# CHAPTER 3

# THE CONCEPT OF TISSUE ENGINEERING

## 3.1 Introduction

In the previous chapter, the current problems associated with heart valve replacement were stated in general. The importance of the hydrodynamic performance and the shortcomings of artificial heart valves were highlighted and a review of VHD and current detection methods were presented. To overcome the problems associated with valvular replacement, the relatively new field of Tissue Engineering (TE) offers a solution to heart valve replacement (Langer and Vacanti, 1993).

In this chapter a TE-approach is presented and reviewed with a particular focus on a polymer strategy. The use of a polymer scaffolding material and cardiac cells to grow a heart valve *in vitro,* mimicking an *in vivo* environment was investigated. The three main integrated elements are all part of the overall research program, currently undertaken at Swinburne University. One of these activities is to develop a polymer heart-valve-scaffold and to seed this scaffold with cardiac cells followed by culture in a Bioreactor (BR). Therefore, an overview of scaffold materials followed by cell sources/types and a review of existing BRs is presented. Experimental procedures were carried out to create a durable heart-valve-scaffold and to establish primary cell lines from ovine cardiac tissue. These procedures are presented and discussed in sections 3.3.1.6 and 3.3.2.3. The remainder of the chapter reviews four recent key-studies, where BRs were used to grow and test Tissue Engineered Heart Valves (TEHVs) *in vitro*.

## 3.2 Background

The term Tissue Engineering (TE) was initially defined by the attendees of the first National Science Foundation (NSF) sponsored meeting in 1988 as the "application of the principles and methods of engineering and life sciences toward fundamental understanding of structure-function relationship in normal and pathological mammalian tissues and the development of biological substitutes for the repair or regeneration of tissue or organ function" (Shalak and Fox, 1988). TE differs from standard therapies in that engineered tissues become integrated within the patient, affording a potentially permanent and specific treatment of the disease state. "TE is an emerging multidisciplinary field that applies the principles of biology and engineering to the development of viable substitutes that restore, maintain or improve the function of human tissues" (Langer and Vacanti, 1993).

The different possibilities of creating replacement parts with TE were explored more than twenty years ago by Bell and colleagues, who investigated the possibility of the reconstruction of living tissues (Bell et al., 1981). During the 90s, TE progressed rapidly and biological substitutes were developed for several tissues in the body. TE-products have reached the market and in little over a decade, the TE-industry has grown to become a $3.5 billion worldwide R&D effort by over forty biotech start-ups and business units (Lysaght et al., 1998). TE has emerged as a potential alternative to tissue or organ transplantation and tissue loss or organ failure may be treated either by implantation of an engineered biological substitute or alternatively with *ex vivo* perfusion systems. TE products may be fully functional at the time of treatment (e.g., liver assist devices, encapsulated pancreatic islets), or have potential to integrate and form the expected functional tissue upon implantation.

Three general approaches

Currently the literature describes three general TE approaches. These principles are closely related to each other and may be applied to create new tissues. These approaches include:

1. Design and grow human tissues *in vitro* for later implantation to repair or replace diseased tissues: The most common example is the skin graft, used for the treatment of burns (Eldad et al., 1987). Skin graft replacements have been grown in tissue culture and used clinically for more than 10 years.

2. Implantation of cell-containing or cell-free devices that induce the regeneration of functional human tissues: "signal" molecules, e.g. growth factors may be used to assist in biomaterial-guided tissue regeneration. Also, novel polymers have been created and assembled into three-dimensional configurations, to which cells attach and grow to reconstitute tissues. An example is the use of a polymer matrix to form cartilage (Carver and Heath, 1999).

3. The development of external devices containing human tissues designed to replace the function of diseased internal tissues: This approach involves establishing primary cell-lines, placing the cells on or within structural matrices and implanting the new system inside the body (Shinoka et al., 1995). Examples of this approach include repair of bone, muscle, tendon and cartilage, endothelial cell-lined vascular grafts and heart valve substitutes.

The overall aim of the study at Swinburne University investigates the third approach: "The development of external devices containing human tissues (heart valves) designed to replace the function of diseased internal tissues".

## 3.3 Tissue Engineering of Heart Valves

No currently used valvular replacement devices provide growth potential, a major restriction that was discussed in the last chapter. TEHVs focuses on the development of a functional identical copy of a healthy heart valve. This is a relatively new technique within the TE-field and it may be possible to overcome all the disadvantages associated with the clinical use of mechanical and bio-prosthetic heart-valves (Hoerstrup et al., 2000a). This technique may be able to improve the medical treatment of patients with heart-valve diseases.

### Overview

A TE-approach for creating valve replacements may be categorized into two general strategies: (1) using degradable *polymeric scaffolds* or (2) acellular *bio-matrices* that support cell growth.

The first strategy is to use degradable polymeric scaffolds moulded into heart valve geometries. Cells isolated from donor tissue are cultured and then seeded onto these scaffolds, resulting in constructs that can be implanted *in vivo* after a specific cultivation period. The cells grow, develop, and produce extra cellular matrix (ECM) as the polymer degrades, ultimately leaving a natural tissue heart valve without any synthetic component (Shinoka et al., 1995). Ideally, autologous cells should be used to eliminate immunological responses to the TE-construct and to facilitate the growth and remodelling processes. The cell-polymer interaction is also critical because the quality and extent of ECM formation will determine the overall structure and mechanical properties of the newly developed tissue structure. Degradable polymeric materials, ideal this purpose are discussed in detail in the next section (3.3.1).

The second TEHV strategy uses acellular, natural bio-matrices. For example, porcine heart valves may be processed to remove their cellular antigens and reduce their

immunogenicity. These constructs are then implanted *in vivo* and repopulated with host cells. This approach requires decellularization techniques that do not adversely affect the mechanical properties of the bio-matrices or the reconstitution of the tissue *in vivo*. Issues involving the stability and resorption of the natural bio-matrices must also be resolved (Steinhoff et al., 2000). Supporters of this strategy argue, however, that in contrast to polymeric scaffold TEHVs, acellular biomatrices retain natural ligands and ECM constituents more suited for cell attachment and endothelialization.

## Decellularized Biomatrices

The overall objective of the current research primarily lies on the polymer-strategy. However, the second strategy, using decellularized biomatrices, also has great potential for fabricating TEHVs. Recent work has focused on the development of alternatives to the decellularization process in order to create scaffolds. Glutaraldehyde has been used since the 1960s to reduce the immuogenicity of xenogenic tissues. Using this agent, collagen fibers of the biomatrices are cross-linked to minimize xenogenic tissue solubility and antigenicity. Also the mechanical properties of the natural biomatrix can be altered (Love, 1997). Glutaraldehyde has been shown to increase the risk of heart valve calcification, and chemical residues from the fixation process can invoke an inflammatory response and reduce the viability of the repopulating cells. Due to this, alternative cross-linking solutions have been developed with different advantages and disadvantages. In the following sections three studies of different decellularation processes are summarized and compared.

Bader et al., (1998) and Steinhoff et al., (2000) decellularized porcine aortic valves and ovine pulmonary valves using a similar approach. In both of these experiments native cells were removed from the biomatrices, which were subsequently repopulated with cultured cells. Additionally, the Steinhoff group implanted its TEHV constructs into lambs. Electron microscopy, histology and immunohistochemistry were used by both groups to evaluate the efficiency of their decellularization processes. The results

suggested that the decellularization processes successfully removed most of the cells while preserving the ECM organization of the biomatrices. Steinhoff et al., (2000) also evaluated the *in vivo* performance of TEHVs with echocardiography. Although the leaflets did thicken and calcify without an apparent loss of function, pulmonary regurgitation was not observed among the TE-constructs.

O'Brien et al., (1999) used a non-detergent-based decellularization solution and did not repopulate the biomatrices with cells prior to implantation into female sheep. Up to six months following implantation of these scaffolds, no pulmonary regurgitation, calcification or gross abnormality was observed. In this period, host sheep cells re-populated the scaffold and the performance did not show significant difference from cryopreserved, cellularized, allogenic sheep aortic heart valves in comparable tests.

The decellularization method used by O'Brien et al., (1999) is the basis for the commercially available SynerGraft pulmonary replacement valve. CryoLife Inc, manufacturer of these valves, received a CE mark in October 2000 to distribute these heart valves throughout the European Union. Nearly six months later, the first successful implant of this valve was announced. A 3-year-old male child in Norway received the TE substitute (O'Brien et al., 1999). In comparison to polymeric scaffold-derived TEHVs, these replacements do not require pre-conditioning or pre-seeding.

### 3.3.1 Scaffold materials

In the beginning of the 19$^{th}$ century, research into synthesized materials such as glycolic acid and other α-hydroxy acids was abandoned, because the developed polymers were too unstable for long-term use. This instability, leading to biodegradation (BD), has proven to be immensely important in medical applications over the last three decades. Polymers prepared from glycolic acid and lactic acid have found a multitude of uses in medical practice. Since BD sutures were first approved in the 60s, diverse products based on lactic and glycolic acid and other related types have been accepted for use as medical devices. In addition to these approved devices, a great deal of research continues on the biodegradability of these polymers. The design and development of TEHVs has benefited from many years of clinical use of a wide range of these polymers. Besides this, newly developed BD polymers and novel modifications of existing materials allow the creation of ideal scaffolds for many TE-applications.

In general, BD polymers can be categorized as biologically derived or synthetically produced. One of the research objectives at Swinburne University is to identify a BD polymer, which can be shaped into a heart-valve-scaffold and can be further cultured inside a BR after seeding. Both types of polymers under consideration for this purpose are reviewed in this section. Furthermore, details of these reviewed polymers are presented in **Appendix A4**, including synthesis and the properties of copolymers. Degradation and a processing technique called Fused Deposit Modelling that can process polymers into valvular scaffolds are reviewed as well. The final part of this section (3.3.1.6) is used to discuss why a particular polymer was selected for the current investigation.

BD Polymers in Tissue Engineering:

One of the current areas for applications of biodegradable polymers is TE. Several companies are investigating in the use of these materials as a scaffold to grow tissue on. Important properties in this regard include porosity for cell in-growth, a surface that balances hydrophilicity (affinity for water) and hydrophobicity (repelling in water) for cellular attachment, mechanical properties that are compatible with those of the tissue, and degradation rate and by-product production. To grow a TEHV, the polymer matrix may be utilized in three different ways:

- May represent the scaffold itself, which will degrade *in vivo,* where autologous cells grow over the structure.
- Can be a scaffold for cell growth *in vitro* that is degraded by the growing cells before the structure is implanted.
- Can be a combination of the first two.

In addition to these three possibilities, the scaffold can also be formulated to contain additives or active agents for more rapid tissue growth (Lanza et al., 1999). In the future, device designers, tissue engineers and physicians will even have a wider choice of BD polymers as scaffolds for TEHV-applications, as biodegradable polymers are under constant development.

Biodegradable polymers are either derived from natural or synthetic sources. In general, synthetic polymers offer greater advantages compared to natural materials. Synthetic polymers can be tailored to give a wider range of properties and more predictable lot-to-lot uniformity than can materials from natural sources. Synthetic polymers also represent a more uniform source of raw materials and are free from concerns of immunogenicity. Naturally occurring hydroxy acids, such as glycolic, lactic and ε-caproic acids have been utilized to synthesize an array of useful BD polymers for variety of medical product applications. The selection of the scaffold material plays a key role in the design and development of a particular TE product. Although the classical selection criteria focus on a safe, stable implant, it is recognized that every material used will elict a different cellular response in terms of degradation (Kohn and

Langer, 1996). One of the current challenges in culturing TEHVs is to select a polymer scaffold that meets the mechanical properties and degradation times required. In **Appendix A4** several combinations of biodegradable polymers are presented, each with different mechanical properties and degradation-times. The ideal polymer for a particular application should be configured so that it possesses the following properties:

- Appropriate mechanical strength to mimic *in vivo* conditions.
- Rate of matrix regeneration close to biodegradability rate of the BD polymer scaffold.
- Does not invoke an inflammatory or toxic response.
- Is metabolised in the body after fulfilling its purpose, leaving no trace (bio-absorbable).
- Is easily processable into the final product form, either porous or compatible with a range of extremely hydrophilic additives (starch, salt) to create porosity.
- Demonstrates acceptable shelf life and is easily sterilized.

In general, the factors affecting the mechanical performance of biodegradable polymers include monomer selection, initiator selection, process conditions, and the presence of additives. These factors in turn influence the specific features of the polymer such as: hydrophilicity, crystallinity, melt and glass-transition temperatures, molecular weight, molecular-weight distribution, end groups and presence of residual monomer or additives. In addition, these properties of BD materials should be evaluated, in order to determine its effect on biodegradation. In general, an unstable backbone of the material leads to biodegradation. The most common chemical functional groups with hydrolytically unstable linkages are esters, anhydrides, orthoesters and amides. A review of natural and synthetic polymers that may be of use as TEHV-scaffolds for the current study is presented in the next section (3.3.1.1).

#### 3.3.1.1 Natural BD polymers

There are many different existing potential biodegradable-scaffold materials that may be used for TE-applications. Five of the most commonly used materials that may be used as a tissue scaffold are discussed.

Type I collagen: Collagen is the major protein component of mammalian connective tissue, accounting for 30% of all protein in the human body. It is found in every major tissue that requires strength and flexibility. Nineteen types of collagens have been identified; the most abundant being type I, which makes up more than 90% of all fibrous proteins. Individual collagen molecules, consisting of a chain of amino acids, that polymerise *in vitro* into strong fibers. These fibers consist of three chains of amino acids that can be subsequently formed into larger organized structures like scaffolds. Cross-linking or chemical bonding can be enhanced after isolation through a number of well-described physical or chemical techniques (Pachence et al., 1987). Increasing the intermolecular cross link's a) increases biodegradation time, b) increases hydrophobicity, c) decreases the solubility, d) and will increase the tensile strength of the collagen fibers.

Glycosaminoglycans (GAGs):
GAGs are highly negatively charged molecules, with an extended conformation that gives high viscosity. GAGs are located primarily on the surface of cells or in the extra cellular matrix (ECM). Structural components of the ECM, such as collagen and GAGs, have major roles in valvular degeneration and calcification of bioprosthetic heart valves. In particular the GAGs located in the spongiosa layer of the heart valves are extremely important for mechanical properties (Schoen, 1997). Their rigidity provides structural integrity to cells and provides channels between cells that permit cell migration. The specific GAGs of physiological significance are hyaluronic acid, dermatan sulphate, chondroitin sulphate, heparin, heparan sulphate, and keratan sulphate (Lovekamp and Vyavahare, in press). Due to its relative ease of isolation and modification and its ability to form solid structures, hyaluronic acid has become the principled GAG investigated for medical device development.

Chitosan:

This biosynthetic polysaccharide can be slowly depolarised *in vivo* with lysozyme. Lysozyme is an enzyme that occurrs naturally in egg white, human tears, saliva, and other body fluids. It is capable of destroying the cell walls of some bacteria and acts as a mild antiseptic. The biodegradation time is determined by the amount of residual acetyl content, a parameter that can be easily varied. Chemical modification of chitosan produces material with a variety of physical and mechanical properties. Like hyaluronic acid, chitosan is non-antigenic and is a well-tolerated implant material. It can be formed into membranes and matrices suitable for several TE-applications (Byrom, 1992).

Polyhydroxyalkanoates (PHA):

PHA polyesters are degradable, biocompatible, thermoplastic materials produced by several different microorganisms. Depending on growth conditions, bacterial strain, and carbon source the molecular weight of these polyesters can range from tens into hundreds of thousands. The most extensively studied PHA is the simplest: Poly-3-hydroxybutyrate (P3HB). Most of these are homopolymers and are highly crystalline, extremely brittle and relatively hydrophobic. Consequently, these polymers can have *in vivo* degradation times in the order of years and therefore not suitable as a scaffold material. P3HB and its copolymers containing $\leq$ 30% 3-hydroxyvaleric acids are currently commercially available. It has been reported that a PHA copolymer of 3-hydroxybutyrate combined with 10% 3-hydroxyvalerate may provide an optimum balance of strength and toughness for a wide range of scaffold applications. It has low toxicity, partly due to the fact that it degrades *in vivo* to d-3-hydroxybutyric acid, a normal constituent of blood (Doi et al., 1990). Poly-4-hydroxybutyrate (P4HB) contains many of the same properties as P3HB and is an easily mouldable thermoplastic that can be formed into functional valve scaffolds (Sodian et al., 2000b).

#### 3.3.1.2 Synthetic BD polymers

This section presents an overview of the synthetic biodegradable polymers that are currently used or being investigated for use in wound closure, orthopaedic fixation devices, dental applications, intestinal applications and cardiovascular applications. Most of the commercially available biodegradable devices consist of polyesters composed of homopolymers or copolymers of glycolide or lactide. The five most commonly investigated synthetic polymers used as matrices for TEHVs are described.

Poly (glycolic acid, poly (lactic acid) and their copolymers:

PGA, PLA and their copolymers are the most widely used BD polymers in medicine. Of this family of linear aliphatic polyesters, PGA has the simplest structure. This group is derived from organic compounds where carbon and hydrogen molecules are arranged in straight or branched chains, a type of hydrocarbon that includes; alkanes, alkenes, and alkynes.

- Polyglycolide (PGA): PGA was used to develop the first totally synthetic absorbable suture, marketed as Dexon in the 1960s. Glycolide monomers are synthesized from the dimerization of glycolic acid. PGA is highly crystalline, with a high melting point and a glass-transition temperature between 35 and 40°C. The glass-transition temperature is the temperature where a plastic changes from being brittle and hard into flexible material without changing phase. Because of its high degree of crystallization, it is not soluble in most organic solvents. Fibers from PGA exhibit high strength and modulus and are too stiff to be used as sutures except as a braided material. Due to its hydrophobic nature, scaffolding materials made of PGA tend to lose their mechanical strength rapidly, over a period of 2 - 4 weeks post implantation and are completely absorbed in 4 - 6 months (Reed and Gilding, 1981).

- Polylactide (PLA): PLA is more hydrophobic because of the extra methyl group in lactic acid and is therefore more soluble in organic solvents than PGA. This polymer exists as two optical isomers, d and l. l-lactide is a naturally occurring

isomer. Dl-lactide is the synthetic blend of d-lactide and l-lactide. The homopolymer of l-lactide (LPLA) is a semi crystalline polymer. These types of materials exhibit high tensile strength and low elongation. Consequently, these materials have a high modulus which makes them more suitable for load-bearing applications such as the cyclic stress experienced in the cardiovascular system. Poly dl-lactide (DLPLA) is an amorphous polymer exhibiting a random distribution of both isomeric forms of lactic acid, and accordingly is unable to arrange into an organized crystalline structure. This material has lower tensile strength, higher elongation, and a much more rapid degradation time, making it attractive as a drug delivery system. The degradation time of LPLA is much slower than that of DLPLA, requiring more than two years to be completely absorbed. Copolymers of l-lactide and dl-lactide have been prepared to disrupt the crystallinity of l-lactide and can accelerate the degradation process (Pitt, 1990).

- Poly (lactide-co-glycolide)**:** Using the polyglycolide and poly (l-lactide) properties as a starting point, it is possible to copolymerize the two monomers to extend the range of homopolymer properties. Copolymers of glycolide with both l-lactide and dl-lactide have been developed for both device and drug delivery applications. It also may be used as a scaffold material. It is important to note that there is not a linear relationship between the copolymer composition and the mechanical and degradation properties of the materials. For example, a copolymer of 50% glycolide and 50% dl-lactide degrades faster than either homopolymer (Figure 3.1).

Poly ε-caprolactone: Poly ε-caprolactone (PCL) is an aliphatic polyester that has been intensively investigated as a biomaterial. The discovery that PCL can be degraded by micro-organisms led to the evaluation of PCL as a biodegradable packaging material (Pitt, 1990). The ring-opening polymerisation of ε-caprolactone yields a semi crystalline polymer with a melting point of around 60°C. The polymer is regarded as tissue compatible and used as biodegradable sutures in Europe. Because the homopolymer has a degradation time in the order of two years, copolymers have been synthesized to

accelerate the rate of bio-absorption. Copolymers of ε-caprolactone with dl-lactide have yielded materials with more-rapid degradation rates (Pitt, 1990).

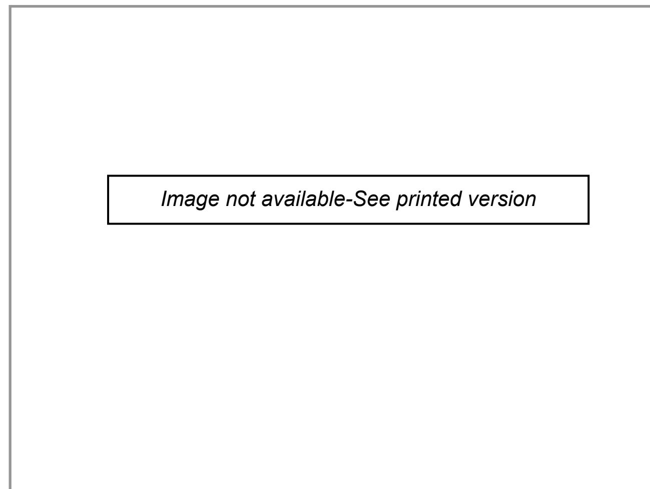



***Figure 3.1***

*Half-life of PLA and PGA homo and copolymers implanted in rat tissue*

(Figure reproduced courtesy of Journal of Biomedical Materials Research, 11:711, 1977.)

Poly-dioxanone: The ring-opening polymerisation of *p*-dioxanone resulted in the first clinically tested monofilament synthetic scaffolding material, known as PDO. This material has approximately 55% crystallinity, with a glass-transition temperature of –10 - 0 °C. The polymer is processed at the lowest possible temperature to prevent de-polymerisation back into a monomer. Poly (dioxanone) has demonstrated no acute or toxic effects after implantation. The monofilament loses 50% of its initial breaking strength after three weeks and is absorbed within six months.

Polyglyconate: Copolymers of glycolide with trimethylene carbonate (TMC), called polyglyconate, have been used as sutures, tacks and screws. These materials have better flexibility than pure PGA and are absorbed in approximately seven months. Glycolide has also been polymerised with TMC and *p*-dioxanone to form a suture that absorbs within 3 - 4 months and offers reduced stiffness compared to pure PGA fibers.

### 3.3.1.3 Degradation

Once implanted, a scaffold material should maintain its mechanical properties until it is no longer needed and then be absorbed and excreted by the body, leaving no trace. There are two types of biodegradation and both are discussed in this section.

A simple chemical hydrolysis of the hydrolytically unstable backbone is the prevailing mechanism for a polymer's degradation. This occurs in two phases. In the first phase, water penetrates the bulk of the device, attacking the chemical bonds and converting long polymer chains into shorter water-soluble fragments. This occurs in the amorphous phase and initially there is a reduction in molecular weight without a loss in physical properties, since the device matrix is still held together by the crystalline regions. The reduction in molecular weight is followed by a reduction in physical properties, as water begins to fragment the device (Figure 3.2). In the second phase, enzymatic attack and metabolization of the fragments occurs, resulting in a rapid loss of polymer mass. This type of degradation, where the rate at which water penetrates the device exceeds that at which the polymer is converted into water-soluble materials, is called bulk erosion. This results in erosion throughout the device. All commercially available synthetic devices and sutures degrade by bulk erosion.

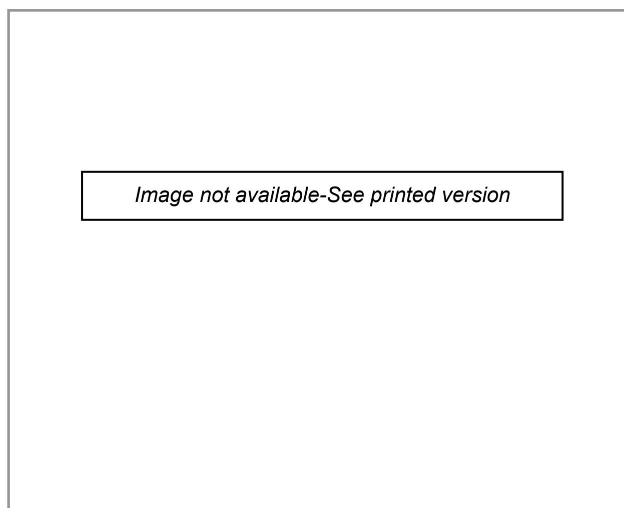



***Figure 3.2***

*Generic absorption curves:*
*Showing the sequence of polymer molecular weight, strength, and mass reduction*

(Figure reproduced courtesy of Journal of Craniofacial Surgery, (8)2:89, 1997.)

A second type of biodegradation, known as surface erosion, occurs when the rate at which the water penetrates the scaffold is slower than the rate of conversion of the polymer into water-soluble materials. Surface erosion results in the device thinning over time while maintaining its bulk integrity. In general, this process is referred to as bio-erosion rather than biodegradation. The degradation-absorption mechanism is the result of many interrelated factors that include:

- Chemical stability of the polymer backbone.
- Presence of catalysts, additives, impurities, or plasticizers.
- The geometry of the device.

Factors that accelerate polymer degradation include an increased hydrophilic backbone, increased number of reactive hydrolytic groups in the backbone, less crystallinity, increased porosity and a larger surface area (Reed and Gilding, 1981; Doi et al., 1990). Balancing each of these factors will allow an implant to slowly degrade and transfer stress at an appropriate rate to surrounding tissues as they heal. This is one of the major challenges facing TE research today.

#### 3.3.1.4 Comparison of scaffolding materials for TEHVs

The following sub-section provides an overview of biodegradable polymers that have been used in attempts to TE heart valves *in vitro*.

Shinoka et al., (1995) attempted to identify a suitable degradable polymer for a fully functional and autologous TEHV. This group successfully constructed a TEHV-leaflet from woven and non-woven PGA mesh sheets. Leaflets from polyglactin sheets sandwiched between PGA mesh sheets were also fabricated. Results from this study suggested that these materials were too thick, non-pliable and therefore could not form non-stenotic, tri-leaflet heart valves. Furthermore, the fibrous PGA meshes had

insufficient strength to withstand *in vivo* flow conditions. As a result, several naturally occurring thermoplastic polymers, like PHA and P4HB, where investigated for TEHV scaffold fabrication. As described in the previous section, all naturally thermoplastic polymers posses biocompatible, resorbable and flexible features. Furthermore, they have high mechanical strength and induce a minimal inflammatory response. The low melting point of these thermoplastics permits moulding into the configuration of a tri-leaflet heart valve, and salt leaching technique can be used to construct a porous scaffold that promotes cell ingrowth (Sodian et al., 2000a and 2000b). These polymers have been used either alone or in combination to fabricate different TEHV scaffolds.

In a comparative study conducted by Sodian et al., (2000b) the *in vitro* performance of TEHVs constructed from tri-leaflet-shaped polymer scaffolds of PHA and P4HB were examined. No significant difference was demonstrated. The superior view of a P4HB-scaffold is shown in Figure 3.3. In the study, increased cellularity and collagen formation was observed when compared to fibrous PGA sheets. Therefore, polymer scaffolds were developed to combine the favourable cell-polymer interactions of PGA with the processability and strength of thermoplastics.

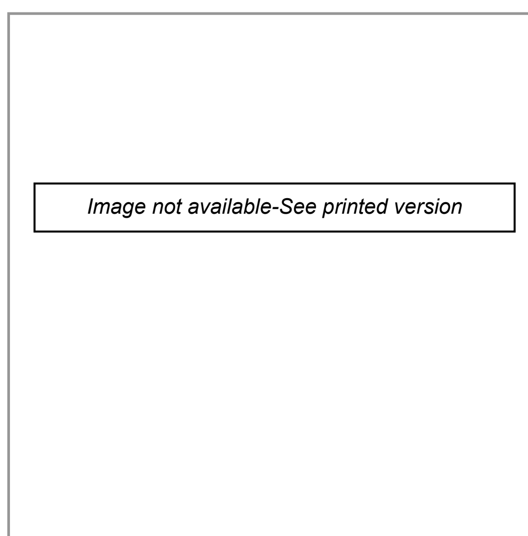



***Figure 3.3***

*Superior view of a trileaflet TEHV- scaffold, fabricated from porous P4HB*

(Figure from: Sodian et al., 2000b)

As shown in figure 3.4, another investigation carried out by Sodian et al., (1999) used scaffolds where PGA was moulded around a softened PHA tube in order to form the conduit wall. After this procedure leaflets constructed from PGA-PHA-PGA sandwiches were attached. Hoerstrup et al., (2000a) presented another approach (Figure 3.5). Here the TEHV-scaffold was created from coated PGA meshes with a thin layer of P4HB. After the solvent evaporated, the P4HB-coating physically bonded with the PGA fibers. Finally, attempts were made to construct TEHVs from a nonporous PHO/PGA.

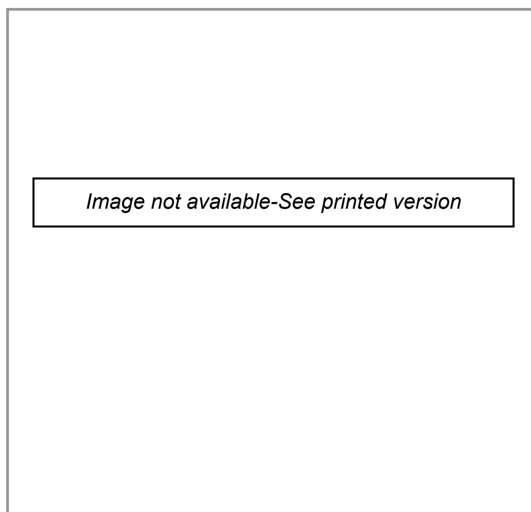



***Figure 3.4***

*Inferior view of a trileaflet TEHV-scaffold:*

*Conduit wall is fabricated from an outer layer of PGA pressed around an inner layer of PHA. Leaflets are fabricated from PGA-PHA-PGA sandwiches.*

(Figure from: Sodian et al.,1999)

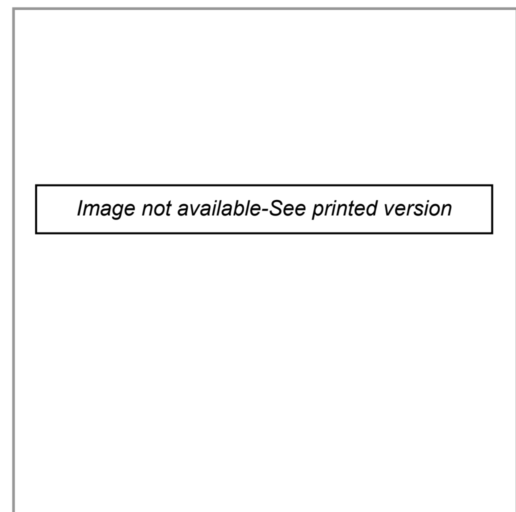



***Figure 3.5***

*Inferior view of a TEHV-scaffold:*

*Fabricated from PGA with a thin coating of P4HB, after 14 days in a bioreactor*

(Figure from: Hoerstrup et al., 2000a)

Stock et al., (2000) fabricated valve conduit walls from nonporous PHO films sandwiched between nonwoven layers of PGA. In Figure 3.6a the superior view of this construct is shown. The leaflets were constructed from porous PHO and sutured to the conduit wall with polydioxanone (PDO) (Figure 3.6b). This construct consisted of four different biomaterials with different biomechanical, biochemical, and degradative properties. The nonporous PHO wall inhibited cell ingrowth, and scar formation was observed on the exterior surface of the TEHV construct. Based on these observations, a new thermal processing technique was developed to replace leaflet suturing and to construct both the conduit wall and leaflets from porous PHO (Sodian et al., 2000d). The resultant porosity permitted cells to grow into the polymer, formed viable tissue, and initiated polymer degradation.

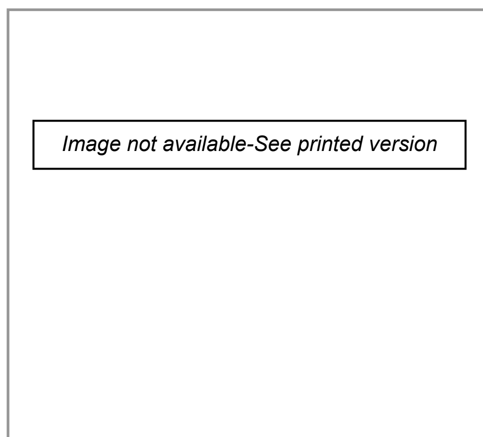



***Figure 3.6 A***

*Superior view of trileaflet TEHV-scaffold:*

*Conduit wall fabricated from nonporous PHO film with 1mm nonwoven PGA felts on both the inside and outside of PHO layer. Porous PHO leaflets are sutured to the conduit wall with PDO.*

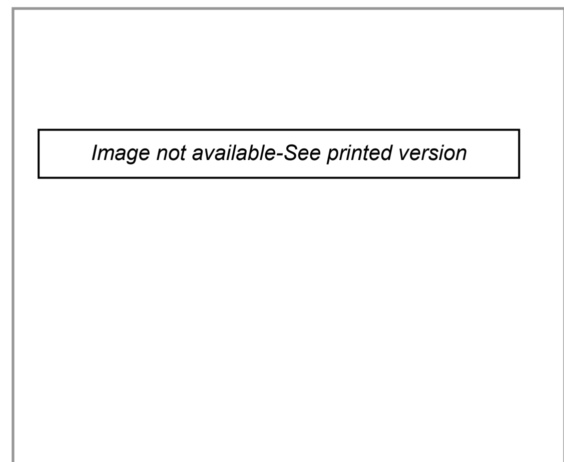



***Figure 3.6 B***

*Schematic of the TEHV:*

*18mm in diameter and 20mm in length*

(Figures 3.6 A and B obtained from Stock et al., 2000)

#### 3.3.1.5 Scaffold Processing

Different processing techniques have been developed for the design and fabrication of three-dimensional (3D) scaffolds suitable for TE implants. Conventional techniques for scaffold fabrication include fiberbonding, solvent casting, particle leaching, membrane lamination and melt molding. One of the newest methods being developed by Therics (Princeton, NJ) uses a system for building three-dimensional devices for use as scaffolds and for drug delivery products. In this system, small spheres of polymer are deposited as thin films. Using technology similar to that found in ink-jet printers, small amounts of solvent are used to fuse particles together. The particles not fused are removed and another layer of particles is deposited. This particle placement and fusing is continued for many layers, until the exact three-dimensional structure is obtained. Because each polymer layer is applied in a separate step, different polymers can be used to obtain different properties of the interior and exterior surface of the device. Swinburne University has a fused deposition-modelling machine that may be used to fabricate porous scaffolds from BD materials.

Fused Deposition Modelling (FDM):

FDM is a rapid prototyping process that integrates Computer Aided Design (CAD), polymer science, computer numerical control, and extrusion technologies to produce 3-D solid objects directly from a CAD model using a layer-by-layer deposition of molten thermoplastics extruded through a very small nozzle (Hayes et al., 2000). This technique has been used to fabricate 3D scaffolds with a honeycomb structure from PCL. These scaffolds have good mechanical properties and a porosity around 60% (Hutmacher et al., 2001). FDM is one of the few commercially available rapid prototyping technologies that offers the possibility of producing solid or porous objects in a range of different materials including metals and composites. The FDM system, developed by Stratasys Inc, fabricates structures from different kind of plastics and BD polymers. One FDM machine, the FDM3000 can be used to fabricate scaffolds for TE applications with layer thicknesses ranging from 0.178±0.127 - 0.356±0.127 mm. The FDM method (Figure 3.7) involves the melt extrusion of filament materials through a heated nozzle and deposition as thin solid layers on a platform.

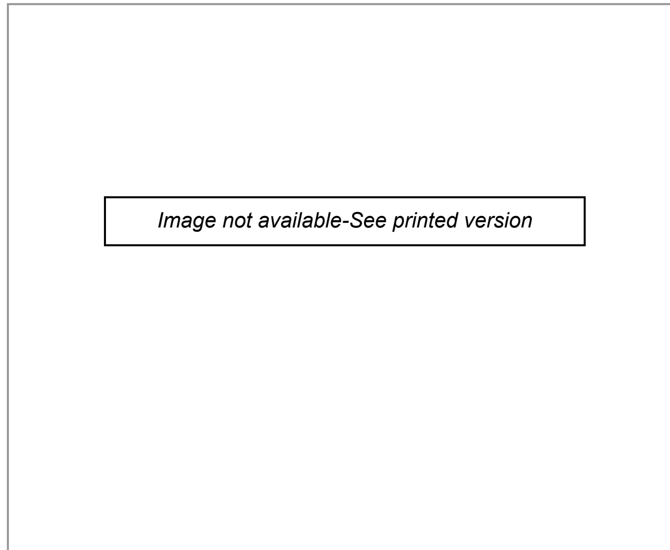



***Figure 3.7***

*The FDM process*

(Figure adapted from: Hutmucher D et al., 2001)

The process begins with the creation of a solid model or a closed surface model with CAD. The model is converted into an STL file using a specific translator on the CAD system. The STL file is then sent to the FDM slicing and pre-processing software called QuickSlice, where the designer selects proper orientation, creating supports and slicing and other parameters to prepare the part program for sending to FDM machine. A proper orientation of STL model is necessary to minimise or eliminate supports. The STL file is then sliced into thin cross sections at a desired resolution, creating an SLC file. Each slice must be a closed curve and any unclosed curves are edited and closed. Supports are then created if required, and sliced. Supports can also be created as part of the CAD model and imported as part of the STL file. The sliced model and supports are then converted into SML file, which contains actual instructions code for the FDM machine tip to follow specific tool paths, called roads, to deposit the extruded material to create each cross section. The designer selects various sets and road parameters to create a SML file. The SML file is sent to the FDM machine and the FDM head creates each horizontal layer by depositing molten extruded material on a foam foundation until the part is completed. The part is then removed, supports are detached carefully, and it is ready for use.

### 3.3.1.6 Discussion: Choice of polymer in this investigation

The BD materials reviewed in the previous sections provide a wide range of options for the fabrication of valvular scaffolds. The combination of different types of BD polymers to provide different degradation times and mechanical properties generate an unlimited number of possibilities (Sodian et al., 1999).

As no previous attempts have been attempted to fabricate a valvular scaffold for this investigation, it was decided to focus on one polymer. Preferably the material would be synthetic so it can be tailored to give a wider range of properties and more predictable lot-to-lot uniformity than materials from natural sources. Synthetic polymers also represent a more reliable source of raw materials (Kohn and Langer, 1996). It was decided to use Poly ε-caprolactone (PCL) as the material is totally bioabsorable, cheap and widely available. It was expected that PCL would have appropriate mechanical strength and it is easily processable into a porous product (Pitt et al., 1990). Moreover, PCL may be able to be processed by FDM (Hutmacher et al., 2001). This possibility is currently being investigated at Swinburne University. For the current study however, several manually fabricated tri-leaflet heart valve scaffolds from Poly ε-caprolactone (PCL) were created with an inner diameter of 24 mm and a porosity of ±84%. This was achieved by using a combined solvent casting salt leaching technique.

Procedure:

The PCL-material was dissolved in chloroform and salt (NaCl) was added. After evaporation the PCL-NaCl-mixture was extruded into a 3 mm thick sheet. The sheet was moulded into the final form with heat. The salt was leached out by placing the construct in water for a 24h period. This process was repeated several times until all the salt was leached out. The fabricated scaffold is shown in Figure 3.8.

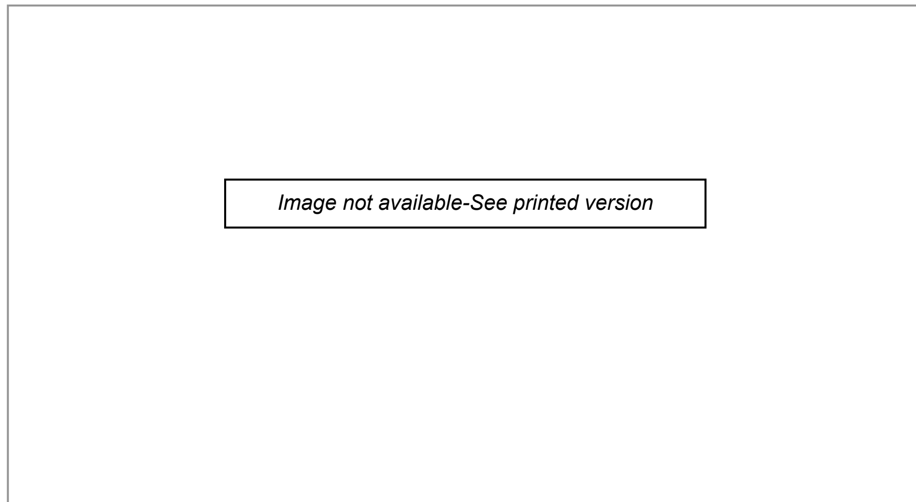



***Figure 3.8***

*Manually fabricated tri-leaflet heart valve scaffold from PCL*
*A: Anterior view B: Superior view*

### 3.3.2 Cells Seeding of Scaffolds

Heart valves are composed of many different types of cells that include endothelial cells, myocytes and fibroblasts. To create a BD-scaffold that is a functional, durable TE-construct, the establishment of cultured cells is a priority. To avoid rejection, cells should be autologous (Shinoka et al., 1995). The use of autologous cells has many advantages, including ethical considerations. Another cell source may be stem cells, but currently ethical issues make it impractical to use these cells for research purposes. In addition to this, to the authors understanding, the present lack of knowledge on how to trigger the mechanism to grow a particular organ or cell makes the use of this source an unrealistic possibility. However, the stem-cell approach shows great potential for future TE-applications. A brief overview is presented of the basics of stem cell engineering. Futhermore, an overview is presented of the different available cell sources and how different cells react *in vitro* to simulated *in vivo* conditions. During this experimental investigation attempts were made to establish primary cell lines.

#### 3.3.2.1 Cell Sources

Cells may be isolated from several sources but to grow a TEHV that can be directly implanted into a patient without rejection, investigators are limited to a few possibilities:

- I) Cells donated by other individuals (allogenic)
- II) Cells obtained from the same individual (autologous)
- III) Universal stem cells

These three cell sources are described and their relevance to the current investigation is discussed.

I Allogenic cells:

Allogenic cells are cells that are isolated from a donor of the same species. Animal cells have been widely used for experimental cardiovascular implants and the use of human cells for *in vitro* investigations possesses ethical constraint. Allogenic dermal fibroblasts have been shown to be acceptable immunologically, and a source is to some extent available, from human foreskins. Dermal fibroblasts have proven inferior to vascular fibroblasts for vascular prostheses. Besides fibroblasts, endothelial cells from the human umbilical vein may be a suitable source for TE arteries (Shinoka et al., 1996 and 1997).

II Autologous cells:

Therapies that use a patient's own cells are safest from an immunologic point of view. However, these methods may not always be available. For example, many surgeons are not enthusiastic about performing two operations, (i.e. one to harvest cells, and another, weeks later, to implant a cell seeded scaffold) because of the additional costs and time issues. Even when harvesting a patient's cells for immediate implantation there are two surgical sites, i.e. the implantation site and the harvest site. In these cases, there may be donor site morbidity, including infection and chronic pain, as well as additional surgical costs. Finally, a very ill or elderly patient may not have sufficient viable cells, to establish useful cell lines. For each of these reasons, there is significant interest in having an "off-the-shelf" supply of donor cells. These cells would be expanded *in vitro* and immortalized. Foetal or neonatal tissues are extremely useful for this purpose since they are non-immunogenic and are a rich source of stem cells. This approach, however, is a very controversial ethical issue.

III Stem cells:

Stem cells have the ability to divide in culture and give rise to specialized cells. In order to understand the potential of stem cells a short overview is presented. A human fertilized egg is a totipotent cell, meaning that its potential is total. In the first hours after fertilization, this cell divides into two identical totipotent cells. Approximately four days after fertilization, these totipotent cells begin to specialize, forming a blastocyst, a hollow sphere of cells. The outer layer of cells will go on to form the placenta and other supporting tissues needed for foetal development in the uterus. The inner layer of cells

in the blastocyst will form virtually all of the tissues of the human body. These cells are pluripotent and give rise to many types of cells and tissues. The pluripotent stem cells undergo further specialization into stem cells that are committed to give rise to cells that have a particular function. Examples of this include blood stem cells that give rise to red blood cells, white blood cells, platelets and skin stem cells that give rise to the various types of skin cells. These more specialized stem cells are called multipotent. While stem cells are extraordinarily important in early human development, multipotent stem cells are also found in children and adults. For example, one of the best-understood stem cells is the blood stem cell. Blood stem cells reside in the bone marrow of humans and perform the critical role of continually replenishing the supply of blood cells throughout life.

Populating a TE scaffold with adult human stem cells may be possible (Young et al., 1998; Alison et al., 2000). Although stem cells have a huge potential for TE applications, the literature review undertaken during this research program did not find references to any research directly related to TE a particular organ or construct from stem cells. To the authors understanding, it appears that researchers do not precisely know how to grow particular organs from universal stem cells.

#### 3.3.2.2 Cell Types

A number of different cell sources have been used to seed various polymer constructs for TEHV fabrication. Most of the experiments used mixed cell sources, usually from sheep, to seed the scaffolds (Shinoka et al., 1995; Sodian et al., 1999 and 2000abc; Hoerstrup et al., 2000a). In this section the most promising results from four different cell types are reviewed and their response to cyclic stress is discussed. This is especially important for cardiac constructs since they have to adapt to peripheral organ demands under the changing conditions of pressure and flow. The aorta, aortic valve and large arteries are able to adapt their thickness due to high collagen and elastin components and function primarily to deliver and distribute blood under high pressure to the various tissue beds.

Endothelial cells (ECs);

ECs form a monolayer that constitutes the primary interface between the bloodstream and all extravascular tissue of the body. The endothelium is strategically located to serve as a sensory tissue that assesses haemodynamic conditions such as blood flow and pressure. In response to haemodynamic factors the endothelium synthesizes and secretes biologically active molecules that control smooth muscle cell tone and the vascular geometry. In recent years experimental evidence has demonstrated the importance of the cyclic stretch on ECs. i.e. cells have been seeded onto combined PHA/PHO scaffolds, following the isolation from segments of ovine carotid arteries. (Shinoka et al. 1995; Hoerstrup et al., 2000a).

Myocytes;

Myocytes are muscle cells that constitute the muscular wall of the heart. Each cell beats independently at different rates and when formed in small clusters they contract spontaneously at a uniform beat rate. Electrical stimulation controls the rate of beats per minute (BPM) of these clusters. Although myocytes are rarely used to seed scaffolds for TE approaches, tests have shown that embryonic cardiomycytes from mice have a significant effect on blood vessel growth (Okamura et al., 2002).

Fibroblasts:

Fibroblasts are found in the connective tissues and secrete fibrillar pro-collagen, which forms collagen that contributes to increased tissue strength. Experiments have shown that the addition of fibroblasts to scaffolds permit secretion of collagen and matrix proteins that maintain structural integrity (Sodian et al., 2000a). In addition, several other cell sources have been proposed for use in creating TEHVs. These include cells from peripheral veins, stem cells, and circulating bone marrow-derived endothelial cells. For example, Stock et al., (2000) reported that myofibroblasts migrated into culture dishes and their number could be expanded separately. When sufficient cell numbers were attained, these myofibroblasts were seeded onto polymer scaffolds after four days of incubation. The cells adhered to the polymer scaffolds, migrated into the pores, and secreted ECM, but the new tissue found was immature, mechanically weak, and lacked structural organization.

Smooth Muscle Cells (SMCs):

Aortic valves originate from the aortic wall during embryonic development and aortic smooth muscle cells (SMCs) are often co-cultured with ECs to engineer TEHVs (Mol et al., 2001). However, SMCs are not found in fully developed heart valves. The media, the middle of three layers in artery walls, contains SMCs that are oriented circumferentially, within an elastin and collagen matrix. The media consists of SMCs and elastin fibers in alternating layers that form lamellar units. The elastin fibers permit distension of the artery while the collagen bundles provide tensile strength, limit distension and prevent disruption. This organization controls to the distribution and magnitude of tensile stress.

#### 3.3.2.3 Establishment of Cell Lines

In order to seed fabricated polymer scaffolds, primary cell line cultures need to be established. In the previous sections, cell types and their sources were reviewed. For the current investigation it was decided that ovine cardiac tissue would be the most logical source as this tissue is easy to obtain and has been widely used in similar investigations (Schinoka et al., 1995; Sodian et al., 2000abc). Thus, at Swinburne University cells from ovine cardiac tissue were used to seed the manually fabricated scaffold.

Experimental Procedure:

Under aseptic conditions a first dissection was made at the anterior surface of the left ventricle of a lamb heart. After exposure of the left ventricle cavity, the aortic valve cusps were exposed. A surface of 5 x 5mm segment of the lamb heart valve leaflet tissue was removed from the aortic semilunar valve and 10 mm of left coronary artery. The tissue was washed three times in phosphate buffered saline (PBS) containing penicillin-streptomycin. After this procedure the tissues were divided in Petri dishes into cubes of 1 x 1 x 1 mm. The ovine tissue-cubes were subsequently allowed to dry in the Petri dishes for approximately 1 hour. After this period 15 ml of Dulbecco's Modified Eagle Medium (DMEM) growth media was added. DMEM is a modification of basal medium eagle that contains four-fold concentrations of the amino acids and vitamins that support primary cultures of cells. The Petri dishes were put into a humidified incubator at 37°C in a 5% of $CO_2$ atmosphere. The medium was changed every 24 hours.

The attempts to create viable cell lines resulted in a negative outcome. Therefore, the following paragraph should be considered as a guideline for future investigators of TEHVs.

Seeding of the scaffold:

After establishing viable cell cultures, cells should be seeded onto the fabricated 3D scaffolds. Scaffolds prepared for this experiment were described in section 3.3.1.6. Seeded scaffolds should be further cultivated and assessed in the developed BR-system. This will be the first step to engineer tissues for heart valve development.

In general, basic seeding requirements include:

1) High yield, to maximize cell utilization.
2) High kinetic rate, to minimize the time in suspension for anchorage-dependent and shear sensitive cells.
3) High and spatially uniform distribution of attached cells, for rapid and uniform tissue growth.

After one to three days the seeded PCL-scaffolds should be transferred into a BR. It is important to ensure that as many cells adhere in a confluent layer around and in the scaffold, before fixating the seeded scaffold into the BR. Although the ideal cell culture conditions are not known for each tissue, research has demonstrated that rotating the scaffold though the culture medium increases the cell attachment rate (Vunjak-Novakovic et al., 1999). However, other parameters such as type of cell and the type of polymer play a significant role in cell-adhesion.

### 3.3.3 Bioreactors

To grow TEHVs under conditions that mimic the *in vivo* environment, seeded scaffold materials should degrade over time. These environments may be created in BRs and can be divided into several categories, depending on the type of tissue required. Much research has been done on the development of skin and cartilage (Eldad et al., 1987; Carver and Heath, 1999) but cardiac TE requires a pulsatile flow of the culture media through the construct. BRs need to incorporate flow parameters in order to simulate the *in vivo* cardiac environment. The following section is divided into two categories: an overview of different BRs for TE in general and for cardiac tissues in particular. Furthermore, four studies where BRs were used to grow TEHVs are presented.

General:

It has been reported that cultured cells grow more like their *in vivo* counterparts if the *in vitro* environment mimics the dynamic physical demands of the *in vivo* environment. Dr. Gail Naughton, President of Advanced Tissue Sciences, Inc. patented a biomimetic culture BR. Cells grown in this BR were resilient and became aligned in the direction of flow. In this BR (Figure 3.9) cells were subjected to a unidirectional flow of media, bringing nutrients in and carrying away metabolites and other wastes. While a BR can enhance culture of cartilage cells and blood vessel cells, the results with TEHVs are particularly encouraging. Grown in a BR, tissue doubled in mechanical strength and secreted increased amount of collagen and elastin compared to cells in petri-dishes (Bubbers et al., 2002).

BRs were developed to mimic the complex *in vivo* environment, including the stimuli that have an impact on cell growth and differentiation. No matter what structure develops in a particular BR, the different stimuli can be divided in four basic categories:

(1) Growth matrix in or on which cells grow.
(2) The chemical and physiological composition of the medium.
(3) Composition of the gas in the incubator.
(4) Incubation temperature.

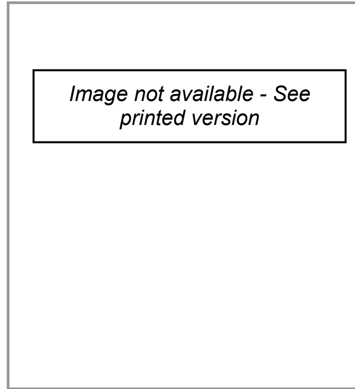



***Figure 3.9***

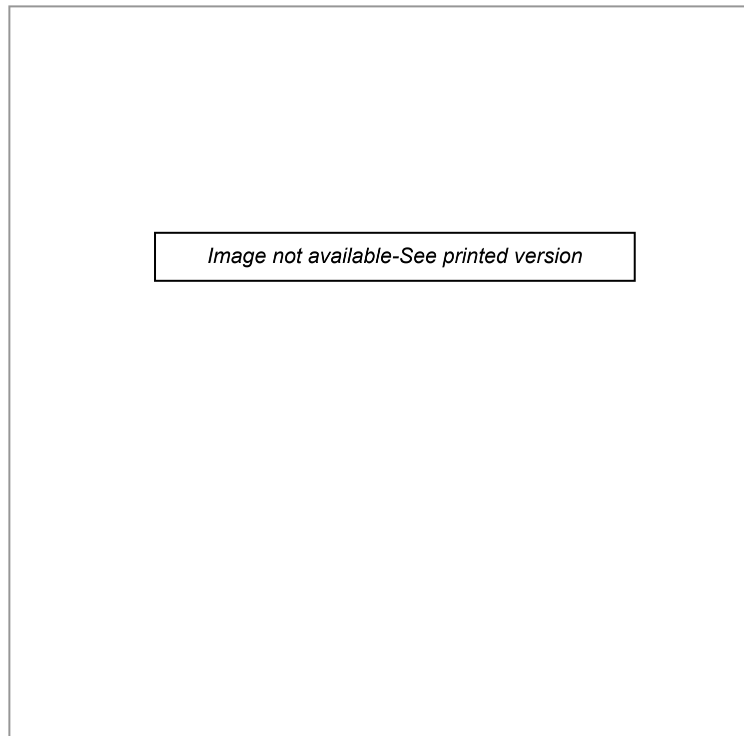
*Gail Naughton's Bioreactor*

(Figure adapted from: http://navier.rug.ac.be/public/biomed/res_kris.html)

However, in order to grow viable heart valve tissue, the four above-mentioned aspects should also include *mechanical stimuli* that mimic *in-vivo* conditions. In addition to this, the BR has to meet several other requirements that include compact size, sterility, low volume, easy refreshment of the medium and access to the TEHV. The use of a pump (heat production) must not disturb the climate in the incubator. The medium should be exposed to a controlled atmosphere (diffusion of $O_2$, $CO_2$, $N_2$) in the incubator. To meet these requirements, a microenvironment must be created with adequate nutrients and without accumulation of metabolites. The BR should allow testing of entire valves as well as discrete parts of it. From a haemodynamic point of view, the BR should be able to provide parameters such as transvalvular pressure gradients, flows and frequencies within certain physiological limits (Bubbers et al., 2002).

#### 3.3.3.1 Overview of Bioreactors (BRs)

Regardless of the type of tissue cultured a particular BR, the enhancement of tissue growth in these environments must fulfil some design principles that were described in the previous section. In this section, several studies are reviewed and presented that demonstrate how BRs can modulate 3D tissue formation *in vitro*. In the second section an overview is presented on the current status of pulsatile BRs and for engineering of cardiovascular constructs. In contrast to a large number of TE studies that focus on scaffold design and *in vivo* tissue repair with cells and/or biomaterials (Sodian et al., 2000d; Hutmacher et al., 2001; Jockenhoevel et al., 2001), there has been relatively little work performed with pulsatile BRs. As described in this chapter, specific BR design features may be used to improve the structure and function of engineered structures.




***Figures 3.10***
*Various Bioreactors:*
*a; flask system, b; Slow Turning Lateral Vessel, c; High Aspect Ratio Vessel d; Rotating Wall Perfused Vessel e; Perfused columns, f; Perfused chambers*

(Figures obtained from: Lanza et al., 1999)

- One of the most basic designs of a BR is a flask system (see Figure 3.10a): It contains 120 ml of culture medium and can contain several TE constructs depending on the size. Flasks are either operated statically or mixed, normally at 50-80 rpm. "Cardiac like tissues cultivated for one week in mixed flasks showed a DNA contents of 16% while the cell-size and the metabolic activity were similar to that in neonatal ventricles" (Bursac et al., 1999). The peripheral region of constructs was electrically excitable and could be captured over a wide range of pacing frequencies (80-270 BPM).
- The same kind of tissue have also been grown in High Aspect Ratio Vessels (HARVs). Slow Turning Lateral Vessels (STLVs) (Fig.3.10b) and HARVs (Fig.3.10c) have been used to engineer cartilage and cardiac tissues. The STLV is configured as the annular space between two concentric cylinders, the inner of which is a gas exchange membrane, whereas the HARV is cylindrical vessel with a gas exchange membrane at its base. Both vessels are operated in a horizontal plane at 15-40 rpm. "Cartilaginous constructs cultured *in vitro* for seven months in STLV's, showed a GAG fraction and equilibrium modulus that reached or exceeded values measured for native cartilage. However, other properties of seven-month constructs remained atypical; i.e. collagen fraction, cross-link concentration were only a third while dynamic stiffness was only half the value as reported in native cartilage" (Schwartz et al., 1992; Prewett et al., 1993). However, engineered tissues grown in rotating vessels were structurally and functionally superior to constructs grown in either static or mixed flasks.

- Rotating Wall Perfused Vessels (Figure 3.10d) were developed by the National Aeronautics and Space administration (NASA) and used to engineer cartilage in a microgravity environment of space and a control study on Earth (Vunjak-Novakovic et al., 1999).

- Medium was continuously re-circulated between the column and an external membrane in perfused columns (Fig.3.10e). Perfused chambers (Fig.3.10f) were designed to allow tissue culture in the microgravity of space. The chambers can contain different volumes of culture media up to 30 ml. and hold up to five

constructs. The medium is continuously circulated between the chamber and an external membrane at rates of 1-30 ml per construct a day (Lanza et al., 1999).

Although the mechanisms underlying these effects are yet to be determined, it appears that hydrodynamic forces affect cultured cells via pressure fluctuations and/or shear stress, stretching the cell membrane. These BR functions may result in increased size and improved structure and function of engineered tissues. The BR systems described promoted mass transfer to the construct surface, but did not enhance the relatively slow diffusion of nutrients to the construct interior. This is not a major consideration for engineering cartilage, an avascular tissue with low cellularity that can be cultivated in BRs to a thickness that exceeds that of native cartilage. However, efforts to engineer tissue that has high vascularity and/or cellularity are limited and in particular, very low tissue formation has been reported for cardiac tissue (Dunkelman et al., 1995).

Other groups have demonstrated advantages using BR systems that provide continuous perfusion and mechanical stimulation during cultivation, such as seeded TEHV-constructs subjected to recirculated culture media though a closed loop (Hoerstup et al., 2000a; Sodian 2000abc). In each of these cases perfusion of media led to increased tissue growth and metabolism. In addition, closed loop perfusion BRs reduce the risk of contamination during long-term cultivation. The finding that physical stimuli modulate tissue development has motivated the design of several BR systems in which growing tissues are exposed to mechanical forces (Hoerstup 2000b).

In summary, each of the above studies showed that the presence of mechanical forces (externally applied or internally generated) during culture stimulated the development of the engineered tissues. This author believes that the stimulation is directly related to the physical stimuli, normally present *in vivo*.

#### 3.3.3.2 Pulsatile Bioreactors (PBRs)

In the development of cardiac tissues and in particular valve leaflets, the use of mechanical forces plays a key-role. The haemodynamic function and performance of TEHVs can be improved by exposing developing tissue to physiological stresses *in vitro*. Therefore, a pulsatile-fluid-flow-BR (PBR) has been developed to provide physiological pressure and fluid flows. PBRs provide mechanical conditioning of constructs in the form of pulsatile culture media flow that mimics *in vivo* conditions. PBRs require a sophisticated pump-system to generate cyclic fluid-flow inside the chamber. The generation of such flows is still experimental, as the ideal conditions for tissue growth are not yet established. Generated flows should be directed through the centre of the constrained cardiac construct, mimicking *in vivo* simulation. This approach, to stimulate cell growth *in vitro,* is different to the mechanical stimuli described in the previous section. The following section describes and compares four investigations where a PBR was used to culture TEHVs.

In addition to the four reviewed studies in the next section (section 3.4), a BR system has been designed for TE blood vessels. This system consists of three tubes assembled into a parallel horizontal flow system where scaffolds may be secured in the tubes (Niklason and Langer, 1997). The culture media is pumped through these tubes by a pulsatile pump that is controlled by a compliance chamber. In order to control the pulsatile flow, the BR was placed on a magnetic stir plate. The BR was also connected to an open medium reservoir to provide gas-exchange. The compliance chamber consisted of a 300-ml plastic reservoir that minimized the transmission of high frequency vibrations to the BRs. The flow of the culture media was applied directly through the BR at 165 BPM with 5% radial distension (strain).

## 3.4 PBRs used for TEHVs

Most PBRs described in the literature are able to provide pulsatile flow and consist of three major components i.e. two chambers separated by a diaphragm (Figure 3.11).

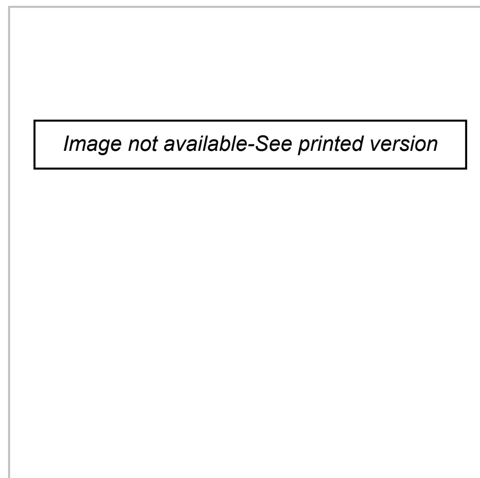



***Figure 3.11***

*Cross-sectional view of the pulsatile flow bioreactor*

(Figure from: Hoerstrup et al., 2000b)

The bottom chamber (1) is filled with air, and the upper chamber (2) is a dual-compartment fluid chamber. A silicone membrane (3) divides the two compartments. A sterilized TEHV construct is mounted onto a removable silicone tube (5), which is then slipped onto the fixed silicone tube (4) in the PBR. The air chamber (1) is connected to a respirator, and when air is cyclically pumped into the lower chamber (2a), the silicone diaphragm (3) is periodically displaced, pushing fluid through the TEHV and into the perfusion chamber (2b). PBR systems like these are particularly appropriate for long term culture of cardiac neo-tissues, in particular blood vessels or heart valves (Hoerstrup et al., 2000b; Dumont et al., 2001).

In the following paragraph the outcomes from four studies involving this type of PBR are described. The generated fluid flow and pressures created inside the PBRs are determined by the pump-system that drives it. In the design proposed by Hoerstrup et al. (2000b) pulsatile flows ranging from 50 – 2.000 ml/min and systemic pressures from 10 – 240 mmHg were generated using a respirator pump. Using this PBR, different TEHVs can be exposed to increasing levels of pulsatile flow and pressure *in vitro*. Following culture, the cellular response to different constructs can be evaluated with histological or biochemical techniques.

- In a study carried out by Sodian et al., (1999) the described PBR was started at a low flow condition of 140 ml/min and a systolic pressure of 10 mmHg. Over time the flow rate was increased to 350 ml/ min and the systolic pressure was set at 13 mmHg. Mixed vascular cells from adult ovine carotid artery were cultured and seeded on a tri-leaflet scaffold prior to the experiment. It was observed that by day 4 a nearly confluent cell layer was formed over the whole construct. Furthermore, leaflet cells oriented in the direction of flow and cells on the conduit wall formed bridges between pores (Sodian et al., 1999). In this study the mechanical strength of the cultured TEHV was not tested, however, ECG-tests showed no pulmonary regurgitation. Furthermore, some thickening of the valve structure was observed without functional loss. Another interesting outcome of this study was that significantly more cells and collagen on the cultured constructs were detected compared to static conditioning.

- Another study conducted by Sodian et al., (2000a), tested the PBR under similar conditions as described in the experiment above. Under these conditions, the fabricated TEHVs were found to open and close synchronously with pulsatile flow. In this study tri-leaflet scaffolds constructed from salt leached PHA were used. According to the Environmental Scanning Electron Microscopy (ESEM) analysis cells were attached to the scaffold in a nearly confluent manner and oriented themselves in the direction of flow. Connective tissue was demonstrated within the pores of the scaffold after 4 days in the PBR. Movat staining demonstrated that the formed ECM contained both collagen and GAGs,

but not elastin. DNA and 4-hydroxyproline assays demonstrated that exposure to flow increased the cell density and the amount of collagen formed in the TEHVs compared to constructs cultured in static conditions (Sodian et al., 2000a). Therefore, it was concluded that the pre-conditioning of the constructs with flow strengthens the constructs.

- In a third study carried out by Sodian et al., (2000b) the BPR was used with low flow conditions of 100 ml/min for 1h. Cells were derived from a mixed population that included fibroblasts, SMCs, and ECs isolated from ovine arteries. Through a salt-leaching process, porous tri-leaflet scaffolds were constructed from PGA, PHA and P4HB. "The PHA and P4HB-constructs showed an almost confluent cell layer by day 8 and more cells attached to the PGA material than the PHA and P4HB constructs". Significantly more collagen was detected on PGA constructs when compared to the PHA & P4HB constructs (Sodian et al., 2000b).

- An experiment with more physiologically relevant pressures was performed by Hoerstrup and colleagues (2000a). In this study TEHVs fabricated from PGA scaffolds were coated with P4HB. These constructs were placed in a PBR for 21 days. The experiment began with a flow condition of 125 ml/min and a systolic pressure of 30 mmHg. During the investigation both the flow rate and systolic pressure were gradually increased to 750 ml/min and 55 mmHg repectively. In addition, these pressures approached the maximum pressures (56 –70 mmHg) sustained by a normal aortic heart valve in the circumferential direction at the points of leaflet attachment to the conduit wall (Silver, 1994). In contrast to findings that TEHVs were fragile and disintegrating after 14 days of culturing in static conditions, TEHVs cultured for a similar time period in the PBR were intact, pliable, and competent in closure.

Following a pre-conditioning regimen, the valve substitutes engineered by Hoerstrup et al. (2000a) were implanted into the supravalvular position of pulmonary arteries in lambs. No rejection was detected as the implanted constructs were engineered from the

same lambs from which the cells were initially harvested (Sodian et al., 2000d). Prior to implantation, each construct was subjected to high-pressure testing, with pressures > 150 mmHg for 1h. *In vivo* valve function was evaluated via a Doppler echocardiogram. The TEHVs functioned for up to twenty weeks without stenosis, thrombosis, or aneurysm formation, but moderate pulmonary regurgitation and inflammation were observed between 16 - 20 weeks. Histological analysis revealed a patchy EC-layer and incomplete polymer degradation. The mechanical strength of the TEHV conduit walls was also determined using an Instron mechanical testing apparatus. The stress-strain curve the TEHV resembled that of a native pulmonary artery (Sodian et al., 2000).

In summary, several studies have been performed with cultured TEHVs under pulsatile flows within the last four years. It was noted that all constructs in these studies were seeded with cells under static conditions prior to culture. A detailed summary of the 4 reviewed experiments using a PBR to culture TEHVs is presented in **Appendix A5**.