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FABRICATION OF GRACILARIA INCORPORATED STRONTIUM SCAFFOLDS FOR BONE REGENERATION

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ABSTRACT:

A vital area of medical research where people are still working to find a better solution is bone tissue regeneration particularly when it comes to healing fractures, abnormalities, or degenerative one disorders. Recent research has stated that tissue engineering can be a better treatment methodology for this problem because it is a combination of cell biology, material science and research which has brought a lot of significant changes in material science that produces new tissues which resemble the native one. Tissue engineering involves growing stem cells or fabricating biomimetic scaffolds that facilitate the new bone formation which increases the life of a human being. In recent years the advancement in this treatment is producing synthetic biomaterials which is a combination of metal particles incorporated into the scaffolds. In that way conjugates of Gracilaria-strontium–chitosan that stimulates both bone growth and inhibits bone resorption. Gracilaria is chosen because it is highly tolerated by the living tissues due to its biocompatibility. The dimensional structure displayed by the Gracilaria helps in cells to multiply and differentiate which is important during the new bone formation to provide a supporting framework. Strontium helps in replacing the calcium ions at the site of hydroxyapatite crystal lattice as it is comparable to calcium in size and charge. Chitosan is a naturally occurring source that is biodegradable and that increase the activity of osteoblast that promotes osteogenesis. The results state that the combination of the algae, chitosan and the strontium particles enhances the expression level of the genes responsible for the osteogenic differentiation. This paper presents the results of fabrication and characterization of the scaffold, In vitro evaluation of biocompatibility properties of the scaffold and In vivo assessment of the scaffold for bone formation.

KEYWORDS: Tissue engineering, Strontium, Gracilaria, Chitosan, Osteogenesis, Biocompatibility

INTRODUCTION:

There are a lot of events like cellular and molecular events happening after a fracture for the bone repair. It involves a series of steps which includes haematoma, inflammation, angiogenesis, chondrogenesis to osteogenesis and finally bone remodeling (1). Osteogenesis is a process that is occurring in the bone marrow grafts which serves as the model of differentiation among the mixture of cells in the body (2). This process is the major event occurring in the bone remodeling

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process. To enhance this process from the recent research tissue engineering is used to promote the differentiation of cells. In the case where tissues of certain organs are affected by cancer or severe congenital anomaly or trauma, artificial tissue or organ transplantation is suggested first but the challenge faced during this treatment is immune rejection though there are a lot of advances made in immunosuppressive agents (3). Thus tissue engineering serves as the best treatment methodology to treat the affected tissues. The main key material involved in the tissue engineering is scaffold fabrication. Cells produce matrices for the development of tissues; these scaffolds serve as platforms for the cells to perform their development(3). In this paper scaffolds are constructed using conjugates of Gracilaria-strontium-chitosan. Gracilaria is the third largest genus in the red algal group, about 150 species are found worldwide and about 28 species are spotted in the Indian coastal regions. The can grow only in the region with high salinity, brackish water, swamps and in the sea which supports 50% of the worlds agar production due to its biocompatibility (4). From the recent studies it is proven that extracts from Gracilaria verrucosa (GE) may influence the osteoclast differentiation process mediated by RANKL. By suppressing the nuclear factor of activated T-cells (NFATc1) and c-fos protein expression, two essential components of RANKL- mediated osteoclastogenesis, GE dramatically reduced the differentiation of RANKL-activated osteoclasts (5). Chitosan is a carbohydrate biopolymer that repairs both hard and soft connective tissues. It is biodegradable, bioactive, biocompatible, non-toxic, inexpensive and non- immunogenic which has the ability to form new complexes with other anionic compounds thus it is majorly used in the biomaterial and biomedical applications. Chitosan material in the form of flims, hydrogels, sponges, scaffolds and membranes has the ability to contribute in the regeneration and healing of bone, cartilage and other cutaneous cartilage lesions (6). Strontium is trace element mainly taken through normal diet. It if found about 0.00044% of body mass and about 0.035% in the overall calcium content present in the body. As it is similar to the calcium
ions in cellular transportation pathways it has great affinity while incorporating it into the bone matrix during mineralization. Strontium is considered to be as a osteoporotic drug that facilitates bone formation through the underlying mechanism stimulating the cell differentiation of the oesteoblast and inhibiting the differentiation and activity of oesteoclast to prevent the process of resorption. Due to the multiple function strontium particles incorporated biomaterials supports thr regeneration of the damaged and diseased tissues in the human body (7).

METHODOLOGY:

2.1 Fabrication and physicochemical characterization of the scaffold:

The biocompatible polymers suitable for scaffold fabrication, such as polycaprolactone (PCL), poly(lactic-co-glycolic acid) (PLGA), or a combination of natural and synthetic polymers have been chosen as the polymer of interest. The Gracilaria extract and strontium-containing compounds were incorporated into the polymer matrix. The fabrication method such as 3D printing, electrospinning, and freeze-drying were chosen for scaffold design. Ensure even distribution of gracilaria extract and strontium within the scaffold during fabrication. Apply crosslinking agents or methods to enhance the stability and mechanical strength of the scaffold. Ensure that the crosslinking process does not compromise the biocompatibility of the scaffold. Sterilize the fabricated scaffold using appropriate methods (e.g., gamma irradiation or ethylene oxide) to ensure it is free from contaminants for in vivo applications.

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Fig 1. Fabricated Scaffolds

2.2 In vitro evaluation of biocompatibility properties of the scaffold:

Use relevant cell types such as osteoblasts, mesenchymal stem cells (MSCs), or other cells relevant to bone tissue. Include a control group with cells cultured on standard tissue culture plates to compare cellular behavior. Stain cells on the scaffold using live/dead cell viability assays. Use fluorescent dyes (e.g., calcein-AM for live cells and propidium iodide for dead cells) and visualize under a fluorescence microscope. Perform MTT (3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide) assay to quantify cell metabolic activity.

2.3 Characterization Techniques:

Scanning electron microscopy (SEM) is used to examine cell morphology and attachment on the scaffold.Evaluate the interaction of cells with the scaffold surface. Use immunofluorescence staining to visualize cytoskeletal components (e.g., actin filaments) and cell nuclei. Observe the arrangement of the cytoskeleton to assess cell attachment and spreading. RT-PCR (Reverse Transcription Polymerase Chain Reaction) is used to Assess the expression of osteogenic markers (e.g., osteocalcin, Runx2) to evaluate the potential of the scaffold to induce osteogenic differentiation. Measure the release of pro-inflammatory and anti-inflammatory cytokines (e.g., IL-6, TNF-α) to evaluate the inflammatory response induced by the scaffold. Quantify the release of strontium ions from the scaffold using inductively coupled plasma mass spectrometry (ICP- MS). Monitor strontium concentration in the cell culture media over time. Assess ALP activity as an early marker of osteogenic differentiation. Quantify ALP enzymatic activity using colorimetric assays. Perform mineralization assays (e.g., Alizarin Red staining) to visualize and quantify calcium deposition by differentiated cells.

2.4 *in-vivo* Assessment of the scaffold for bone formation:

Choose a relevant animal model for bone tissue regeneration studies (e.g., rat, rabbit, or mouse). Ensure ethical considerations and compliance with relevant regulations. Sterilize the scaffold using appropriate methods. Implant the scaffold into the bone defect site using surgical procedures. Use imaging techniques such as X-rays or micro-CT to monitor the integration of the scaffold with the

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surrounding bone tissue. Assess changes in scaffold morphology over time. Use advanced imaging techniques (e.g., scanning electron microscopy, micro-CT) to analyze the microstructure of the regenerated bone and the scaffold's role in supporting bone formation.

2.5 Mechanical property assessment:

The scaffolds (without hydration) were cut into as per the specifications and tensile strength was investigated in an uniaxial load test machine with a weight loading rate 5 N at extension rate of 12mm/min.(8)

3.RESULTS AND DISCUSSION

3.1PHYTOCHEMICAL STUDY OF *Gracilaria verrucosa*:

The phytochemical studies involve screening for various bioactivities such as antioxidant, antimicrobial, anti-inflammatory, anticancer, antidiabetic, and other pharmacological properties and the identification of bio-active compounds are listed below in Table 1:

BIOACTIVE COMPOUNDS	OBSERVATION	RESULTS
Saponins	Formation of foam	-ve
Flavonoids (Shinnoda test)	No colour changes	-ve
Steroids (Salkowski test)	Reddish brown colour formation	+ve
Tannins (Ferric chloride test)	Dark green colour precipitation occurs	+ve
Phenols (Ferric chloride test)	Dark blue colour formation	+ve

Table 1

3.2 GEL FRACTION STUDY AND FT-IR ANALYSIS:

Average weight of the scaffold is 0.175 gm. Initially dry weights of the scaffolds were measured and the scaffolds were soaked in double-distilled water for 24 h. After that samples were freeze- dried for 24 h. Finally, the dry weights of the twice freeze-dried samples were

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measured three times per sample, and the results are reported as average ± standard error. The FT-IR analysis of the bio-composite frameworks and the individual components are shown in Figure. In the chitosan

spectrum, the peak at 3436 cm^{-1} indicated the presence of NH2 and OH groups, the peak at 1639 cm^{-1} attributed to the presence of an amine group, and the peak at peak 1423 cm^{-1} showed the presence of a hydroxyl group. The spectrum of the Gra-CS-Sr skeleton retained all the

characteristic peaks of CS, Sr indicating the presence of CS in the skeletons, and the complexation of the metal Gra-Sr possibly occurred with the NH2 group of chitosan, resulting in a change in the characteristic led peak of NH2 at 3436 cm^{-1} and the appearance of the peak at 3459 cm^{-1} of Gra-Sr-CS and GRA-Sr.

GEL FRACTION	WEIGHT OF SCAFFOLD SAMPLE (IN DRY CONDITION)	WEIGHT OF SCAFFOLD SAMPLE (AFTER FREEZE DRIED CONDITION)
1st time	0.143 gm	0.176 gm
2nd time	0.143 gm	0.174 gm
3rd time	0.143 gm	0.177 gm

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Table 2

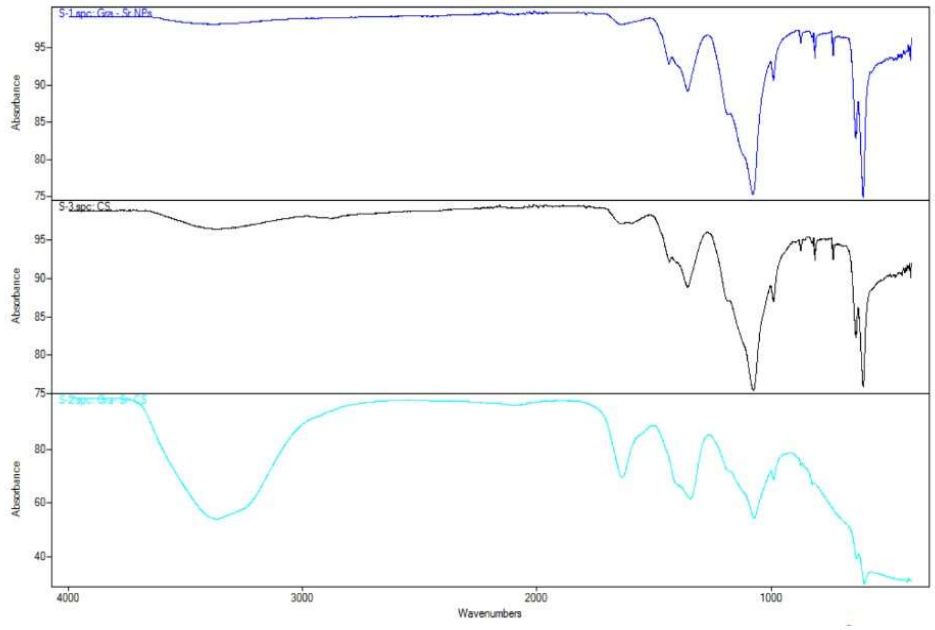

Fig 2. FT – IR ANALYSIS

3.3 SWELLING INDEX INTERPRETATION:

The swelling index provides information about the material's ability to absorb and retain liquids, which can be crucial in various applications, such as in the development of medical dressings, diapers, or absorbent materials in general. The initial weight of the samples(3 different proportions of scaffolds) is taken in the 0^{th} hour. After soaked in 1x Phosphate Buffered Saline (PBS), the weight of the samples at 2^{nd} hour, 4^{th} hour and 6^{th} hour is noted.

Swelling Index = (Final weight-Initial weight)/(Initial weight)

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Fig 3. Swelling Index

S.NO	Weight of sample at 0th hour (mg)	Weight of sample at 2nd hour (mg)	Weight of sample at 4th hour (mg)	Weight of sample at 6th hour (mg)
SAMPLE 1	43.4	265.7	315.6	530.1
SAMPLE 2	44-4	320.9	360.2	636.4
SAMPLE 3	45.6	390.5	410.5	690.8

Table 3

3.4 UV-VISIBLE AND SWELLING INDEX INTERPRETATION:

The absorption peak of Sr (supported nitrate) exhibited at 250nm in the UV region confirms the presence of Strontium in GRA-Sr solution. The swelling index is gradually increased through all the proportions of the scaffold and it is because of the hydrophilic nature of the chitosan. This
hydrophilic nature makes chitosan suitable for various applications, including drug delivery systems, wound dressings, where its ability to interact with water is beneficial.

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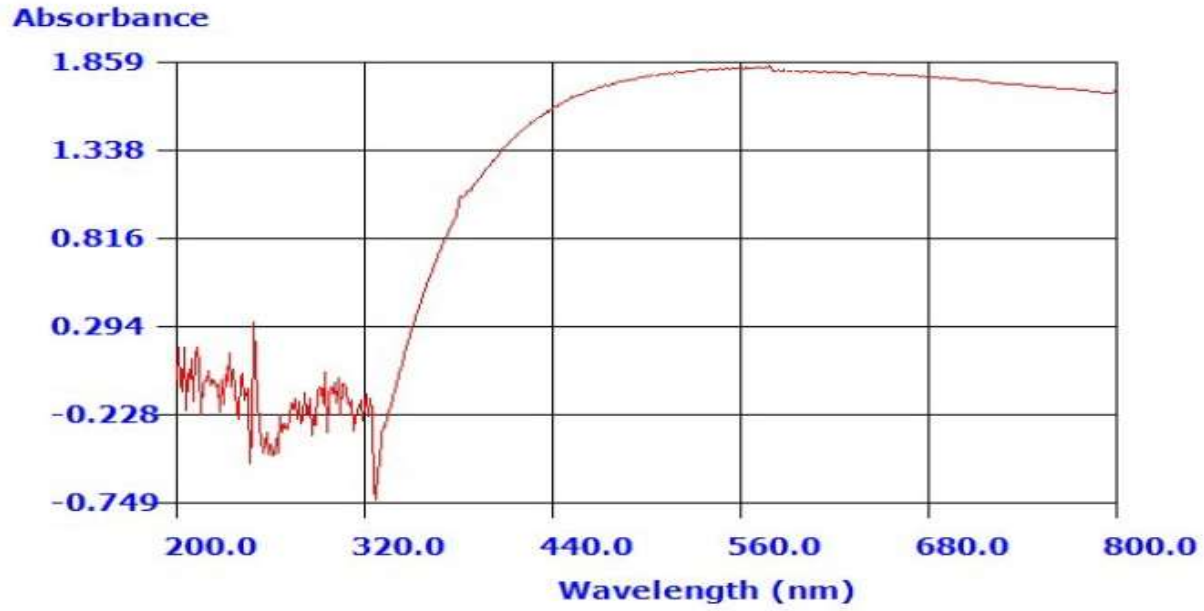

Fig 4. UV – VISIBLE ANALYSIS

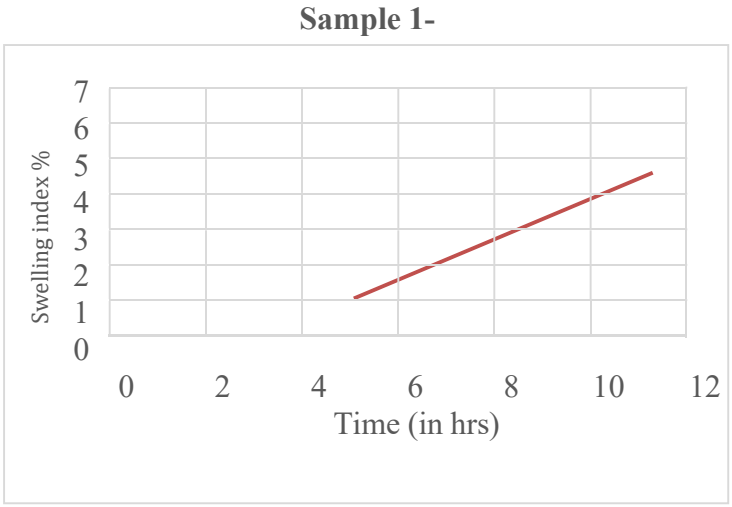

Fig 5. SAMPLE 1 SWELLING INDEX

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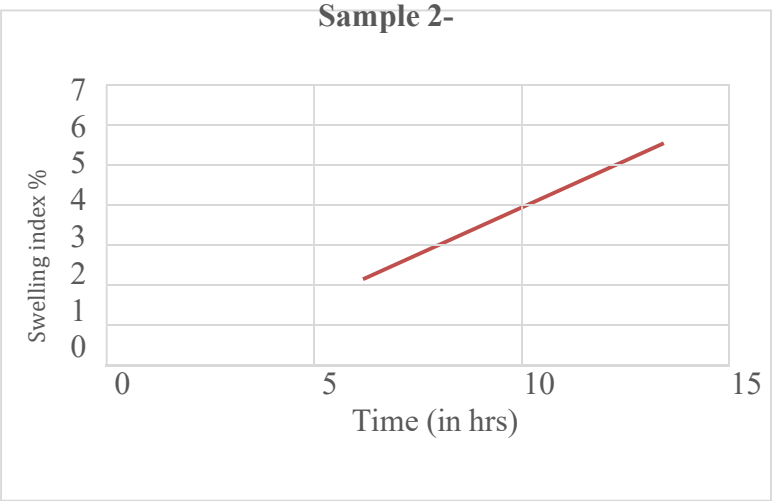

Fig 6. SAMPLE 2 SWELLING INDEX

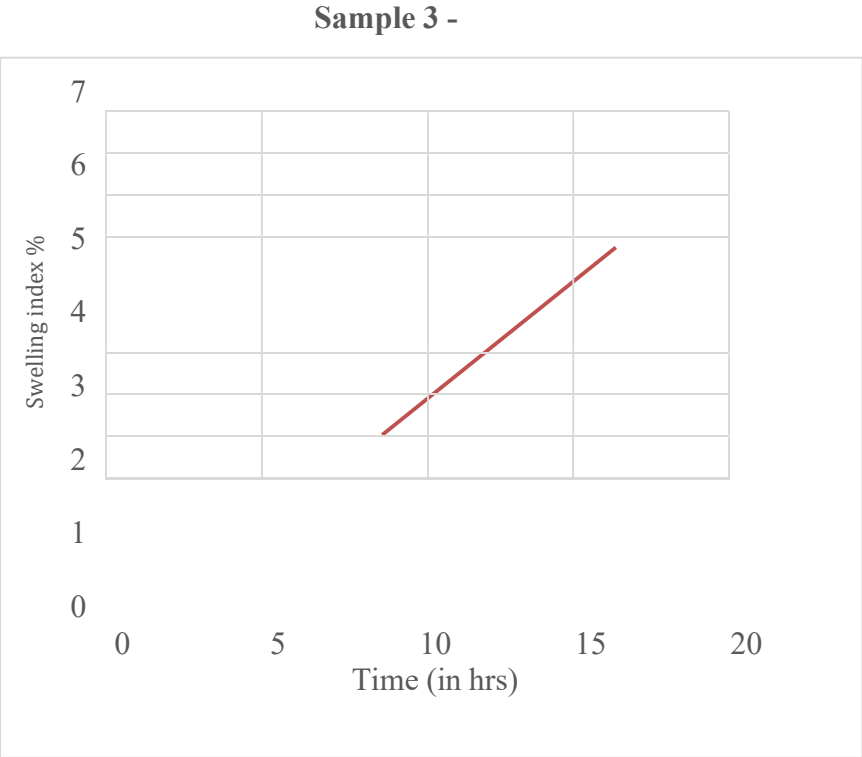

Fig 7. SAMPLE 3 SWELLING INDEX

DPPH RADICAL SCAVENGING ASSAY AND pH STUDY:

Assessing the antioxidant capacity of the scaffold through the DPPH assay can provide valuable information on their potential to scavenge free radicals and protect cells from oxidative damage. This is particularly important in tissue engineering applications, where oxidative stress can negatively

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impact cell viability and tissue regeneration. A lower IC50 value suggests higher antioxidant activity because it indicates that a lower concentration of the scaffold is needed to neutralize half of the free radicals. IC50 value is determined and found to be 43.77104. The pure Gra-Sr-CS sample's pH progressively decreased to 6.12. This decrease in pH is caused by the
presence of additional hydroxyl groups in the polymer structure, which increases its hydrophilicity, speeds up its breakdown over extended periods of time, and creates short polymer chains with acidic ends. Using NaOH, the pH is increased from 6.12 to 7.2 as the osteoblasts (new bone tissue development) works best at 7.200

$$y = 0.594x + 24 \quad R^2 = 0.9737$$

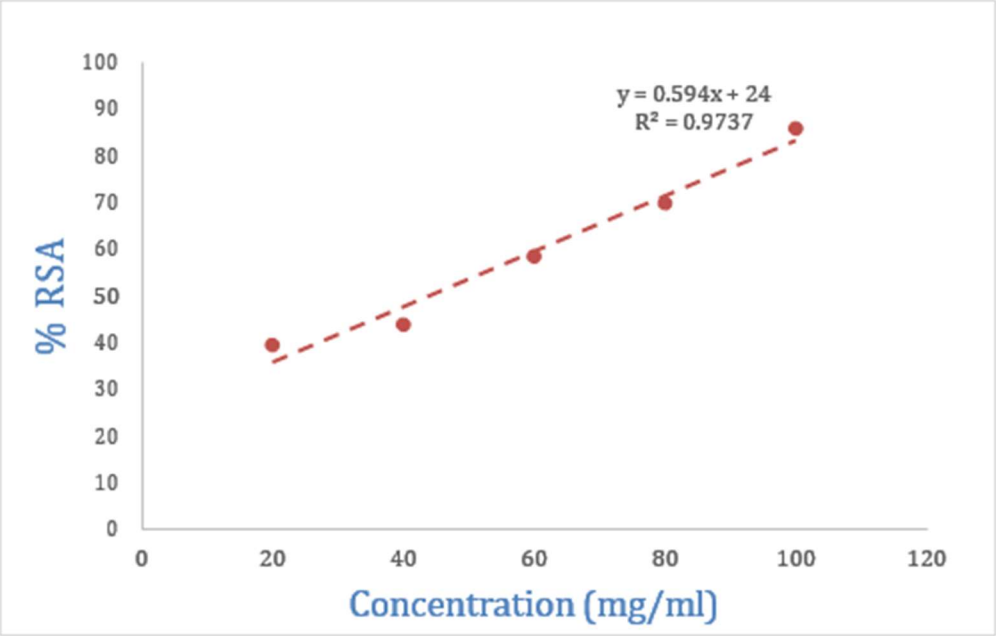

Fig 8. DPPH ASSAY

4.CONCLUSION AND SUGGESTIONS FOR FUTURE WORK:

The study successfully fabricated gracilaria-incorporated strontium scaffolds for bone tissue regeneration. Physicochemical characterization confirmed the structural integrity and composition of the scaffolds. In vitro biocompatibility assessments demonstrated high cell viability, proliferation, and osteogenic differentiation on the scaffolds. The release kinetics of strontium ions contributed to a favorable environment for bone-forming cell activity. The scaffolds exhibited promising physicochemical characteristics, including controlled strontium release, which is vital for bone regeneration. Mechanical testing results met or exceeded standards for bone tissue engineering applications. Further optimize scaffold properties based on the observed results. Investigate additional fabrication parameters to enhance mechanical strength and structural features. Conduct long-term in vitro studies to assess the sustained biocompatibility and osteogenic

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potential of the scaffolds. Progress to in vivo studies for a more comprehensive evaluation of the scaffold's performance. Investigate the synergistic effects of combining the scaffold with growth factors or other bioactive agents to enhance bone regeneration. Address scalability and manufacturing considerations for potential clinical applications. Explore the translational potential of the gracilaria-incorporated strontium scaffolds for clinical applications. Consider the environmental impact of scaffold fabrication and degradation.

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