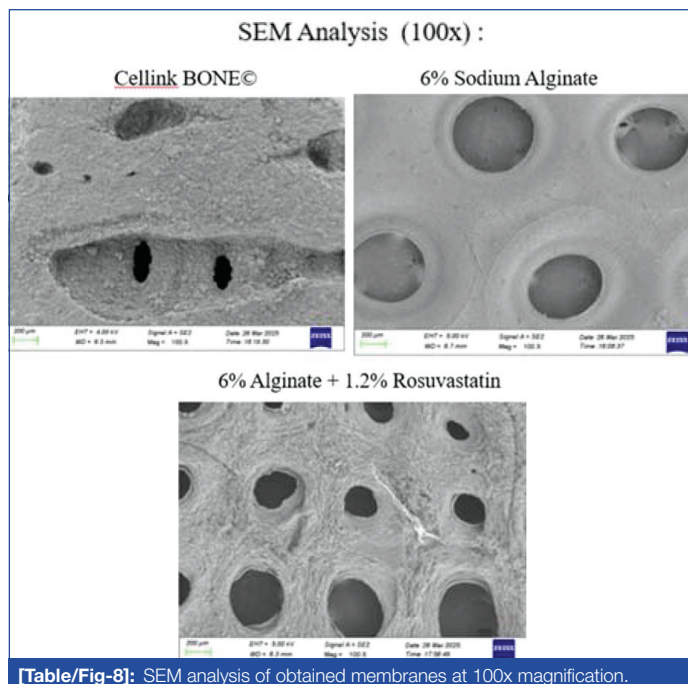
[Table/Fig-7]: Crosslinking of 3D bioprinted membranes.

Characterisation

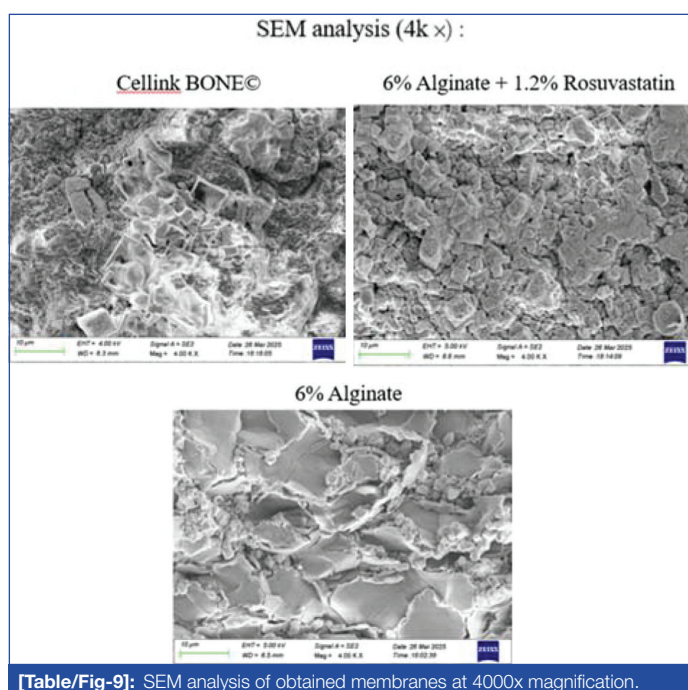
FTIR Analysis [21]: A FTIR spectrometer (BRUKER) was employed to evaluate the cross linking of the membranes [21]. Attenuated Total Reflectance Fourier Transform Infrared (ATR-FTIR) spectra were recorded using dried film samples. Analysis of the polymeric bonding patterns enables assessment of the membrane's chemical interactions and crystalline structure, particularly through identification of bonds between amide and carboxylic functional groups.

Morphology of the membrane: The surface morphology of the membranes was examined using SEM (JEOL-JSM-IT800 Electron Microscopy) at 1 kV. Prior to imaging, samples were prepared using a critical point dryer (Leica EMCPD300). Micrographs were obtained at two different magnifications [Table/Fig-8,9].

Haemocompatibility assessment [18]: Fresh blood was collected in EDTA tubes and centrifuged at 1500 rpm for 10 minutes to separate RBCs. The plasma and buffy coat were removed and the RBC pellet was resuspended in physiological saline to obtain 10% RBC suspension. Test group solution contained Scaffold + 10% RBC suspension. Positive control solution contained 10% RBC suspension + distilled water (100% haemolysis) and negative control solution was 10% RBC suspension and physiological saline (0% haemolysis)



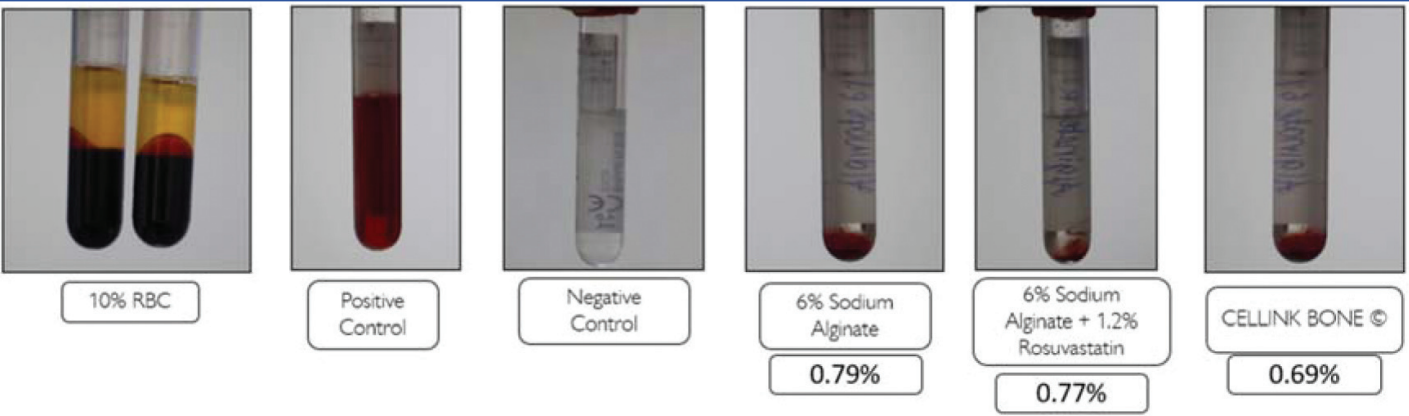
[Table/Fig-8]: SEM analysis of obtained membranes at 100x magnification.



[Table/Fig-9]: SEM analysis of obtained membranes at 4000x magnification.

[18]. All the tubes were incubated at 37°C for 1-3 hours with gentle shaking. After incubation, the solutions were centrifuged at 3000 rpm for 10 minutes to separate intact Red Blood Cells (RBCs) from the haemolysed supernatant. Haemolysis was evaluated by comparing the test samples with positive and negative controls. Complete RBC rupture and haemoglobin release in the positive control resulted in a crimson-coloured supernatant, while the negative control showed minimal haemolysis due to mechanical stress alone [Table/Fig-10].

Contact angle - Wettability [22]: Membrane wettability was assessed using sessile drop technique with freshly prepared physiological saline. A 100 μ L droplet was dispensed using a calibrated mechanical pipette and gently placed onto the sample surface. High-magnification images were captured once the droplet reached equilibrium at 30 seconds using a high-resolution camera equipped with a microlens system (D90, Nikon, Tokyo, Japan). All measurements were conducted under controlled environmental conditions ($T=23 \pm 2^\circ\text{C}$). The acquired images were analysed using dedicated image analysis software (ImageJ 1.47v; Wayne Rasband, National Institutes of Health, Bethesda, MD, USA) to determine the contact angle [Table/Fig-11].



[Table/Fig-10]: Haemocompatibility assessment.



[Table/Fig-11]: Contact angle - wettability of each membrane.

Drug release [23]: The medium used for this method was Phosphate Buffered Saline (PBS). The two membranes (6% alginate and CELLINK BONE®) containing 1.2% RSV were placed in the medium and 500 µL samples were taken at predetermined times (every 50 minutes) and the amount of solution taken was replaced by fresh PBS to maintain sink condition.

Formula for determination of percentage of release of drug from in-vitro dissolution testing:

Concentration of drug (µg/mL)=(slope × absorbance) ± intercept
Amount of drug released mg/mL = Concentration × Dissolution bath volume × dilution factor/1000.

Cumulative percentage release (%) = Volume of sample withdrawn (mL)/bath volume (v) × P (t - 1) + Pt

Where Pt = Percentage release at time t

Where P (t - 1) = Percentage release previous to 't'

RESULTS

Disintegration of CELLINK BONE® with 1.2% RSV membrane: During the airdrying membranes, all the membranes except CELLINK BONE® with 1.2% RSV membrane were obtained. The CELLINK BONE® with 1.2% RSV membrane underwent complete disintegration during air-drying, resulting in a powdered residue upon removal after the airdrying process. Hence, no further analysis was carried out for this membrane.

Morphological analysis by SEM: The SEM analysis under 100x magnification revealed intact and homogenized nature of all the three membranes. It also demonstrates rough surface which aids in increase in surface area suitable for tissue regeneration. The honeycomb pattern and rectilinear patterns of the membrane are not prominently defined. The patterns appeared similar and had macro pores on the surface. A 6% alginate + 1.2% RSV membrane demonstrated more microfoldings and surface microroughness compared to 6% alginate and CELLINK BONE® membranes. Under 4000x magnification, homogenised mixture of drug with

alginate was evident in 6% alginate + 1.2% RSV and no major discrepancies were noted in CELLINK BONE® membranes. All the three membranes exhibited fissures and cracks on the surface [Table/Fig-8,9].

Contact Angle - Wettability: The values of contact angle of each membrane are given in [Table/Fig-12].

Membrane type	Contact angle in degrees
6% alginate	22.60
6% alginate + 1.2% Rosuvastatin (RSV)	23.60
Cellink BONE ©	45.54

[Table/Fig-12]: Contact angles of 6% alginate, 6% alginate + 1.2% Rosuvastatin (RSV), CELLINK BONE®.

All three membranes exhibited contact angle less than 90 degree, hence they are concluded to be wettable in nature, which is an essential feature of the membranes used for periodontal regeneration [Table/Fig-11].

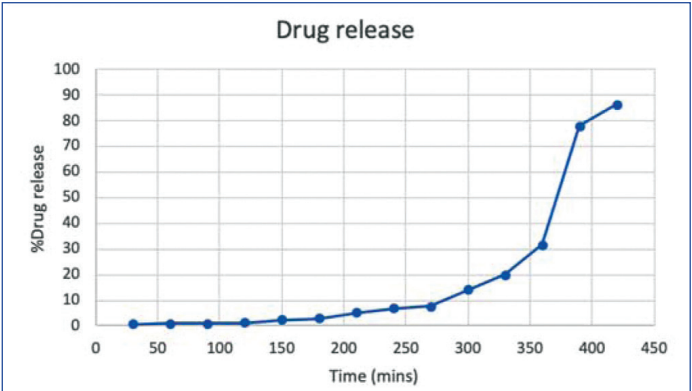
Drug release: The drug release was evaluated at every 50th minute. The 6% alginate + 1.2% RSV membrane showed irregular yet sustained release of drug. The drug release peaked from 10% at 200th minute to around 85% at around 420th minute [Table/Fig-13].

FTIR analysis: The peaks and their group assignments of all the three membranes are enlisted in [Table/Fig-14].

Haemocompatibility Assay: According to American Standard testing for materials, the lysis rate should be less than 5% for a material for it to be deemed to be haemocompatible [19]. The 6% alginate membrane showed haemolysis of 0.79%, 6% alginate + 1.2% RSV showed 0.77% of haemolysis, CELLINK BONE® showed haemolysis of 0.69%. All three membranes showed haemolysis of less than 5%, hence they are concluded to be haemocompatible in nature [Table/Fig-10].

DISCUSSION

The GTR involves the use of a barrier to prevent epithelial ingrowth along the exposed root surface, and it also has a role as a carrier



[Table/Fig-13]: Drug release of 6% alginate + 1.2% Rosuvastatin (RSV) membrane.

CELLINK BONE® bioink bioprinting cartridges. Its Alg-Gel matrix demonstrates viscoelastic and shear thinning fluidity, in combination with nanoparticles of HA that provide enhanced bioactivity, mechanical robustness, and upstream bio-mineralisation. Animal model and in-vitro [17,21] studies have demonstrated that CELLINK BONE® Bioink supports scaffold fidelity, cell attachment, and new bone formation. Additives like β -TCP or bioglass can further improve mechanical stability and calcium release [18,25]. Overall, CELLINK BONE® Bioink is a biomimetic, biocompatible, and clinically translatable material suitable for guided tissue and bone regeneration.

Characterisation of the regenerative periodontal membranes is critical to establish functionality, biocompatibility, and effectiveness. FTIR is employed to authenticate the chemical composition, validating the

Peak position (cm-1)	6% Sodium alginate + 1.2% Rosuvastatin (RSV)	6% Sodium alginate	CELLINK BONE®	Probable assignment
~3860-3880	3860.79	3880.08	-	O-H stretching (Hydrogen-bonded hydroxyl groups)
~3771	3771.12	3771.12	3782.69	O-H stretching (Water content)
~3361-3362	3362.28	3361.32	-	N-H stretching (Amine group)
~2978	-	-	2978.52	C-H stretching (Aliphatic groups)
~2372-2373	2372.98	2369.12	2372.01	CO ₂ asymmetric stretching (Possible carbonate content)
~2305-2309	2305.48	2308.37	2309.34	C≡N or C≡C stretching (Possible nitrile or alkyne presence)
~1729	-	-	1729.83	C=O stretching (Ester or carboxyl group)
~1607-1624	1608.34	1607.38	1623.77	C=C or N-H bending (Aromatic or Amide presence)
~1420-1421	1421.28	1420.32	1402	C-H bending (Alkanes or aromatic ring)
~1322-1327	1326.79	1321.96	-	C-N stretching (Amines)
~1080-1081	1079.94	1080.91	-	C-O stretching (Carbohydrates or ethers)
~1024-1053	1024.98	1024.98	1052.94	C-O stretching (Polysaccharides)
~534-533	534.185	533.221	-	Metal-Oxygen or skeletal vibrations

[Table/Fig-14]: Peaks and their group assignments of 6% sodium alginate, 6% sodium alginate + 1.2% Rosuvastatin (RSV), CELLINK BONE®.

for anabolic agents that promote regeneration [18]. Bioprinting is a revolutionary tool that has been developed in the last few decades for manufacturing of membranes for GTR. Bioprinting provides precise control over the architecture and bioactive component of scaffolds that promotes periodontal regeneration [9].

Alginate bioinks rank among the most popular biomaterials in 3D bioprinting periodontal regenerative membranes owing to their high biocompatibility, viscoelasticity, and mild gelation conditions. Isolated from brown seaweed, alginate readily forms hydrogels through ionotropic gelation with calcium ions. Wu YF et al., (2024) showed that 6 wt% sodium alginate bioink blended with β -TCP resulted in 3D bioprinted scaffolds that were highly osteoconductive and mechanically stable alveolar bone grafts [24]. The processability, degradability, and modifiability of alginate make it one of the preferred hybrid sources for preparing tissue and bone engineering grafts [18].

The RSV is an artificial inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG CoA) reductase. Additionally, the anti-inflammatory and osteogenic efficacy of the medication validates its therapeutic property as an anabolic agent for bone augmentation. Moreover, the use of RSV as an anti-periodontal agent has been investigated with its local delivery system related to gel formulation. Promising results and effectiveness have been stated with the use of 1.2% RSV in aiding substantive CAL gain, PD, and infra-bony defects as demonstrated by randomized controlled trials that outperformed the efficacy of 1.2% atorvastatin and also as an adjuvant with NSPT [15-17].

CELLINK BONE® bioink is an alginate-based hydrogel biomaterial designed to target the application in bone and periodontal scaffolds. Its formulation includes the use of alginate, gelatin, Hydroxyapatite (HA), combining to create biomaterials that form ECM of bone. The bioink is ISO 13485-compliant, sterile, packaged in ready-to-use

polymer interactions, bioactive molecules, or both in the materials. In the present study, the FTIR results demonstrated the presence of all essential functional groups needed in the formulation of the periodontal regeneration membranes. Hydroxyl (-OH), amine (-NH), carboxyl (-COOH), and carbonyl (C=O), saccharide, are central to the structural role in the three-dimensional bioprinted membranes. For the purpose of mimicking the structure in the ECM, the CELLINK BONE® has demonstrated the three fundamental peaks (C≡N stretching, Ester -C=O, Carboxyl group. Ester, C-H bending-polypeptide saccharide structure) that has been the best in mimicking the ECM amongst the three membranes used.

The SEM was used to assess membrane surface morphology, pore size, and interconnectivity, which are critical for cell attachment, nutrient diffusion, and tissue integration. At 100x magnification, clear structural differences were observed between the membranes. The CELLINK BONE® surface was rough with micropores indicative of mineralisation due to the inclusion of HA in the alg-gel scaffold. Such surfaces promote more efficient osteoblast adhesion and osteoconduction [24,26]. In comparison to other membranes, the 6% sodium alginate surface was smoother with more homogenous pores representatives of hydrophilic polymer networks with less surface bio-utility. Incorporation of RSV (6% Alginate + 1.2% RSV) resulted in increased surface roughness and pore interconnectivity, indicating successful drug dispersion and enhanced microstructural heterogeneity, which may favour drug diffusion and cellular colonisation, in agreement with El-Nablaway M et al., (2024) [27].

At higher magnification (4000x), the CELLINK BONE® membrane demonstrated dense agglomerations of plate-like HA crystals, confirming its mineralised composition and osteoconductive potential [28]. The RSV-loaded alginate membrane showed a granular, roughened surface with uniformly distributed microdomains, suggesting strong drug-polymer interactions and potential for

sustained drug release. Conversely, the plain alginate membrane exhibited smoother flakes and fewer granular features, indicating lower bioactive potential. These findings are consistent with studies by Miao G et al., (2023) and Tahmasebifar A et al., (2023), which reported increased surface roughness and bioactivity following incorporation of bioactive molecules or ceramic fillers [29,30]. Overall, SEM analysis confirms that RSV incorporation enhances the structural complexity and surface characteristics of the alginate membrane, supporting its suitability for osteogenic and periodontal regenerative applications.

Haemocompatibility test analyses the compatibility of the membrane with blood components, preventing haemolytic reactions and platelet aggregation, and is essential to avoid any reaction that might occur upon implantation [18]. In this study, all three membranes have appropriate haemocompatibility. Contact angle measurement test is carried out to check the wettability and hydrophilicity of the membrane surface, playing a key role in protein and cell adhesion and proliferation on the membrane surface. The desirable contact angle of periodontal membrane is between 30-45 degree values [22]. Among all three membranes used, the appropriate contact angle value that favours periodontal regeneration has been showed by the "CELLINK BONE®" membrane. Franz diffusion test for drug release is used to study the controlled and sustained release of drugs incorporated into the membrane, allowing the prediction of pharmacokinetics and bioactivity duration of drugs within the periodontal environment. The drug released from the alginate and RSV membrane was sustained but not uniform at each 50th minute. The sudden increase to a peak value of 85% was not gradual but incremental. They indicated the degradation rates of alginate, where the drug is freely dispersed within the membrane.

To the best of our knowledge, this is the first study that has evaluated the physical properties of CELLINK BONE® 3D bioprinted membrane and first study to have explored the incorporation of RSV in alginate based bioink for 3D bioprinted periodontal membranes.

Limitation(s)

The limitation of the study is the disintegration of CELLINK BONE® + 1.2% RSV membrane after heat drying process. The membrane was not stable and resulted in flaky form. This might be due to the cross reaction of ethanol solvent (for RSV) with the components of the CELLINK BONE® bioink such as alginate. Further optimisation is needed for incorporation of drug in CELLINK BONE® bioink. Other limitations include the lack of cell viability tests, cytotoxic assays, degradation kinetics of this membrane are yet to be determined for all the three membranes.

Further in-vivo studies with defined sample size and biodegradation of these membranes are to be evaluated in animal models. Drug release has to be optimised for uniform and consistent release from the membrane.

CONCLUSION(S)

Formulated 6% Alginate, integrating RSV with Alginate & CELLINK BONE® Bioink in 3D bioprinting offers a promising avenue for creating advanced regenerative scaffolds tailored to patient specific needs. Further research and optimisation in concentration of bioink and drug incorporation is needed for future applications.

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