CHAPTER II
LITERATURE SURVEY

2.1 Electrospun Fibers for Tissue Engineering

Tissue engineering approaches are currently being developed to successfully repair and restore the function of damaged or diseased tissues. The basic principle involves the use of an appropriate cell source and a biocompatible and biodegradable scaffold to produce a construct that structurally and functionally mimic the targeted tissue. An additional challenge is that most tissues and organs are multiphasic in nature and contain multiple cell types. Thus, an ideal biomaterial scaffold should be capable of supporting multilineage cell types. To date, few attempts have been made to engineer tissues consisting of multiple cell types by using electrospinning process which was originally developed to produce ultrafine polymeric fibers, and has recently been used as a novel technique to synthesize scaffolds that mimic the architecture and mechanical properties of the extracellular matrix (ECM) of native tissues (Li *et al.*, 2005).

It may now be possible to construct a truly ''biomimicking'' fibrous scaffoldings for tissue engineering. Jamil *et al.* (2003) used electrospun collagen type II fibers to form a variety of composite structures for engineering the articular cartilage. One of the attractive aspects of the electrospinning process is that scaffolds of various shapes and sizes can be constructed while, at the same time, provides precise control over fiber orientation, composition, and dimension. Complex constructs can be fabricated to closely replicate the structural and chemical composition of the native articular cartilage.

Xu *et al.* (2003) suggest that synthetic aligned poly(l-lactide-co-ε caprolactone) [P(LLA-CL)] (75:25) copolymeric electrospun nanofibrous scaffold combines with the advantages of synthetic biodegradable polymers, nanometer-scale (500 nm) dimension mimicking the natural ECM and a defined architecture replicating the in vivo-like vascular structure (the circumferential orientation of cells and fibrils found in the medial layer of a native artery), may represent an ideal tissue engineering scaffold, especially for blood vessel engineering. They investigated the

interaction between this scaffold with human coronary artery smooth muscle cells (SMCs) via MTS assay, phase contrast light microscopy, scanning electron microscopy, immunohistology assay and laser scanning confocal microscopy which separately demonstrated that SMCs attached and migrated along the axis of the aligned nanofibers and expressed a spindle-like contractile phenotype. The distribution and organization of smooth muscle cytoskeleton proteins inside SMCs were parallel to the direction of the nanofibers and the adhesion and proliferation rate of SMCs on the aligned nanofibrous scaffold was significantly improved than on the polymer films.

Yoshimoto *et al.* (2002) described that microporous, non-woven PCL mat made by electrospinning is a potential candidate scaffold for bone tissue engineering. Mesenchymal stem cells (MSCs) derived from the bone marrow of neonatal rats were cultured, expanded and seeded on electrospun PCL scaffolds with osteogenic supplements under dynamic culture conditions for up to 4 weeks. The results were demonstrated by scanning electron microscopy (SEM), histological and immunohistochemical examinations. They performed that penetration of cells and abundant extracellular matrix were observed in the cell-polymer constructs after 1 week, which maintained the size and shape of the original scaffolds during cultivation period. In addition, SEM showed that mineralization and type I collagen were observed and surfaces of the cell-polymer constructs were covered with cell multilayers at 4 weeks.

A novel poly(D,L-lactide-*co*-glycolide) (PLGA) electrospun structure ranging from 500 to 800 nm in diameters which featured a morphologic similarity to the extracellular matrix (ECM) of a natural tissue. A unique architecture, which acts to support and guide cell growth for tissue-engineering applications, had been described by Li *et al.* (2002). PLGA nanofibers are characterized by a wide range of pore diameter distribution, high porosity, and effective mechanical properties. Such a structure meets the essential design criteria of an ideal engineered scaffold and being capable of supporting cell attachment and proliferation. Cells seeded on this structure tend to maintain phenotypic shape and guided growth according to nanofiber orientation.

Jeshke *et al.* (2002) demonstrated that mimicking the physiological adhesion process of chondrocytes to the extracellular matrix is expected to improve cell adhesion of in vitro cultured chondrocytes. Their approach involves coating synthetic scaffolds with tailor-made, cyclic RGD-peptides, which bind to specific integrin receptors on the cell surface. They investigated the expression pattern of integrins on the cell surface of chondrocytes and their capability to specifically bind to RGD-peptide-coated materials in the course of monolayer cultivation. Human chondrocytes expressed integrins during a cultivation period of 20 weeks. Receptors proved to be functionally active as human and pig chondrocytes attached to RGD-coated surfaces. A competition assay with soluble RGD-peptide revealed binding specificity to the RGD-entity. Chondrocyte morphology changed with increasing amounts of cyclic RGD-peptides on the surface.

Skeletal muscle tissue engineering represents an attractive approach to overcome problems associated with autologous transfer of muscle tissue and provides a valid alternative in muscle regeneration enhancement. Riboldi *et al.* (2005) investigate the suitability of electrospun DegraPol® membranes as scaffolds for skeletal muscle tissue engineering. Scaffolds were characterized with reference to their morphological, degradative and mechanical properties. The membranes exhibited absence of toxic residues and satisfactory mechanical properties (linear elastic behavior up to 10% deformation, E modulus in the order of magnitude of MPa). Subsequently, cell viability, adhesion and differentiation on coated and uncoated DegraPol® matrices were investigated using cells (C2C12 and L6) and primary human satellite cells (HSCs) which adhered, proliferated and fused on differently coated electrospun membranes. By positive staining for myosin, heavy chain expression indicated that differentiation of C2C12 multinucleated cells occurred within the porous elastomeric substrate.

Li *et al.* (2005) tested a three-dimensional nanofibrous scaffold fabricated from poly(ε-caprolactone) (PCL) for its ability to support and maintain multilineage differentiation of bone marrow-derived human mesenchymal stem cells (hMSCs) in vitro. hMSCs were seeded onto pre-fabricated electrospun nanofibrous scaffolds, and were induced to differentiate along adipogenic, chondrogenic, or osteogenic lineages by culturing in specific differentiation media. Histological and scanning electron

microscopy observations, gene expression analysis, and immunohistochemical detection of lineage-specific marker molecules confirmed the formation of three-dimensional constructs containing cells differentiated into the specified cell types. These results suggest that the PCL-based nanofibrous scaffold is a promising candidate scaffold for cell-based, multiphasic tissue engineering.

Fujihara *et al.* (2004) studied the new type of guided bone regeneration (GBR) membranes which were fabricated by polycaprolactone (PCL)/$CaCO_3$ composite electrospun nanofibers with two different PCL to calcium carbonate ($CaCO_3$) ratios (PCL:$CaCO_3$=75:25 w/w and 25:75 w/w). In order to achieve mechanical stability of GBR membranes, composite nano-fibers were spun on PCL nano-fibrous membranes which has high tensile strength, i.e., the membranes consist of two layers of functional layer (PCL/$CaCO_3$) and mechanical support layer (PCL). Osteoblast attachment and proliferation of GBR membrane (A) PCL:$CaCO_3$ 75:25 w/w + PCL and (B) $CaCO_3$ = 25:75 w/w + PCL were discussed by MTS assay and scanning electron microscope (SEM) observation. As a result, absorbance intensity of GBR membrane (A) and tissue culture polystyrene (TCPS) increased during 5 days seeding time. In contrast, although absorbance intensity of GBR membrane (B) also increased, its value was lower than membrane (A). SEM observation showed that no significant difference in osteoblast attachment manner was seen on GBR membrane (A) and (B). Because of good cell attachment manner, there is a potential to utilize PCL/$CaCO_3$ composite nano-fibers to GBR membranes.

Kenawy *et al.* (2002) fabricated electrospun poly(ethylene-co-vinyl alcohol) or EVOH mats with the diameters of the fibers being about 0.2–8.0 mm. These mats have been shown to support the culturing of smooth muscle cells and fibroblasts. Even EVOH solutions are not stable at room temperature and eventually the polymer precipitates after several hours, electrospinning is extensive and rapid, allowing coverage of fibers on various substrates prior to precipitation.

Luu *et al.* (2003) presented the first successful demonstration of plasmid DNA incorporation into a polymer scaffold using electrospinning for therapeutic application in gene delivery. Synthetic poly(lactide-co-glycolide) (PLGA) random copolymer /DNA and poly(D,L-lactide)–poly(ethylene glycol) (PLA–PEG) block copolymer/DNA composite scaffolds is investigated. Release of plasmid DNA from

the scaffolds was sustained over a 20-day study period, with maximum release occurring at ~2 h. Cumulative release profiles indicated amounts released were approximately 68–80% of the initially loaded DNA. Variations in the PLGA to PLA–PEG block copolymer ratio vastly affected the overall structural morphology, as well as both the rate and efficiency of DNA release. Results indicated that DNA released directly from these electrospun scaffolds was indeed intact, capable of cellular transfection, and successfully encoded the protein β-galactosidase. When tested under tensile loads, the electrospun polymer/DNA composite scaffolds exhibited tensile moduli of ~35 MPa, with ~45% strain initially which were approximate those of skin and cartilage.

2.2 Polyhydroxyalkanoates (PHAs)

2.2.1 Introduction

Polyhydroxyalkanoate (PHAs) is biodegradable and biocompatible. Both properties can best be achieved by production in bacteria, thus, guaranteeing complete stereospecifity (all chiral carbon atoms in the backbone are in the R(-) configuration), which is essential for their biodegradability and biocompatibility.

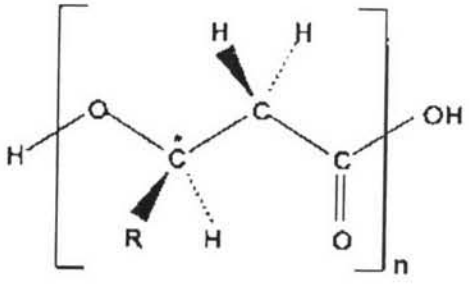

Figure 2.1 Chemical structure of poly(3-hydroxyalkanoate). All monomers have one chiral center (*) in the R position. Poly(3-hydroxybutyrate): R=CH3; poly(3-hydroxyvalerate): R=C2H5.

To date, PHA monomer with straight, branched, saturated, unsaturated, and also aromatic monomers were found. Despite the excitement of

more than 90 different constituents of biosynthetic PHA, it should be pointed out that the commercial exploitation of this variety remains limited, since, at present, only very few PHA are available in sufficient amounts to allow the evaluation of the physical, chemical and biological properties of these polyesters

The length of the side chain and its functional group considerable influence the properties of the bioplastic such as melting point, glass transition temperature and crystallinity (stiffness/flexibility). Also the average molecular weight and the molecular weight distribution are dependent on the carbon source. Some typical glass transition temperature of PHAs has been summarized in Table 2.1. The plastic deformation of polymers listed in Table 2.1 is interesting because it occurs at temperatures of -100°C or lower, which is far below the Tg of most of the polymers.

Table 2.1 Thermal properties of some biosynthesized polymers (3HB, 3-hydroxybutyrate; 3HV, 3-hydroxyvalerate; 4HB, 4-hydroxybutyrate

Polymer	Melting temperature, Tm (°C)	Glass-transition temperature, Tg (°C)
P(3HB)	180	4
P(3HB-co-71%3HV)	83	-13
P(4HB)	53	-48
Polyacrylate	-	-106
Polypropylene	176	-10
Polystyrene	240	100

2.2.2 PHB

The first PHA, poly(3-hydroxybutyrate) (PHB) was discovered in *Bacillus megaterium* by the French scientist Lemoigne in 1926. This bacterium accumulated intracellularly a homopolymer (see Figure 2.2) that consisted of 3-

hydroxybutyric acids that were linked through ester bonds between the 3-hydroxy group and the carboxylic group of the next monomer. Today, PHAs are separated into three classes: Short chain length PHA (sclPHA), Medium chain length PHA (mclPHA, and long chain length PHA (lclPHA).

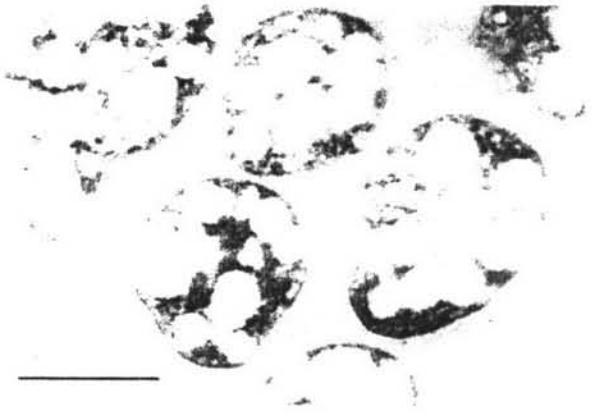

Figure 2.2 Electron microscope view of the accumulation of polymer granules, PHB in cells of the species *Alcaligenes entrophus*. Bar = 1 μm.

The type of bacterium and growth conditions determines the chemical composition of PHAs and the molecular weight, which typically ranges between 2 x 10^5 and 3 x 10^6 Da. A wide range of organisms have been reported to store PHB or PHB-like materials. They include Gram positive and Gram negative species and cyanobacteria. The occurrence of PHB in microorganism species are, for examples, Actinomycetes, Alcaligenes, Azotobactor, Azospirillum, Bacillus, Beijerinckia, Chlorogloea, Chromatium, Chromobacterium, Derxia, Ferrobacillus, Hyphomicrobium, Lampropaedia, Methylobacterium, Micrococcus, Nocardia, Pseudomonas, Rhizobium, Rhodopseudonomas, Rhodospirillum, Sphaerotilus, Spirillum, Streptomyces, Vibrio, and Zoogloea.

2.2.3 PHBV

The materials are polyesters. In PHB, a homopolymer, the repeating unit is 3-hydroxybutyric acid. The chemical formular is:

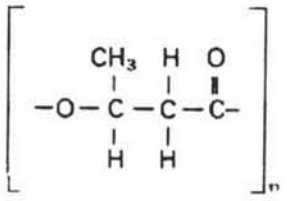

Figure 2.3 Chemical structure of Poly(3-hydroxybutyric acid).

Copolymers with hydroxyvaleric acid can be made by some organisms. In fermentation, PHB/PHV copolymers are made by supplying mixed glucose/propionic acid substrate the hydroxyvaleric content of the polymer is controlled by variation of the ratio of glucose to propionate in the feed. Good control of propionate feed rate is essential, since a supernatant concentration in excess of 0.1% is toxic and prevents polymer synthesis. These are usually random arrangements of hydroxyvaleric and hydroxybutyric acid residues. The structure of polyhydroxyvaleric acid (PHV) is:

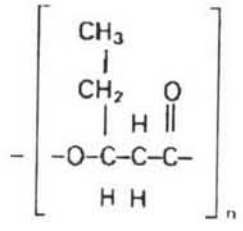

Figure 2.4 Chemical structure of polyhydroxyvaleric acid (PHV).

The copolymers have superior mechanical properties to the homopolymer. They are more flexible and tougher which extends their versatility in end use to applications such as bottle and film manufacture which are not possible with PHB.

2.2.4 Applications

2.2.4.1 Recombination of PHA and Blends with Other Polymers

A very interesting application of PHA is their use as starting chemicals for other chemicals taking advantage of their uniform chirality. Since ester bonds can be easily split either chemically with acid or base, enzymatically or through in vivo degradation under conditions where 3-hydroxybutyric acid dehydrogenase activity is low and the degradation products are excreted.

Blend polymer can be achieved specific properties such as DegraPol, a block-copolyesterurethane chemically synthesized from PHB-diol and α,ω–dihydroxy-poly(ε-caprolactone-b-diethylene-glycol-b-ε-caprolactone) showed a good biocompatibility.

Often, the mixture changes the crystallinity of the plastic and the crystallization rate and finally also the mechanical properties of the material. Urakami *et al.* (2004) studied the blending of PHB and poly(carprolactone) (PCL). A mixture of 40-60% of PCL in PHB improved the mechanical properties over PHBV. A 40% PCL and 60% PHB mixture had a decreased oxygen permeability that was only 5% that of polyethylene.

Maekawa *et al.* (2003) reported that blends of PHB and cellulose propionate were completely miscible since they had a single glass transition, a depression in the equilibrium melting temperature of PHB and a decrease in the spherulitic growth rate of the PHB component. Also, the tensile strength was better for the blend PHB/cellulose propionate than for PHB only. However, Yoon et al. demonstrated that not all blends are compatible such as PHB blended with poly(L-lactide) (PLLA) form a compatible mixture only when the molecular weight of PLLA is smaller than 11700 Da.

Moreover, PHB can be modified by addition of plasticizers which these low molecular weight compound give the plastic a better ductility, but may alter the biodegradation of the polymer, as shown by Savenkova *et al.* (2001).

2.2.4.2 Medical Application of PHA

PHA represents a class of polymers that has immense potential for medical applications (see Table 2.2) and is, therefore, attracting

increasing attention. Williams *et al.* (1997) defined five key parameters that scaffolds need to fulfill for successful tissue engineering: (i) biocompatibility; (ii) support of cell growth and cell adhesion; (iii) guide and organize the cells, (iv) allow ingrowth of cells and passage of nutrients and waste products, and (v) biodegradable without formation of toxic compounds. In addition, tissue engineers have determined that the surface structure is also an important factor. Therefore, porous surfaces can be produced by the leaching technique, which is done by blending of PHA with a salt or sucrouse that can be washed out with water. Moreover, the surface of PHA materials can be rendered more hydrophilic as was shown by the treatment of PHBV with allyl alcohol gas plasma, hyaluronan acid that led to an increase of wettability. Further modifications may be obtained by blending with other polymers which alter the physicochemical properties.

Table 2.2 Potential applications of PHA in medicine

Type of application	Products
Wound management	Sutures, skin substitutes, nerve cuffs, surgical meshes, staples, swabs
Vascular system	Heart valves, cardiovascular fabrics, pericardial patches, vascular grafts
Orthopaedy	Scaffolds for cartilage engineering, spinal cages, bone graft substitutes, meniscus regeneration, internal fixation devices (e.g. screws)
Drug delivery	Micro- and nanospheres for anticancer therapy
Urology	Urological stents
Dental	Barrier material for guided tissue regeneration in periodentitis
Computer assisted tomography and ultrasound imaging	Contrast agents

Biomaterials with optimum properties could be obtained by blending with suitable compounds or by selecting the appropriate PHA. Thus, a wealth of options is available for cell carriers for specific engineering applications. In a study, when PHBV was implanted in rabbit tibia as a carrier of antibiotics cefoperazone and sulbactam, there were no significant symptoms of chronic inflammation or toxicity as judged by histology, scanning electron microscopy

(SEM), microbiology and X-ray studies implying the suitability of the material for in vivo use. In another study, patches of PHB were used to close experimentally induced atrial septal defects in calves (Malm *et al.*, 1992). At the end of 12 months no polymer material was found. Although small particles of polymer with persisting foreign body reaction were observed by polarized light microscopy, PHB appears to be suitable material for human applications. PHBV is now being studied intensely as a tissue engineering substrate. Rivard *et al.* (1996) demonstrated that PHBV (9%) sustained a fibroblast proliferation rate similar to that observed in collagen sponges for at least 35 days. In addition, the PHBV materials maintained their integrity during the culture period while the collagen foams contracted substantially. Moreover, the total protein production after 4 weeks in culture was found to be twice as high in the PHBV foam as in the collagen foam. It thus showed that porous PHBV materials could be more than adequate as polymeric substrates for cell cultures.

Bone formation was investigated in vitro by culturing rat marrow stromal osteoblasts in biodegradable, macroporous PHBV matrices over a period of 60 days (Kose *et al.*, 2003). PHBV solutions with different concentrations were prepared in chloroform: dichloromethane (1:2, v/v). In order to create a matrix with high porosity and uniform pore sizes, sieved sucrose crystals (300–500 mm) were used. PHBV foams were treated with rf-oxygen plasma (100W 10 min) to modify their surface chemistry and hydrophilicity with the aim of increasing the reattachment of osteoblasts. The cell density on and in the foams was determined with MTS assay. MTS results showed that osteoblasts proliferated on PHBV. Twentyone days after seeding of incubation, growth of osteoblasts on matrices and initiation of mineralization were observed by confocal laser scanning microscopy. Increasing ALP and osteocalcin secretion during 60 days confirmed the osteoblastic phenotype of the derived stromal cells. SEM, histological evaluations and confocal laser scanning microscopy showed that osteoblasts could grow inside the matrices and lead to mineralization. Cells exhibited spindle-like morphology and had a diameter of 10–30 mm.

Hydroxyapatite (HA), a mineral quite similar in composition to the inorganic component of bone, is used to modify the mechanical properties of polymeric implants for certain medical applications. Composites of PHBV and HA

with partial biodegradability and high mechanical strength and osteoconductivity were reported to be suitable for fracture fixation (Galego *et al.*, 2000). A composite of PHB reinforced with HA particles was tested as a bone analog and new bone growth at the interface of implantation site was observed after 6 months (Luklinska *et al.*,1997). It thus appears to be suitable filler for improving mechanical properties and bone healing. In this study, the preparation, degradation behavior, surface morphologies and surface modification of porous poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) foams are described. This work also shows osteoblast growth on PHBV foams by SEM indicating the suitability of the material for in vivo use.

Random copolyesters consisting of 3-hydroxybutyrate and 3-hydroxyhexanoate (PHBHHx) is a new member of PHA family, PHBHHx has recently been produced in large scale (Chen *et al.*, 2001). Its mechanical and process properties have been shown to improve over PHB and PHBV that are also available in large scale (Doi, 1995; Kai, 2002). PHBHHx was also evaluated for its biomedical applications. The results showed that PHBHHx had better biocompatibilities for fibroblast (Ya-Wu, 2004; Kai, 2002), chondrocyte (Ying, 2003; Zhong, 2004) and osteoblast (Ya-Wu, 2003; Ming, 2003) compared with PHB and PLA.

All above, the foams were prepared by solvent evaporation and solute leaching technique. In this work, for the first time, the new method which is electrospinning will be applied for increasing the biocompatibility and biodegradability of scaffold construction. It should be realized that most of these applications have not reached their industry level, but just at a laboratory research and development stage. However, their promising potential is believed to be attracting attentions and investments from academia, governments, and industry all over the world.