Chapter 4

Discussion

This study is the first study investigating biocompatibility of chitosan-collagen sponges on growth and differentiation of osteoblasts in long term three-dimensional (3-D) cell cultures of 27 days duration. The 3-D osteoblast culture is a suitable *in vitro* system for studying cell-matrix interactions as the 3-D structure of scaffolds imitates the natural structure of bone cells (Acil et al., 2000). Interconnected pores and 3-D structures are attributed to a large surface area for contact dependent cells such as osteoblasts which attach and grow resulting in a greater growth and differentiation of cells on the 3-D structure than on the two-dimensional surface (Ishaug et al., 1997). The biomaterials should be able to be processed into the 3-D porous structure and biodegradable. Biodegradation products should be non-toxic substances and easily excreted. An excretion of biodegradation products and perfusion of nutrients will be enhanced in porous structures (Ishaug et al., 1997).

A clonal osteoblast cell line has advantages over primary cells in that that their differentiation stages are homogenous and they are sources of cells with consistent phenotypes. It can sustain the expression of a particular trait over extended periods in a continuous culture (Majeska et al., 1980). In contrast, primary cells are usually heterogenous and less susceptible to time-dependent changes that occur in cultures as cells adapt to the *in vitro* environment and grow at different rates (Ankrom et al., 1998). There are many studies using osteoblast cell lines in bone metabolism and biomaterial/cell interactions including, osteoblast-like cell lines from rats, mouse or human osteosarcoma (UMR-106, MC3T3-E1, ROS 17/2.8, SAOS-2, HOS) (Elgendy et al., 1993;Ahmad et al., 1999;Lo et al., 1996;Matsuura et al., 2000).

Mouse calvarial MC3T3-E1 osteoblast cells were chosen for this study because this preosteoblastic cell line undergoes osteoblastic differentiation into mature osteoblasts in 3-D cell cultures as they were reported in many studies of the 3-D cell cultures (Sudo et al., 1983;Wada et al., 1998;Norman et al., 1994;Simon et al., 2002;Isama and Tsuchiya, 2003;Ehara et al., 2003;O'Brien et al., 2005;Tachibana et al., 2005).

Collagen has been widely used as scaffolds in bone tissue engineering but instability of structure and a fast degradation rate of collagenous scaffolds *in vitro* and *in vivo* are shortcomings of the materials (Friess, 1998;Lee et al., 2001). Chitosan is one of another natural polymers which has been fabricated as porous scaffolds for tissue regeneration. Advantageous properties of chitosan are its non-toxicity, biodegradability, osteoconductivity, biocompatibility and availability in various forms (Muzzarelli et al., 1988a;Muzzarelli et al., 1993b;Klokkevold et al., 1996). However, chitosan has poor physical properties which is brittle in a dried state (Oungbho, 1997).

Many investigators (Shahabeddin et al., 1990; Saintigny et al., 1993; Shanmugasundaram et al., 2001) demonstrated that chitosan-collagen substrates could be used as scaffolds for cell cultures of different cell types. Stability of chitosan-collagen scaffolds has proven to be much higher than that of the individual chitosan or collagen (Shanmugasundaram et al., 2001). The results of those previous studies were supported by the findings in this study in that chitosan-collagen sponges were biocompatible and osteoconductive and a supplementation of collagen in chitosan suspension improved porous structures and osteoconductive properties of the scaffolds.

A previous study reported that the chitosan-collagen sponge ratio 1:2 had a homogeneous porous structure that was very efficient at releasing drugs (Oungbho, 1997), which suggests a potential to apply chitosan-collagen 1:2 sponge as a scaffold in bone regeneration. Therefore, the growth and differentiation of osteoblasts on the scaffold and microstructure of the scaffold were investigated in this study to demonstrate the biocompatibility of the composite material and the effects of ratio of supplemented collagen on the properties of chitosan-collagen composite scaffolds. A 3-D cell culture of MC3T3-E1 osteoblast cells was selected as an *in vitro* study model to investigate the biocompatibility and osteoconductivity of the scaffolds.

Cell seeding is a prime step in building *in vitro* tissue constructs. Optimization of cell seeding on 3-D scaffolds requires: (a) high yield, to maximize the utilization of donor cells, (b) high kinetic rate, to minimize the time in suspension culture, and (c) spatially uniform distribution of attached cells, to provide a basis for uniform tissue regeneration (Qi et al., 2004). In addition, an initial seeding of a high number of cells is essential to ensure the growth and differentiation of cells on the construct. This may directly correlate with a promotion of co-operative cell-cell and cell-matrix interactions in a higher matrix production condition of a higher cell density.

Cell seeding methods can be categorized into two methods, static and dynamic cell seeding. A static cell seeding can load a limited number of cells onto the scaffolds. This is because a migration of cells into the internal structures of scaffolds is limited resulting in the local accumulation of cells on the upper and middle portions of the construct. A low seeding efficiency of a static method causes asymmetrical tissue formation on the construct (Wood et al., 2003), while dynamic cell seeding produces an even distribution of cells within the constructs creating a favorable environment for tissue formation (Qi et al., 2004).

However, in this study, a static cell seeding technique was applied because it is a simple technique and the size of scaffolds was small. It was expected that the small size and porous structure of the scaffolds would allow the cells to proliferate into the center of the scaffold and the diffusion of nutrients and gases would not be interfered.

The size of a scaffold is about 3x5 millimeters with a surface area of approximately 5 cm^2. This surface area has been proven to be appropriate for cell seeding at a density of $1x10^5$ cells per scaffold. The surface area and volume of the scaffolds were sufficient for the growth and differentiation of cells on the sponges (Ang et al., 2002;Zhao et al., 2002). Thus, in this study the dimension of scaffolds was suitable for 3-D cell culture of MC3T3-E1 cells, $1x10^5$ cells, in a conventional cell culture system. Additionally, cells were seeded on the moist sponges which were neutralized and incubated in a complete culture medium supplemented with a 10% fetal bovine serum. This procedure would allow for the sponges to be saturated with a culture medium at pH 7.4 and ensure a maximum swelling of the sponges for cell seeding. Thus, an adverse effect of hydrophobic property of chitosan on the efficiency of cell seeding was minimized.

The results of SEM suggest that collagen and 1:1 and 1:2 chitosan-collagen sponges have the 3-D porous structures which serve to mimic the condition of bone structure. The chitosan-collagen sponges with relatively uniform pore size should be applicable in bony tissues because the range of the pore sizes (111.34-262.29 μm) attained in the present study (Table 3) has been demonstrated to be optimal pore size for bone tissue regeneration (Hutmacher, 2000;Hench, 1998). The result is consistent with the previous study emphasizing that the pore sizes of collagen-glycosaminoglycan scaffolds supporting adhesion and growth of MC3T3-E1 cells ranging from 95.9–150.5 μm (O'Brien et al., 2005). In contrast to this study, chitosan sponges had a

collapsed internal structure. The evidence suggests that chitosan-collagen composites stabilize the internal folds of the sponges improving the microstructure of chitosan-collagen sponges, as compared to chitosan and collagen sponges.

The average size of osteoblasts is 30 μm in diameter (Ishaug-Riley et al., 1998;Kose et al., 2003b), and osteoblasts need to establish cell-to-cell contact to create and maintain the differentiation stage. An average pore size should be at least 3 times (>100 μm) as large as a cell so that a single cell can establish contact with the others (Kose et al., 2003a). Scaffolds with a range of pore size between 200-400 μm are preferable for supporting growth and differentiation of osteoblasts (Boyan et al., 1996). Thus, osteoblasts are expected to easily migrate into the porous structure of collagen and 1:1 and 1:2 chitosan-collagen sponges and be able to create a favorable arrangement of intercellular contact within the internal pores of sponges (Figure 13).

The factors influencing pore size and structure of scaffolds are multi-factorial, including crosslinking agents, freezing temperature and cooling rate (Mao et al., 2003). In this study chitosan, collagen and 1:1 and 1:2 chitosan-collagen sponges were crosslinked with 0.05% glutaraldehyde (GA). Chitosan and GA cross-linking to collagen are essential for improving the scaffold biostability. The presence of chitosan significantly improves biostability of the chitosan-collagen scaffold under the GA treatment, where chitosan might function as a cross-linking bridge (Ma et al., 2003). In this study an incorporation of chitosan into collagen increases the porosity of the chitosan-collagen sponge (Figure 12).

Freezing conditions are an important factor controlling microstructure and rigidity of the whole structure. Mean pore diameters can be controlled within a range of 40-250 μm by varying the freezing temperature and the cooling rate. Temperature gradient controls the growth of ice crystals, increasing pore diameter. A formation of porous microstructure is dependent on a formation of ice crystals and pore diameter is increased significantly from the edge towards the center of the sample (Madihally and Matthew, 1999). To minimize effects of the temperature gradient on the non-homogenous formation of ice crystals on the large surface of the matrices, the suspensions were poured into cylindrical syringes (small diameter) with a diameter of 5 mm for the freeze-drying process. The suspension cooling rate and freezing temperature were constantly controlled. It was expected that these conditions would contribute to the homogenous pore structure of the sponges. O'Brien and coworkers

(O'Brien et al., 2004) reported that a production of scaffolds using the constant cooling rate technique by controlling the suspension freezing rate produced a homogeneous pore structure.

The SEM of MC3T3-E1 osteoblast-like cells on culture-day 9 demonstrates changing of cell morphology from a fibroblast-like cell to a polygonal appearance (Figure 13) suggesting the biocompatibility of the surface of scaffolds in supporting attachment and growth of osteoblasts. The cells attach and spread their cytoplasmic process and had intercellular contact with other cells on the surface of all sponges and proliferate into pore structures and grew in a multi-layer fashion on all sponges at later time points (Figure 14-16). This evidence suggests that the porous structure of the sponges supports the 3-D growth of cells and increases the surface area for cell attachment and intercellular communication. These results support previous reports that the 3-D structures of scaffolds mimic the natural structure of bone and promote growth and differentiation of osteoblasts enabling terminal differentiation of osteoblasts *in vitro* (Laurencin et al., 1996;Ishaug-Riley et al., 1998;Ishaug et al., 1997;Ignatius and Claes, 1996;Morrison et al., 1995). However, growth of cells into the internal structure of sponges is limited by static seeding of a small volume of cell suspension on to the sponges. A thorough investigation of cell attachment and infiltration within the structure of the scaffolds is needed to be researched in a further study.

The proliferation rate of cells can be measured directly and indirectly using various techniques such as the measuring of metabolic activity of cells using MTT or WST-1 assays and measuring of DNA content (Zund et al., 1999;Mueller et al., 1999;Peskin and Winterbourn, 2000;Ishaug et al., 1997;Tachibana et al., 2002). In this study, MTT assay was selected as a method of detecting the proliferative activity of cells, because the MTT assay has been demonstrated as a sensitive, precise, convenient, rapid and economical test method by various studies (Hongo and Igarashi, 1990;Wan et al., 1994;Heo et al., 1990). MTT assay was originally described by Mosmann as a useful method for the measurement of *in vitro* cytotoxicity and cell proliferation in relation to mitochondrial metabolism (Mosmann, 1983). A major advantage of this colorimetric assay is the lack of any radioisotope. It was successfully applied to quantitatively assess the viability of cells growing on the 3-D scaffolds, as it is demonstrated in previous studies. For example, PLGA film (5x3 mm) with pore size 100-150 μm (Ryu et al., In press) polyesterurethane foam (20×5×1 mm) with pore size

150-200 μm (Yang et al., 2003) , a PLLA scaffold (7x1.48mm) (Wan et al., 2003), and a keratin sponge (10x2 mm) with pore size 100 μm (Tachibana et al., 2002) were tested. These studies showed excellent results without any limitations when MTT assay was used in 3-D cell culture. An alternative method for detecting numbers of cells on 3-D structure is determining amount of DNA contents (Tachibana et al., 2002).

Cell growth in proliferative and plateau phases were demonstrated by a typical pattern of growth curve in all of the groups of study. An active proliferation phase was found during the first two weeks and cell growth was sustained in a late cell culture stage. It is postulated that the accumulation, maturation and mineralization of the ECM decrease growth rate while they increase differentiation activity of cells (Owen et al., 1990;Malaval et al., 1994).

During the proliferation phase, cells on the chitosan sponge had the highest growth rate and reached plateau phase (on day 15) earlier than the other groups. Growth rate among the other groups were not significantly different throughout the cell culture (Figure 8). This might be because positive charges of chitosan attracted the negative charges of cell surfaces creating a tight bond between chitosan and cells and this condition facilitates growth of cells on chitosan surface (Ma et al., 2001). This finding supports a previous report that chitosan sponges have good cellular adaptability and support cell proliferation (Lahiji et al., 2000). Fakhry and coworkers (Fakhry et al., 2004) reported that chitosan preferentially supports the initial attachment and spreading of osteoblasts at 24 hours after cell seeding.

In the plateau phase, the growth of cells in all groups were at similar levels. It was suggested that although collagen and an incorporation of chitosan in collagen sponges did not support growth of cells in a proliferative phase as highly as chitosan sponges. Collagen and chitosan-collagen sponges were able to support the growth of cells in a long term cell culture in a similar manner to chitosan sponges. This might be because an addition of chitosan into collagen sponges provides much more amino groups for cell adhesion and the composition of chitosan-collagen sponge is closer to the native tissue (Wang et al., 2003a). A larger surface area on the porous structure of collagen and chitosan-collagen sponges might be another factor contributing to the patterns of cell growth and differentiation in this study. It could be seen that chitosan stimulates the proliferation of MC3T3-E1 in the proliferative phase and collagen and

chitosan-collagen matrices are able to support growth of cells into a terminal stage of differentiation (Figures 8).

Osteoblastic differentiation of MC3T3-E1 cell was demonstrated by expressions of ALP, as a marker of the differentiation in an early stage, and a deposit of calcium in ECM or mineralization of ECM, as a marker of the differentiation in a terminal stage (Aubin and Liu, 1996;Franceschi and Iyer, 1992). In analytical processes determining ALP activity and calcium content in this study, cell lysis and demineralization process were performed. These processes ensure that ALP activity on surface of cell membrane and calcium deposition in ECM of all cells within the scaffolds are measured in the analysis. Therefore, the levels of ALP activity and calcium concentrations in this culture system reliably represent the activities of cells in the study.

A significant increase in the ALP levels during the second week suggests that the cells shifted to higher differentiation stages (Aubin and Liu, 1996). This result corresponds to a previous report that after the cultures underwent growth arrest owing to the attainment of confluence, ALP activity and mineralization of extracellular collagenous matrix would be expressed (Quarles et al., 1992) (Figure 8 and 9).

A reverse relationship between proliferation and differentiation stages of cells are clearly demonstrated in Figures 8 and 9. It can be seen that during the first 15 culture-days, cells in the chitosan group had the highest proliferation rate while the ALP activity was in the lowest level compared to the other groups. These results suggested that MC3T3-E1 in the proliferative phase underwent DNA synthesis and cell division resulting in a downregulation of osteoblastic differentiation. Conversely, an upregulation of osteoblastic differentiation during the plateau phase of cell growth was clearly shown on days 18–27. On days 18–27, levels of ALP activity in all groups were increased to the highest levels on day 27, while cell proliferation was in the plateau phase (Figures 8 and 9). These findings emphasize a previous study's results stating that the proliferation rate of cells in the confluence state was downregulated while an expression of the osteogenic phenotype, ALP, was increased (Sudo et al., 1983).

The most advances in the osteoblastic differentiation stage of MC3T3-E1 cells were found in the collagen group, where the highest level of ALP activity and calcium content were expressed (Aubin and Liu, 1996). This might be because osteoblasts have specific affinity to collagen fibrils of collagen scaffolds and this osteogenic effect was further enhanced by the stimulating effects of 3-D structure of collagen matrix (Casser-

bette et al., 1990). A similarity of collagen matrices to collagen fibrils in ECM of bone might be another factor that further enhanced osteoblastic differentiation (Aronow et al., 1990; Owen et al., 1990;Lynch et al., 1995;Masi et al., 2000;O'Brien et al., 2005). Positive effects of collagen on osteoblastic differentiation and mineralization of the matrix were also shown in chitosan-collagen scaffold groups. Levels of ALP activity of cells on chitosan-collagen sponges were higher than chitosan sponges (Figure 9). This study supports the findings of a previous study by Oungbho which indicates that collagen has enhanced the osteoconductive property and porous structure of chitosan (Oungbho, 1997).

A lower level of calcium contents in chitosan-collagen sponge groups compared to collagen sponge group found in this study (Figure 10) was in consistent with previous studies. It was found that the addition of glycosaminoglycan (similar structure to chitosan) to collagen sponges inhibited or decreased mineral deposition (Hunter et al., 1985; Tenenbaum and Hunter, 1987). It might be because chitosan and collagen formed a polyanion-polycation complex that stabilizes the triple helix structure of collagen (Taravel and Domard, 1995) and this complex is different from those of collagen and chitosan alone (Taravel and Domard, 1993) resulting in decreasing the degradation of collagen in the culture system and probably decreases in the calcium deposition. A changing of triple helix structure might interfere with the mineralization process in which the configuration of the a triple helix facilitates a heterogenous pattern of mineralization found in the mineralization process of the bone matrix (Ten Cate, 1994). The results indicated that collagen was the optimal matrix in promoting mineralization of ECM in the osteoblast cell culture.

This study demonstrated the chitosan-collagen group, particularly chitosan-collagen 1:2 sponge and collagen sponge groups were able to support growth and differentiation of osteoblasts *in vitro*. However, to develop these scaffolds to be bioactive scaffolds in skeletal defects, behaviors of osteoblasts on structures and especially physical properties of the scaffolds are needed to be futher investigated.

Limitations of the study and suggested further investigations are studies of the distribution and depth of the penetration of cells on the sponges, demonstration of physical image of mineralization of the matrix and expression of other biochemical marker of mature osteoblasts and physical and mechanical properties of the sponges.

In order to demonstrate the efficiency of cell seeding and growth of cells within the structure of the scaffolds, investigations can be performed by embedded scaffolds in paraffin and stained by hematoxylin and eosin to examine distribution of cells under a light microscope (Ishaug et al., 1997;Lee et al., 2000).

To reciprocate the evidence demonstrating that cells differentiate into mature osteoblasts besides detection of calcium contents, other markers of late osteoblastic differentiation stage such as the expression of osteocalcin should be detected (Aubin and Liu, 1996). In this study, if a physical image of mineralization of ECM were demonstrated, it would confirm biochemical results of calcium contents and would increase the validity of the results indicating progress of osteoblastic differentiation into mature osteoblasts. In order to demonstrate mineralization of ECM accumulated within the scaffolds, the paraffin embedded section of the scaffold can undergo von Kossa staining, in which matrix mineralization will be demonstrated as black area and mineralized areas can be measured using computer image software (Kolbeck et al., 2003).

As it is well known that physical and mechanical properties of scaffolds play significant roles in determining applicability of the scaffolds in tissue engineering of each specific area. Scaffolds for bone tissue engineering must be able to withstand compressive force of functional loading while supporting growth and differentiation of cells on the structure (Butler et al., 2000;Sikavitsas et al., 2001), therefore the physical properties of scaffolds such as porosity, pore size, strength, degradation rate and swelling rate should be fully tested in further study.