CHAPTER 5

DISCUSSION

The use of animal models to investigate the potential of biomaterials for bone regeneration is well documented. A study done by Neyt *et al.* back in 1998 already mentioned that rabbits are one of the most commonly used animals in studies that involve the musculoskeletal system. In this study, rabbit was the chosen animal model for several reasons. This animal model offered the advantage of easy surgical access compared to rats. Hollinger and Kleinschmidt (1990) encountered not only difficulties in accessing the surgical site of the mandibular defect but also the implanted materials had the tendency to fall into the fascial spaces when using rats as animal models. In addition, rabbits are inexpensive and easy to maintain compared to larger animals such as dogs or goats. Further selection criteria for choosing rabbit in this study include its' life span which is compatible with the period of study, animal availability, acceptability to society, tolerance to captivity and ease of housing.

Careful considerations of certain factors including the sufficient quantity of PHA, time frame, the maintenance of the animals have to be taken into account since we are dealing with live animals in this study. As with most studies, a higher number of replicates would produce better statistical results. In this study, the sample size was limited to three replicates per time interval (with three controls). It was easier to maintain a consistent base-line (i.e. start conditions) with a smaller and yet suitable quantity of replicates at the right time interval for e.g. the weight of the rabbits. Similarly, rabbits were used with two replicates for each time interval of 3-, 6-, 9-, 12- till 68- weeks in a study done by Suuronen (1992) to investigate the strength retention of self-reinforced poly-L-lactic acid (SR-PLLA) plates and screws *in vivo*. In addition, Thomaidis *et al*. (2008) have reported that 10-week healing period is long enough for bone cells to proliferate into the defect of the animal.

A critical size defect refers to an intraosseous defect that will not heal by bone formation during the lifetime of the animal (Hollinger and Kleinschmidt, 1990). Various sites of critical size defects in rabbit models have been described. These include the cranial cavitational defect (Blum *et al.*, 2003; Dean *et al.*, 2005) and long-bone segmental defect (Hedberg *et al.*,

2005a, 2005b). However, all these models do not procure the oral and maxillofacial environment which is the concern in this study. The unique masticatory stresses and cell populations during bone healing in this region could be observed in the defect at the mandibular site.

As mentioned in the results, the mandibular incisors in three out of twelve rabbits were deformed and lengthened. Lennox (2008) commented that rabbits are species which commonly hide clinical signs of illness and hence the dental abnormalities could only be seen when the mandible was harvested during euthanasia. It was observed in this study that there were no unusual signs of the animals before euthanasia other than those mentioned in the results. According to Lennox (2008), dental diseases in rabbits can be generally divided into congenital or acquired diseases. Nutritional deficiencies could be the cause of abnormalities observed postoperatively. Surgical trauma interfered with normal eruption of or wearing of continuously growing teeth, resulting in the rabbits not being able to masticate with their incisor teeth normally. Nevertheless, there was no evidence indicating any clinical disease signs for the cheek teeth. It was reported that rabbits were still able to eat normally

without the incisors teeth because food is manipulated by the lips and tongue into the oral cavity for the crushing by the cheek teeth (Lennox, 2008).

The sterilization methods of PHA must also be considered as it would affect the outcome in the results of this study. As mentioned earlier, PHA in this study have been sterilized using ethylene oxide as previous study done by Williams and Martin (2002) noted that ethylene oxide did not cause any significant changes to the physical and chemical properties of the polymers. Besides that, beta- and gamma- rays can also be used to sterilize PHA. However, Suuronen (1992) pointed out that beta- and gamma- rays can degrade the macromolecules of polylactic acid and polyglycolic acid to a certain extent depending on the form of the sample.

All biomaterials must meet certain criteria and regulatory requirements. The PHA membrane produced in this study is a medium-chain-length PHA. Generally, these biomaterials are elastomers with a low degree of crystallinity, a low glass transition temperature and a low melting point (de Koning, 1995) which makes it physically suitable as a membrane for bone grafting purposes compared to its other derivatives. In terms of guided bone regeneration, this bioabsorbable membrane was able to prevent soft tissue invasion into the bone

defects created. Another advantage of using PHA from SPKO is that the PHA is amenable to modification after isolation because of its long side-chain fatty acids and attached functional groups. The presence of double bonds in some fatty acids results in unsaturated monomers that provide sites for chemical modification of the mcl-PHA produced. This aspect is discussed as it is important to take note that the raw ingredients (palm kernel oil) used to produce mcl-PHA in this study is easily obtained. Furthermore, the mcl-PHA may be modified to meet the criteria of better tissue engineering material. A study done by Li *et al*. (2005) stated that the surface modification of poly (3-hydroxybutyrate-*co*-3-3hydroxyhexanoate) (PHBHHx), a new member of the PHA family, has better osteoblast cell response and for application in bone-tissue engineering compared to poly (3-hydroxybutyrate) (PHB). The criteria of better tissue engineering material are; the material should be biocompatible, it should be able to support cell growth, it should have a surface which promotes cell adhesion, it must be able to act as a guide or an organizer to the cells in a desired order, it should have the tendency to allow ingrowth of a significant number of cells and lastly, it should be able to degrade once it has fulfilled its purpose in generating new tissues (Williams *et al.*, 1999).

Since mcl-PHA may be a good candidate for tissue scaffoldings, it is necessary to discuss its future outlook in terms of its production. So far, only the scl-PHAs have been commercially produced (up to 500 tons per year) by manufacturer Monsanto (Kellerhals *et al.*, 2000). Hence, the recent challenge will be to produce mcl-PHA in large scale due to the fact that it is very expensive to produce this polymer in bulk amount compared to synthetic plastics. The production of PHA in this study was derived from the preliminary studies of the production of mcl-PHA using renewable and cost-effective substrates e.g. SPKO and its major free fatty acids reported by Tan *et al.* (1997). Various factors that will affect the production of this material have been studied. For example, Annuar *et al.* (2007) have reported that fed-batch fermentation produces a higher mcl-PHA yield compared to batch fermentation. Besides that, the concentration of SPKO in the aqueous medium and the amount of ammonium as the growth limiting substrate were taken into consideration and it was reported that higher concentrations of SPKO can significantly reduced the production of mcl-PHA. Another study done by Alias *et al.* (2005) which aimed at obtaining bacterial strains which could directly utilize palm oil as the carbon source for growth and PHA production without having to saponify the

oil, which would mean a reduction in the number of steps and cost in the production process for bacterial PHA.

Two sample t-tests for a difference in mean can be either unpaired or paired. The unpaired t-test, also known as Student's t-test, is used when two separate independent and identically distributed samples are obtained, one from each of the two populations being compared. On the other hand, paired t-tests typically consist of samples of similar units that have been tested twice or study subjects who have been measured at two time points - usually the measurement of before and after a treatment intervention. Both unpaired t-test and paired t-test would assume that analyzed data is from a normal distribution. In this study, the unpaired Student's t-test was performed with a confidence level of 95% ($p < 0.05$).

The unpaired t-test was performed as opposed to paired t-test because there were two independent groups i.e. the average mean new bone volume for PHA group and the control group. The sample size from these two groups is equal and they are from two different sites i.e. the left mandible or the right mandible.

The histological and histomorphometric findings in this study demonstrated that PHA possesses both biocompatible and osteoconductive properties. Microscopic examination of the PHA grafted sites harvested at different healing time intervals did not show any adverse host tissue response. The host bone surrounding the critical sized defect was vital. The grafted PHA remained inert throughout the experimental phase, and did not evoke any chronic inflammatory cell reaction or foreign body giant cell granuloma formation within the osseous environment. This observation is crucial because it indirectly indicates that the process of PHA biosynthesis as performed in this study where *Pseudomonas putida* was utilized for bacterial fermentation of saponified palm kernel oil, palm oil and their fatty acids as a carbon source did not yield any pyrogen or endotoxin which would contraindicate this method of PHA production for bone grafting purposes. It also suggests that PHA and its degradation by-products did not incite any adverse tissue reaction in bone. An early study done by Ong *et al.* (2005) showed that PHA demonstrated higher compatibility compared to polylactic acid (PLA) in the subcutaneous location in a hamster model. Galego *et al.* (2000) and Luklinska and Schluckwerder (2003) have shown that materials derived from PHA polymers have

biocompatibility and osteoconductive behaviour which is suitable for bone implant or bone substitute.

Osteogenesis was far more advanced in the PHA groups than in the control groups. The significantly higher mean bone volume percentages score at the PHA grafted site supports this. In addition, there is a positive correlation between the extent of bone formation within the PHA defect and time. From the results obtained, the mean new bone volume for PHA groups gradually increased with increasing time interval. This pattern could have arisen due to the duration of time the PHA film was left inserted in the animal. It is worthy of note that the time interval is one of the manipulating variables in this study. The longer the time interval permits, the higher yield of the new bone formed i.e. the average of the new bone volume. It could also be seen certainly that the average mean new bone volume for PHA group for every time interval is higher than the average mean new bone volume for negative control respectively (Figure 4.9). However, the overall new bone yield in this study is relatively low compared to studies done by Doyle *et al.* (1991) and Luklinska and Schluckwerder (2003). This could be due to the surface properties of the PHA used or the fabrication of the PHA used in this study which is a

rectangular membranous film of size 19 mm × 27 mm.

The biodegradability of PHA is an important issue in relation to its applicability as bone-forming medical devices since the polymer will disappear as bone tissues regenerate. The biodegradation of PHA can be influenced by various factors such as the polymer compositions, which include the surface area, its membranous shape and thickness of the film, surface texture, porosity and crystallinity, site of implantation, the presence of blood, interstitial fluids and multiple cell types in various activation states. In the current study, at 12 weeks PHA residual grafting material was still evident. PHAs may have longer degradation time *in vivo* and the implants are likely to undergo a progressive change rather than an abrupt change (Williams and Martin, 2002). This might be an advantage in tissue regeneration applications where a sudden loss of a mechanical property is undesirable for the material is still needed to promote maximum osteogenesis. This is supported by the study done by Qu *et al.* (2006) where the PHA derivatives implanted into the back of rabbits were still present after six months although degradation have taken place. Besides that, a study done by Lim *et al.* (2005) which utilized the similar mcl-PHA in this study, has reported that the material buried in mangrove soils and forest soils

indicated signs of degradation in the soils after 112 days. The physical changes of mcl-PHA (from a transparent and elastomeric film to an opaque and brittle film) may suggest that the process of biodegradation might have taken place. Modifying the PHA chemically has been suggested as one method to reduce degradability times. This can be done by several techniques such as trans-esterification, graft polymerization or effective crosslinking by gamma-irradiation (Chodak, 2002).