Fabrication, Characterization and *In-vitro* Biological Evaluation of Microporous 3D Composite Scaffolds Containing nano-Silica/nano-Bioglass for Bone Tissue Engineering

SYNOPSIS OF THE THESIS

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DECLARATION

I Ms. Kavya. K.C (KH.NS.D* NMS08004), hereby declare that this synopsis of the thesis titled “Fabrication, Characterization and In-vitro Biological Evaluation of Microporous 3D Composite Scaffolds Containing nano-Silica/nano-Bioglass for Bone Tissue Engineering” is a bonafide record of original work done by me under the guidance of Dr. Krishnaprasad Chennazhi, Associate Professor, Amrita Centre for Nanosciences and Molecular Medicine, Kochi and to the best of my knowledge and belief, it contains no material previously published or written by another person, no material which has been accepted for the award of any other degree or diploma of the university or other institute of higher learning, except where due acknowledgment has been made in the text.

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Chapter 1: Introduction

Defects in skeleton that occur as a result of acquired injuries or other factors remain a major and challenging health concern. Though bone has the capacity for inherent regeneration, a critical size defect if left untreated is impossible to heal. The idea that a biocompatible and biodegradable matrix inserted into the defect will support, guide and enhance bone healing has come into the picture. A suitable carrier device in the form of scaffold is required to facilitate the delivery of cells and extracellular matrix material to the site of bony defect. This proposal has provided the arena for tissue engineering. Tissue engineering aims to generate functional tissue thereby raising the possibility of in vitro engineering of bone. Several materials have been proposed for this purpose. The ideal candidate has to be biocompatible and osteocompatible in addition to demonstrating mechanical strength and resilience. Besides, the scaffold should be adequately porous allowing bone tissue to regenerate within. A relatively slow degradation profile is necessary to provide the needed mechanical and structural framework, prior to complete native bone regeneration.

Among numerous materials evaluated for scaffold fabrication, chitosan is considered promising, attributing to its non-toxicity, excellent biocompatibility and biodegradability. It has good moldability and can form porous structures upon lyophilization. But, the hydrophobic property of chitosan may lower cell attachment and proliferation. To enhance its performance, gelatin can be utilized to modify and prepare composites with better hydrophilicity and biological compatibility. Gelatin, a natural polymer, has good cell-interactive properties besides being non-toxic, biodegradable and non-immunogenic. Chondroitin sulfate, an acidic mucopolysaccharide can also be utilized to enhance the performance of chitosan, in terms of better hydrophilicity and biological compatibility. Besides, this acidic macromolecule is said to promote calcification. With these observations, composite scaffolds of chitosan/gelatin and chitosan/chondroitin sulfate were fabricated by lyophilization technique.

Bone is a unique composite of polymeric biological molecules (collagen) and inorganic minerals (calcium phosphate). Taking hint, the fabrication of scaffolds mimicking similar composition comprising polymeric phase and mineral phase could offer better performance. Moreover, the advent in nanotechnology has led to the development of many novel and ingenious nano sized scaffold materials. Decreasing the particle size increases the surface area, resulting in higher exposure of bioactive compounds to the surrounding tissue, enhancing the bioactivity. Nano-sized particles can form tighter interface with polymer matrix and can enhance mechanical properties of the composites. Several materials in their nano form influences better osteoblast adhesion and differentiation of MSCs in to osteogenic lineage. Several ceramic nanoparticles have found application in bone tissue engineering due to its innate property in enhancing the bioactivity and osseoinductive ability of scaffold, upon integration. Among them nano-silica and nano-bioglass have gained considerable attention.

Silica is essential in skeletal development, is involved in early stage of bone calcification, and is found to up-regulate osteoblast specific genes. It is said to promote crystallization of
apatite, cell adhesion and collagen formation. It is found that, bioactive glass, on contact with physiological solution, could also crystallize hydroxyl carbonated apatite similar to the mineral phase of bone. These nanoparticles if present in the composite can provide nucleation sites to enhance scaffold mineralization. In light of these details, in this study, we combined the composite scaffolds of chitosan/gelatin and chitosan/chondroitin sulfate with nano silica and nano bioglass to fabricate scaffolds of chitosan/gelatin/nano SiO₂, chitosan/chondroitin sulfate/nano SiO₂ and chitosan/ chondroitin sulfate/nano-bioglass, by lyophilization. The thus fabricated nano-composites were evaluated for their potential as scaffolds for bone tissue engineering.

**Main objectives of the study include:**
- Fabrication and characterization of chitosan, chitosan/gelatin and chitosan/gelatin/nano SiO₂ scaffold
- Fabrication and characterization of chitosan/chondroitin sulfate and chitosan/chondroitin sulfate/nano SiO₂ scaffold
- Fabrication and characterization of chitosan/ chondroitin sulfate/nano-bioglass scaffold
- Evaluation of porosity, swelling, protein adsorption, degradation, cytocompatibility, mineralization and mechanical characteristics of the prepared nano-composites and control scaffolds, *in vitro*
- Evaluation of cell attachment, proliferation and maturation on the prepared nano-composites and control scaffolds, *in vitro*
- Analysis of osteoblast specific gene expression in cells grown on the prepared nano-composites and control scaffolds, *in vitro*

**Review of Literature**

Tissue repair and regeneration is an interdisciplinary field focusing on development of biological and bioactive substitutes.\(^1\) The substitute in the form of supporting matrix plays a crucial role as an artificial ECM to provide temporary environment for cells to infiltrate, adhere, proliferate, and differentiate thereby guiding the repair and regeneration of tissue. An ideal matrix material need to have biocompatibility, suitable microstructure, desired mechanical strength and degradation rate, and the ability to support cell residence allowing retention of metabolic functions.\(^2\)[3] The scaffold as a substratum should allow cells to attach, proliferate, differentiate and organize into normal, healthy bone while it degrades.\(^4\)

A major hurdle in the design of tissue engineering scaffolds is that most materials are not simultaneously mechanically competent and bioresorbable.\(^5\) From a biological perspective, it makes sense to combine polymers and bioceramics to fabricate scaffolds because native bone is the combination of a naturally occurring polymer (organic phase) and biological apatite (inorganic phase). From materials science point of view, a single material does not usually provide the necessary mechanical and/or chemical properties required. Hence, by combining two or more materials, their individual properties could be integrated into the composite. Polymers
and ceramics/glasses that have the ability to degrade in vivo are ideal candidates for composite scaffolds as they gradually degrade while new tissue is formed.\textsuperscript{[6],[7]}

Chitosan is of particular interest as biomaterial and supporting matrix for tissue repair and regeneration and it has been a widely used natural polymer for bone tissue engineering application.\textsuperscript{[8]} It can be easily formulated into matrices of various forms. By a simple freeze-drying method, chitosan-based 3D scaffolds with interconnected porous structures can be prepared.\textsuperscript{[9]} Chitosan, under acidic conditions gains a pH-dependent cationic nature, thereby imparting the ability to interact with anionic glycosaminoglycans.\textsuperscript{[10]} Chitosan can also be modified to improve its biological performance by combining with several natural macromolecules.\textsuperscript{[11]} Crosslinking helps modulate a broad spectrum of characteristics of chitosan-based biomaterials for tissue repair, typically, mechanical strength and biostability. Covalent crosslinking of chitosan scaffolds is performed utilizing aldehydes like glutaraldehyde.\textsuperscript{[12],[13]} To improve mechanical strength and biological performance of chitosan, it has been a widely accepted approach to incorporate other biopolymers or inorganic materials into chitosan scaffolds. Chitosan has been combined with other natural polymers like gelatin\textsuperscript{[14]} and chondroitin sulfate (CS)\textsuperscript{[15]} for bone tissue engineering applications.

Gelatin is a partially hydrolyzed product of collagen.\textsuperscript{[15]} Research shows that chitosan/gelatin hybrid scaffold facilitated hepatocyte growth in vitro.\textsuperscript{[16]} In addition, chitosan/gelatin nanofibers fabricated by electrospinning showed remarkably higher tensile strength.\textsuperscript{[17]} Chondroitin sulfate is a glycosaminoglycan that occurs naturally in the body.\textsuperscript{[18]} Mi et al. has successfully prepared a crosslinked chitosan/CS porous scaffold.\textsuperscript{[19]} Chondroitin sulfate can be linked to chitosan framework by glutaraldehyde.\textsuperscript{[20]} CS is also expected to improve cytocompatibility. Research shows that the composites of chitosan/CS exhibits increased cell-seeding efficiency and proliferation. Freeze-drying is the most commonly used technique to fabricate chitosan-based porous scaffolds where the pore parameters can be regulated by controlling solution concentration and freezing temperature.\textsuperscript{[21]}

It is understood that bone ECM is composed of organic phase and HA-containing inorganic phase. For that reason, composite materials of chitosan and inorganic bioceramics are paid considerable attention in bone regenerative studies.\textsuperscript{[9]} Incorporation of nanotopographic features mimicking the nanostructure of natural bone is gaining interest.\textsuperscript{[22],[23]} Silicon is an essential initiator of bone mineralization.\textsuperscript{[24],[25]} It is concluded to have an effect on collagen, to make bone matrix more calcifiable.\textsuperscript{[26]} Silicon binds to glycosaminoglycans and helps in the formation of cross-links between collagen and proteoglycans.\textsuperscript{[27]} Chemically, silica is an oxide of silicon (SiO\textsubscript{2}) and is found in numerous tissues including bones. Silica deficiency leads to detrimental effects on the skeleton, including skull.\textsuperscript{[28]} A silica xerogl that was hybridized with chitosan for bone regeneration showed rapid induction of calcium phosphate in SBF and higher ALP activity of osteoblastic cells.\textsuperscript{[29]} The glass-ceramic, nano-bioglass (nBGC) is reported to cause an increase in protein adsorption and mineral deposition.\textsuperscript{[30]} Bioactive glass offers remarkable advantages as the inorganic component of composite scaffolds due to their high bioactive index, and their ability to bond to both soft and hard connective tissues.\textsuperscript{[31]} Reactions
on bioactive glass surfaces release critical concentrations of soluble Si, Ca, P and Na ions, which induce intracellular and extracellular responses.\[^{32}\]

Mechanically, bioceramics and glasses are stronger than polymers and play a critical role in providing mechanical stability to scaffold constructs prior to synthesis of new bone matrix by cells. However, ceramics and glasses are fragile and prone to catastrophic failure due to their intrinsic brittleness and flaw sensitivity. The formation of composites thus capitalises the advantages of both material types and minimize their shortcomings.\[^{33}\] There is growing interest in using nanomaterials for bone tissue engineering to mimic the structure of natural bone tissue which possesses a nanocomposite structure interwoven in a 3D matrix. The inclusion of nanoparticles into the biopolymer matrix has the dual objective of improving mechanical properties and incorporation of nanotopographic features mimicking the nanostructure of natural tissue.\[^{34}\][^35]

Chapter 2: Materials and Methods

Preparation of bioactive glass ceramic nanoparticles (nBGC)

1.56g TEOS and 25g Ca(NO)\textsubscript{3}.4H\textsubscript{2}O were dissolved in 240ml deionized water and 80ml 100% ethanol, stirred at room temperature for 4 hours, and pH adjusted to 2 using dilute HNO\textsubscript{3}. 3.96g of NH\textsubscript{2}H\textsubscript{2}PO\textsubscript{4} was dissolved in 3000ml ddH\textsubscript{2}O and 30g of PEG (20,000 MW) and pH adjusted to 10 using NH\textsubscript{4}OH. The TEOS - Ca(NO)\textsubscript{3}.4H\textsubscript{2}O mixture, taken in a burette was added drop wise in to NH\textsubscript{2}H\textsubscript{2}PO\textsubscript{4}, under constant stirring. The solution was stirred for 24 hours and filtered (0.2µm filter paper). The precipitate was washed thrice with 100% ethanol and ddH\textsubscript{2}O, frozen overnight at -20°C, lyophilized for 48 hours and sintered at 700°C for 7 hours to obtain nano-bioglass.

Preparation of chitosan, chitosan/gelatin, chitosan/gelatin/ nano-SiO\textsubscript{2}, chitosan/chondroitin sulfate, chitosan/chondroitin sulfate/nano-SiO\textsubscript{2} and chitosan/chondroitin sulfate/nano-bioglass scaffolds.

For chitosan scaffold, 2% chitosan solution was prepared by dissolving chitosan in 1% acetic acid and cross-linked with 0.25% glutaraldehyde (1:32, 2h). For chitosan/gelatin scaffold, chitosan-gelatin blend was prepared by dissolving 1% gelatin flakes in 2% chitosan solution at 40°C and cross-linked with 0.25% glutaraldehyde (1:32, 2h). For chitosan/gelatin/nSiO\textsubscript{2} scaffold, chitosan/gelatin blend was prepared, 1% nSiO\textsubscript{2} powder added and cross linked with 0.25% glutaraldehyde (1:32, 2h). For chitosan/chondroitin sulfate scaffold, the blend was prepared by dissolving 0.5% chondroitin sulfate powder in 2% chitosan solution, and cross-linked with 0.25% glutaraldehyde (1:32, 2h). For chitosan/chondroitin sulfate/nano-SiO\textsubscript{2} scaffold, chitosan-chondroitin sulfate blend was prepared, 1% nano-SiO\textsubscript{2} powder added and cross-linked with 0.25% glutaraldehyde (1:32, 2h). For chitosan/chondroitin sulfate/nano-bioglass scaffold, chitosan-chondroitin sulfate blend was prepared, 1% nano-bioglass powder added and cross-linked with 0.25% glutaraldehyde (1:32, 2h).
All scaffold blends were transferred into 24-well plate, frozen overnight at −20°C and lyophilized (48h) to obtain dry, porous scaffolds. For scaffold neutralization, the lyophilized scaffolds were treated with 2% NaOH (1h), washed with distilled water, treated with 5% NaBH₄ solution, again washed with distilled water, frozen overnight at −20°C, and lyophilized for 48h.

**Fibrin coating of scaffolds**

Scaffold samples were incubated in thrombin suspension (5 units/mL in 35 mM CaCl₂) for 30 min at 37°C. The scaffolds were then dipped in fibrinogen suspension (2 mg/mL in sterile distilled water) for 3–5 sec and incubated at 37°C for 30 min, to obtain a fibrin coat.

**Characterization of scaffolds**

The particle size of nano-bioglass was estimated using DLS. The scaffolds were characterized using SEM, XRD, FTIR and TGA. Porosity, apparent density, swelling in PBS, *in vitro* biodegradability, protein adsorption, *in vitro* biomineralization in SBF, and mechanical strength of the scaffolds were evaluated.

**In vitro biological evaluation of scaffolds**

*In vitro* biocompatibility, cell attachment, spreading, proliferation and maturation on the scaffolds were tested using MG63 cells. The cell differentiation and maturation studies on scaffolds were analyzed using hMSCs. Bone specific gene expression in MSCs cultured on the scaffolds was analyzed by checking the expression levels of Collagen Type I, Alkaline Phosphatase, Osteocalcin and Osteopontin.

**Chapter 3: Results and Discussion**

**Section 1: Fabrication and characterizations of Chitosan/Gelatin/nSiO₂ scaffold**

3D nano-composite scaffold of chitosan/gelatin/nSiO₂ developed by lyophilization exhibited an interconnected porous architecture with pore size ranging from 200-250µm. Nano-silica incorporation reduced the mean pore size in the nano-composite. FTIR, XRD, and TG analysis confirmed the formation of crosslinked, amorphous and thermally stable nanocomposite.

![SEM images](image1)

**Fig.1.** SEM images of: chitosan (A), chitosan/gelatin (B), and chitosan/gelatin/nSiO₂ scaffolds.

The prepared nano-composite also exhibited significant reduction in porosity, swelling and degradation in PBS-Lysozyme compared to control scaffolds. There is significant increase in apparent density and serum protein adsorption. The nanocomposite is also confirmed as
cytofriendly. Improved density can contribute to high mechanical strength and optimal porosity is a measure of permeability. Reduction in swelling helps avoid prominent increase in pore size. Adsorbed protein is said to facilitate cell adhesion and delayed degradation of scaffolds gives more time for new tissue formation before the support it provides for cells is lost.

Fig.2. (A) Porosity, (B) Apparent density, (C) Swelling in PBS, (D) Degradation in PBS-Lysozyme, (E) Serum protein adsorption, and (F) Biocompatibility of scaffolds.

Biomineralization of the scaffolds in SBF demonstrated increased mineral deposition on the nano-composite. The rate of mineralization is faster and more prominent in nSiO₂ containing scaffold. The deposited mineral is confirmed as hydroxyapatite. Mechanical testing showed a significant increase in compressive strength of the nano-composite. The strength increased further after mineralization. These in vitro characterization results are indicative of the performances that this nanocomposite would display in vivo for bone tissue engineering application.

Fig.3. (A → F) SEM images of scaffolds biomineralized in SBF for 7 days and 14 days, (G → I) XRD of biomineralized scaffolds for 7 days and 14 days, and (J) Mechanical testing of scaffolds.
Section 2: Fabrication and characterizations of Chitosan/Chondroitin sulfate/nSiO$_2$ scaffold

Chitosan/chondroitin sulfate/nSiO$_2$ scaffold was developed by lyophilization and displayed interconnected porous architecture (pore size $\rightarrow$ 150–200 µm). Nanosilica incorporation has caused the reduction in mean pore size in this nano-composite as well. FTIR, XRD, and TG analysis confirmed the formation of a crosslinked, amorphous and thermally stable nanocomposite.

Fig.4. SEM images of: (A) chitosan, (B) chitosan/CS, and (C) chitosan/CS/nSiO$_2$ scaffolds.

Chitosan/chondroitin sulfate/nSiO$_2$ exhibited significant reduction in porosity, swelling and degradation when compared to control scaffolds. There is significant increase in apparent density and serum protein adsorption. The prepared nanocomposite is confirmed as biocompatible. Biomineralization of in SBF demonstrated increased and prominent hydroxyapatite deposition on the nano-composite, confirmed by XRD. Mechanical testing showed significant increase in compressive strength of nanocomposite, which increased further on mineralization. The in vitro characterization results indicate the performance that this nanocomposite would display in vivo for bone tissue engineering application.

Section 3: Fabrication and characterizations of Chitosan/Chondroitin sulfate/nano-Bioglass scaffold.

Chitosan/Chondroitin sulfate/nBGC displayed interconnected porous architecture with pore size ranging from 150–200 µm. Nano-bioglass incorporation has also caused the reduction in mean pore size in the nano-composite. FTIR, XRD, and TG analysis confirmed the formation of a well crosslinked, amorphous and thermally stable nanocomposite contributed by chitosan, chondroitin sulfate and nano-bioglass.

Fig.5. SEM images of: (A) chitosan, (B) chitosan/CS, and (C) chitosan/CS/nSiO$_2$ scaffolds. (D) DLS of synthesized nano-bioglass
In vitro characterization of chitosan/chondroitin sulfate/nano-bioglass showed that the scaffold exhibited significant reduction in porosity, swelling and degradation in PBS-lysozyme as compared to control scaffolds. The nanocomposite also exhibited significant increase in apparent density, serum protein adsorption and is confirmed to be cytocompatible. Biomineralization in SBF and mechanical testing of chitosan/chondroitin sulfate/nBGC was also performed. Biomineralization demonstrated increased and prominent hydroxyapatite deposition on the nano-composite by day 7. The nano-composite displayed improved mechanical property (compressive strength), which increased further after mineralization. These in vitro analysis results are indicative of the performances that this nanocomposite would display in vivo for bone tissue engineering application.

Section 4: In vitro biological evaluation of Chitosan/Gelatin/nSiO$_2$, Chitosan/CS/nSiO$_2$ and Chitosan/CS/nano-Bioglass scaffolds

During biological evaluation, it was understood that the morphological and structural changes to chondroitin sulfate containing scaffolds were minimal as compared to gelatin containing scaffold. The chondroitin sulfate based system stayed relatively unaffected even after 28 days of incubation in serum-containing media, as observed by SEM.

![Fig.6](image_url)

Fig. 6. Images of fabricated nano-composite scaffolds (A-C) and SEM images of scaffolds incubated in serum containing media for 28 days (D-F).

Based on the above observation, chondroitin sulfate based system was used for further biological studies. Comparative cell-based biological evaluations were performed between chitosan/CS/nSiO$_2$ and chitosan/CS/nBGC scaffolds. Prior to these cell-based experiments, the scaffolds were coated with fibrin to enhance initial cell attachment during seeding. Fibrin coating allowed cells to achieve a well spread morphology on the scaffolds within 12 h in culture. Cell proliferation was also significantly higher on fibrin-coated scaffolds.
Studies using MG63 pre-osteoblast cells showed that bone-cell specific characteristics like ALP activity and cell-based mineralization were significantly higher on bioglass containing nano-composite when compared to silica-based composite and other control scaffolds. In addition, the expression pattern of osteoblast specific genes of ALP, Collagen-I, Osteocalcin and Osteopontin in MSCs cultured on the nano-composite scaffolds were significantly better on chitosan/CS/nano-bioglass as compared to chitosan/CS/nano-silica and other control scaffolds.

Fig. 7. SEM of uncoated (1, 2) and fibrin-coated scaffolds (C, D). SEM of cells on uncoated (A, B) and fibrin-coated scaffolds (C, D), 12 h after seeding.

Fig. 8. Alamar blue assay to analyze MG63 proliferation on scaffolds after 12h (A) and 72 h (B) in culture. CPC assay to analyze cell-based mineralization on uncoated (C) and fibrin-coated (D) scaffolds, in osteogenic media. ALP activity in MG63 grown on uncoated (E) and fibrin-coated (F) scaffolds, in osteogenic media.
Chapter 4: Conclusion

Micro porous, 3D nano-composite scaffolds of chitosan/gelatin/nano-silica, chitosan/chondroitin sulfate/nano-silica and chitosan/chondroitin sulfate/nano-bioglass were developed. In comparison to the conventional chitosan-based control scaffolds, the presence of nano-silica and nano-bioglass significantly enhanced scaffold’s properties. Chitosan-chondroitin sulfate composite was found to be more favorable for long term cell-based studies than gelatin based system. Fibrin coating of the scaffolds resulted in a superlative effect on cell attachment, spreading and proliferation in vitro. Nano-composite scaffold containing bioglass was found to enhance osteoblast maturation and cell-based mineralization, as observed by the studies conducted using MG63 pre-osteoblast cells. Also, the expression pattern of osteoblast specific genes suggested the efficacy of chitosan/chondroitin sulfate/nano-bioglass scaffold in significantly enhancing the osteogenic differentiation and maturation of stem cells into functional osteoblasts, as compared to silica containing nano-composite and control scaffolds. These in vitro results are indicative of the performances that the chitosan/chondroitin sulfate/nano-bioglass scaffold would display in vivo, making it the best choice as an alternative scaffold favorable for bone tissue engineering.

References

List of Publications


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