DEVELOPMENT AND EVALUATION OF SCAFFOLDS FOR BONE TISSUE ENGINEERING

THESIS SUBMITTED IN PARTIAL FULFILMENT FOR THE AWARD OF DEGREE

DOCTOR OF PHILOSOPHY

By

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CHAPTER 5

CONCLUSION AND FUTURE PERSPECTIVE

4. CONCLUSIONS & FUTURE PERSPECTIVE

Bone tissue engineering has emerged as a promising alternative approach for repairing and replacing damaged or diseased bone tissue. A critical challenge in this field is the design and development of orous and high mechanical strength, scaffold architecture using suitable biomaterials that possess key properties such as biocompatibility, biodegradability, osteoconductivity, surface characteristics, and the ability to support bone matrix formation. Chitosan derived from chitin and gelatin applied as a collagen alternative is a potential biomaterial blend with several advantageous scaffold properties. However, they alone does not fully meet all the necessary criteria for bone tissue regeneration due to their lack of strength and stability, flexibility, and porosity. To address these limitations, blending Ch/G with different bioceramics and nanoparticles presents an attractive option, as it can enhance the overall performance of the scaffold and support bone tissue regeneration. In addition to material properties, the fabrication of scaffolds with a porous architecture is crucial, as it can closely mimic natural tissue and provide a higher surface area. In this regard, freeze-drying has emerged as the most effective method for producing porous nanocomposite scaffolds to date.

Therefore, the current thesis demonstrates the development and evaluation of three-dimensional Ch/G based porous biomaterials with novel composition and enhanced mechanical strength for bone tissue engineering research and development.

The most encouraging results achieved from this research are summarized as follows:

1. In the first phase of the thesis work, efforts were focused on developing Ch/G/CN nanocomposite scaffolds using freeze drying process. The scaffolds were fabricated by blending solutions of Ch/G in varying compositions (50/50, 25/75 and 75/25 (w/w)) and then adding synthesized copper nanoparticles in different percentages (0.01%, 0.02% and 0.03% (w/v)) into it. The incorporation of CN with Ch/G solution led to significant improvements in various physicochemical, mechanical, and biological properties compared to pure Ch/G scaffolds. Among the different composites, the Ch/G/CN scaffold with a 0.02% CN demonstrated the most promising set of superior properties for tissue engineering applications. Scaffold with 0.03% CN exhibited highest mechanical strength but showed a cytotoxicity effect on cell which was seen in the MTT assay as well.

   The scaffolds were evaluated for morphological (SEM), structural (XRD, FT-IR), mechanical strength, biodegradation, porosity (Liquid displacement method), swelling ratio and cell viability (MTT assay). The scaffold's average pore diameter was found to be 71μm after the addition of copper nanoparticles. In comparison to pure Ch/G scaffold, the

scaffold showed enhanced porosity of 80%, better degradation , swelling index and high mechanical strength of 3.0MPa in case of 0.03% CN scaffold which did not show enhanced metabolic activity in comparison to the original Ch/G scaffold.

Nonetheless, the scaffolds continue to have a strength of 2.3MPa, which is adequate to sustain various forms of tissue regeneration. The ability of skin cells obtained from cell line L979 to adhere to, proliferate, penetrate, and engage in cellular metabolic activity on top of the scaffold has been validated by an *in vitro* cell culture investigation. Consequently, the structure for the created Ch/G/CN scaffolds was chosen for more research.

2. From the previous work we obtained scaffold with enhanced mechanical strength and therefore we wanted to study them further for bone tissue engineering applications. Therefore in this phase, we synthesized nano-hydroxyapatite (nHAP) using the sol-gel method. It was anticipated that the addition of nHAP and CN will enhance the bioactivity, osteogenic, and mechanical properties of the developed Ch/G scaffold, all of which are critical for bone tissue regeneration. Then further it was utilized in different combinations to develop Ch/G/nHAP/CN scaffolds. The Ch/G concentration was fixed at 2% and 8% respectively while nHAP concentration was fixed at 3.5% and the concentration of CN utilized was varied (0.01%,0.02% and 0.03%) as in the previous study.

   The morphological, structural, and physical characteristics of the synthesised nanohydroxyapatite were evaluated via characterization. HRSEM, EDX, FTIR and XRD investigation showed that the produced nano-hydroxyapatite was amorphous in nature which was further confirmed by FTIR and XRD investigation. Furthermore, the SEM and FTIR was performed for the developed scaffolds which showed the porous architecture of the Ch/G/nHAP/CN scaffold developed with average pore size between 121 μm to 146 μm which was very close to Ch/G/nHAP scaffold with 157 μm. It was also improved in comparison to the previous study where the average scaffold pore size was 71 μm only. Then further porosity measurement showed 80-85% porosity while the test scaffolds possessed 87%, this was also like the previous study where we achieved 80% porosity after copper addition. Swelling studies showed that CN-based scaffolds possessed optimum swelling capacity which was required for bone tissue regeneration. 0.01% and 0.02% CN concentrations of CN showed good cell cytocompatibility and physicochemical properties except for mechanical strength which was the highest in the case of 0.03% concentration of CN (3.5MPa) scaffolds. This study proved again that 0.02%CN is beneficial for tissue engineering applications.

Nonetheless, the scaffolds continue to have an average compressive strength of 3.2 MPa, which was higher in comparison to the previous study. Yet the results were considerably greater than that of the Ch/G/CN scaffold (2.3MPa) indicating that it can promote cell penetration into the matrix. The ability of MG63 cells used here showed good cellular metabolic activity which was validated by an *in-vitro* cell culture investigation. As demonstrated by ALP activity, the biomineralization ability of the developed scaffold Ch/G/nHAP/0.02CN also has good osteogenic properties. Consequently, it also states that the combination of nHAP and CN makes an ideal choice for bone tissue engineering applications and therefore its combinatorial effect must be studied further for *in-vivo* applications in bone tissue-related applications. However, the mechanical strength achieved by this combination of materials was adequate to sustain various forms of bone tissue regeneration, we still need to improve the strength for bone tissue-related applications.

3. In this section of the thesis, efforts were made to enhance the Ch/G blend scaffold's porosity, mechanical strength and osteogenic capacity by adding nanobioglass (6 w/w%) to beta tricalcium phosphate in three different ratios (1:0, 0:1 and 1:1), which produced Ch/G/NBG/TCP composite scaffold with different compositions. Four designations for the created composite scaffolds were Ch-G, Ch-G-NBG, Ch-G-TCP and Ch-G-NBG-TCP where Ch-G served as the control. It was observed that the scaffold developed in this study possessed good pore size (220-265μm) which might be due to freeze-drying of the freshly prepared polymeric solution. With the addition of 6% TCP into the scaffold, the developed composite scaffolds have demonstrated decreased average pore diameter (222 to 250μm), and for Ch/G/NBG/TCP (1:1)average pore diameter decreased to (215-265μm). The swelling ratio of Ch-G scaffolds was significantly higher than that of Ch-G-TCP, TCP/NBG scaffolds and Ch-G-NBG scaffolds. This could be due to the reduced surface area as the inorganic material attached over the pore walls couldn’t absorb fluids, unlike polymers. The degradation also decreased from 53-55% for NBG and TCP scaffolds which was lesser in comparison to the Ch/G scaffold (71%) but was better in comparison to the previous study for the Ch/G/nHAP/CN scaffold. The compressive modulus for the Ch/G scaffold improved after the addition of NBG and TCP to 2.7MPa from 2.1MPa. It was observed that after the addition of NBG and TCP, the porosity was almost similar to the previous study around 80% but the compressive strength decreased which showed that adding metallic nanoparticles plays an essential role in enhancing the mechanical strength

of the fabricated scaffold. The developed NBG and TCP integrated Ch/G composite scaffold's cell-supporting properties were observed by MTT assay which showed good cytocompatibility in the case of all the scaffolds yet it was maximum in the case of 1:1 ratio of NBG and TCP. Further ALP activity was evaluated for the developed scaffolds which proved their osteogenic ability and suggested that these scaffolds can be utilized for BTE applications with enhanced degradation and mechanical strength.

4. We already found one successful biomaterial for bone tissue engineering from the subchapter 4.2 (Ch/G/nHAP/0.02%CN) scaffold. But besides the porosity and osteogenic qualities, the designed scaffolds in the previous study (subchapter 4.3) did not attain higher mechanical qualities with NBG and therefore this final study aimed to improve the mechanical strength with the help of Graphene oxide(GO) and Nanobioglass (NBG). They both also play a significant role in bone tissue regeneration. Therefore, an attempt was made to incorporate GO nanoparticles and NBG nanoparticles in Ch/G scaffolds in subchapter 4.4 to increase the mechanical properties of Ch/G scaffolds in this phase of research work. Different batches of Ch/G porous scaffolds with GO and NBG incorporation (Ch-G, Ch-G-NBG, Ch-G-NBG-GO30%, Ch-G-NBG-GO60%, and Ch-G-NBG-GO90%) were made and physicochemical characterization was performed for them. SEM showed porous architecture of scaffold with average pore size between 90-120μm. The scaffolds with different compositions of GO (30% (w/v), 60% (w/v) and 90%(w/v)) and NBG (12%w/v) showed that among the other scaffolds with different percentages of GO, Ch-G-NBG-GO90% exhibited excellent mechanical strength of 8.5MPa which was best until now when compared to all the other previous scaffolds while scaffolds with 60%GO and 30% GO exhibited 7.6MPa and 7.2MPa strength respectively. However, the porosity studies revealed 75-77% porosity for GO-based scaffolds which was approximately the same for all three of them. The swelling index was also reduced in the 90% GO-based scaffold from 4.9 to 4 when compared to the control scaffold. Furthermore, the cell viability of the scaffolds revealed cell attachment and metabolic activity was increased after the addition of NBG into the scaffold, and after adding 30% GO it was approximately, but with the addition of an increased percentage of GO (60%), the cell viability increased slowly again but with 90% GO addition it slightly reduced. But overall the cell metabolic activity was similar in GO-based scaffolds except 90% and this type of pattern we have also observed in Ch/G/nHAP/CN scaffold. The thermal stability of the GO-based scaffold was also determined to know the shelf life of the developed scaffolds in this phase which suggested

that scaffold defunctionalization did not occur here due to the specific interaction between Ch/G/NBG and GO present in different concentrations. It was also proved by the XRD and FTIR studies of the developed scaffold. ALP activity also suggested enhanced osteogenic potential in GO-based scaffolds. Even though the 30% GO composite scaffold somewhat showed higher ALP activity than 60% GO scaffold the difference in activity was not that significant. This suggests that the two composite scaffolds have similar osteogenic potential. Therefore, it was determined that the created composite scaffold Ch/G/NBG containing 60%GO proves to be a good option for bone tissue engineering just like Ch/G/nHAP/0.02CN and they both should be further studied through *in-vivo* studies.

Overall, in this dissertation, novel nanocomposite porous chitosan and gelatin-based scaffolds were developed, showing significant potential as a substrate for bone tissue engineering applications. The scaffold's, mechanical properties, porosity, bioactivity, and osteogenic properties were enhanced by incorporating bioceramics like nanobioglass and nanohydroxyapatite into the matrix, resulting in Ch/G/NBG and Ch/G/nHAP scaffolds that facilitated the recruitment and growth of MG-63cells. The scaffold's mechanical properties and bioactivity were further improved by adding CN and GO nanoparticles, leading to the creation of the Ch/G/NBG/60%GO composite and Ch/G/nHAP/0.02CN composites. In conclusion, the results suggest that the Ch/G blend scaffold could serve as a promising base polymeric scaffold for tissue engineering, particularly in bone tissue regeneration.

Suggested Future Work

The following research directions are recommended for future study using the developed composite scaffolds:

1. Conduct detailed *in-vivo* animal studies to evaluate their suitability for potential future clinical applications.
2. More focus can be given on immunological and molecular aspects of the developed tissue in *in-vivo* studies.
3. Three-dimensional scaffolds with optimized design and structure can be fabricated using advanced techniques such as rapid prototyping.
4. Additionally, future research should focus on developing aqueous-based composite scaffolds to minimize the risk of toxicity associated with organic solvents.

Nano-porous scaffolds possess a nano-architecture that closely mimics the natural extracellular matrix of collagen fibres (50–500 nm). However, fabricating a 3D scaffold with precise geometry and controlled pore size using the lyophilization technique presents challenges. In

this regard, rapid prototyping (RP) and 3D bioprinting can offer a promising alternative, making it worthwhile to explore scaffold fabrication using 3D printing techniques.