Evaluation of bone repair using chitosan-hydroxyapatite biomaterial for Bone tissue engineering

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by

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Chapter 5. Summary and Conclusion

The successful scaffold-based bone regenerative approach had been strongly dependent on the structural and functional performance of the materials utilized for scaffold fabrication.

The most encouraging observations obtained from this research work are summarised below:

In the scaffold material based studies, the combinatorial design strategy was employed for the scaffold fabrication from the chemically synthesised-hydroxyapatite, gelatin and chitosan. Chemically cross-linked scaffolds with multiscale pore size and interconnected porosity by controlled gelate freeze-drying technique. The ‘gelate freeze-drying’ was the method of choice depending on the requirement of the morphological, physiochemical, biological, thermal and mechanical properties of the natural bone extracellular matrix. Therefore, peculiar properties of the GH and CGH scaffold were highlighted in terms of their ability to mimic the natural bone extracellular matrix and cell supporting behaviour. From AFM, the average surface roughness was best optimised in CGH scaffold group of 14.9nm and maximum was recorded by GH (~40nm). Hence, CGH would promote highest proliferation among the scaffold groups, whereas increased cell adhesion would be favoured by GH. The interconnected pores with minimum and maximum pore

diameter of 70µm and 375µm, respectively with optimised porosity of 80% was noted for GH and CGH. The morphology and thickened pore walls provided 3D topography for cell proliferation. The atomic weight of calcium and phosphate in the mineral deposits and crystal spots corresponded to both gelatin and hydroxyapatite in contributing to the crystallinity in the scaffolds with either gelatin, hydroxyapatite or both. The crystallinity based observations were cumulatively supported by XRD, EDX and SAED. After the crystal plane are designated in detail, the peak broadening in the scaffold corresponds to decrease in crystal spots in CH and GH. Minimum reduction in crystalline behaviour was observed in CH. Indicating the role of gelatin in reducing the hydroxyapatite induced crystallinity in the GH, CG and CGH scaffolds.

The degradation to mineralization aspect of all the four scaffolds was studied. With little progress in mineralization, CG was first to degraded completely. Omitting its selection for cell-seeding and subsequent *in vitro* and *in vivo* studies. Also, CH displayed poor degradability but encouraged quick mineral deposition when immersed in SBF for mineralization study. Hence, the CH scaffold geometry become unfit for the defect site implantation because the osteoblast proliferation and expansion after cell seeding on the scaffold is affected by its unbalanced degradation to mineralization ability. GH and CGH displayed prominent degradation and mineralization were considered for cell studies. CGH exhibited innate antibacterial property against both gram positive and negative bacterium with ZOI of 1.4mm and 2.2 mm, respectively. Remaining three of the scaffolds have antibacterial property for either Gram positive and negative but not both as found from the agar-diffusion method.

From the compression studies carried-out in one direction along the diameter, highest stiffness was found for CH, making it brittle and unfit for cell seeding. Chitosan addition reduced the scaffold flexibility over inherent brittle behavior of hydroxyapatite. While,

CG displayed least Load bearing potential. Hence, only GH and CGH scaffold were considered for cell studies with Y of $1.22x10^{8}$ and $6.54x10^{8}$ Pa.

After the thermal studies with Tg at 120°C, GH displayed the scaffold with the highest shelf life. Shelf life of the product did not have any effect on the porosity and mechanical properties of the scaffolds. Overall these found results elucidated, the structure-property relationships in the fabricated GH and CGH scaffolds suitable for cell seeding. The structure stability was determined degradation during the 30 days study degradation, sufficient for tissue regeneration. Longer periods of investigation are currently underway. Overall, evidences supporting the capability of CGH scaffold to be used as a promising substitute at the bone defect site.

In the rabbit osteoblast based *in vitro* studies, the biological performance of the selected humidity-set scaffolds i.e. GH and CGH was evaluated by carrying out separate culture of the rabbit MSCs derived osteoblast and tissue derived osteoblast in the selected scaffolds. The MSCs were identified using battery of cell surface markers before seeding them in the GH and CGH scaffolds. At the Cell concentration of $5x\ 10^{5}$ cells/ml cells were seeded in the scaffolds. From the growth curve the specific growth rate of rM and rT was found to be 3.854 h^{-1} b and 6.058 h^{-1}, respectively. The PDT of rM was found to be double of that of the rT. From the cell cycle analysis, the Go/G1 phase population were selected for seeding in both GH and CGH. The cell-seeded scaffolds were studied for the changes in their properties after seeding with the cells every week till culture day 28. The surface roughness was observed to rise with chitosan inclusion (2.5 to 4.6 in rMHG and rMHCG). Mineral deposits noted from Ca/P was 1.67 for rMHCG. This resembles the natural bone Ca/P of 1.69. The attachment, metabolic activity and proliferation rate was analysed using the proliferation and mineralization assays. Highest DNA and cell viability was observed for rMCGH and rTCGH, respectively. CGH displayed maximum

differentiation supporting ability when seeded with rT over rM as found after ALP and ARS assays. The gene expression levels obtained after this cell in scaffold culture was tested from the expression of the osteogenic markers. Mineralization mainly due to type I collagen were observed at 4 weeks, with collagen concentration of 2.231 µg/100 µl HCl. Non-protein matrix component, Osteocalcin concentration was 0.0056µg/ µl DPBS upto culture day 14. After cell seeding, crystallinity observed to be highest in rTCGH, making it mechanically stable and fit for load bearing implantation. These findings also suggest that rabbit bone tissue derived osteoblast represents readily obtainable and highly proliferative invasive cell source for regenerative therapeutics. Osteoblast cells *in vitro* studies within the synthetic materials as bone substitutes shows that there are specific parameters set for future clinical studies.

In the human cell based *in vitro* studies, the natural process of biomineralization with the freeze-dried chitosan infused gelatin-hydroxyapatite biomaterial was studied in the assessment of its biocompatibility and bioactivity using bone cells of human-origin obtained either from the differentiated bone marrow MSC (hM) or bone tissue (hT). Additional crystalline behaviour proved to be more prominent in CGH as observed from XRD. The chitosan infusion showed improved biomineral activity when seeded with hM. The incorporation of chitosan into GH based composite increased the hM viability and activity over GH with hT. From DSC, highest shelf life was observed for hCH. Ca-P deposition with the increase in culture duration was reflective of the stimulated differentiation ability of hM. In future, the in vivo cell studies over time would reflect the properties of the substrate suitable for bone tissue engineering involving hM. From the *in vitro* experiments allowed to conclude that both GH and CGH scaffolds seemed to possess the osteoconductive property required for colonization of osteoblastic progenitor

cell colonization and their subsequent differentiation into mature osteoblasts. Thus, CGH scaffolds able to act as the 3D framework to guide tissue formation.

From the *In vivo* study, it was observed that in the 12 week of implantation, the cytocompatibility and osteoconductive and osteoinductive properties of rTCGH scaffold. It resulted in bone reconstruction probably by the recruitment, proliferation and finally differentiation of the osteoprogenitor cells.

To conclude, the natural process of bio-mineralization was a success when gelate freeze-dry chitosan infused gelatin-hydroxyapatite scaffold was seeded with the osteoblast from rabbit bone tissue, as assessment of the biocompatibility and bioactivity of the scaffolds directly influences the healing step in bone tissue. Ca/P ratio was closest to that of natural bone in 'CGH'. The porosity, mineralization and degradation properties were also improved. Among all the scaffolds, crystalline behavior was more prominent in CGH. The incorporation of chitosan into GH based composite increased the surface roughness only after cell seeding. Ca-P deposition with the increase in culture duration was reflective of the stimulated differentiation ability when checked for reproducible results with human bone tissue derive osteoblast i.e. hT. In future, the *in vivo* cell studies over time would reflect the properties of the substrate suitable for bone tissue engineering involving rTCGH.

- Scope for further work:

  With the designed and in vitro and in vivo evaluated rTCGH scaffold, following future studies can be carried out:

  A. Scale-up of the culture studies involving 'cell seeded scaffold' in the bioreactor.

  B. Reproducible *in vitro* studies with the human derived bone tissue osteoblast.

C. Clinical trials need to be undertaken for the hTCGH scaffold to decide the therapeutic potential on mass scale.