IRIS, Industrial Research Institute Swinburne
Swinburne University of Technology

Design, Development and Optimisation of a Tissue Culture Vessel
System for Tissue Engineering Applications

By

BAS STEFAAN DAMEN

B.E. (Biomedical)
Hanzehogeschool, Hogeschool van Groningen
The Netherlands

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Statement of Originality

I hereby declare that, the submission is my own work. To the best of my knowledge it contains no previous published material written for any award, degree or diploma except where due acknowledgements or references are made in the text.

Signed:

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Bas Stèfaan Damen
(November 2003)
Abstract

A Tissue Engineering (TE) approach to heart valve replacement has the aim of producing an implant that is identical to healthy tissue in morphology, function and immune recognition. The aim is to harvest tissue from a patient, establish cells in culture from this tissue and then use these cells to grow a new tissue in a desired shape for the implant. The scaffold material that supports the growth of cells into a desired shape may be composed of a biodegradable polymer that degrades over time, so that the final engineered implant is composed entirely of living tissue. The approach used at Swinburne University was to induce the desired mechanical and functional properties of tissue and is to be developed in an environment subjected to flow stresses that mimicked the haemodynamic forces that natural tissue experiences. The full attainment of natural biomechanical and morphological properties of a TE structure has not as yet been demonstrated.

In this thesis a review of Tissue Engineering of Heart Valves (TEHVs) is presented followed by an assessment of biocompatible materials currently used for TEHVs. The thrust of the work was the design and development of a Bioreactor (BR) system, capable of simulating the corresponding haemodynamic forces in vitro so that long-term cultivation of TEHVs and/or other structures can be mimicked. A full description of the developed BR and the verification of its functionality under various physiological conditions using Laser Doppler Anemometry (LDA) are given. An analysis of the fluid flow and shear stress forces in and around a heart valve scaffold is also provided.

Finally, preliminary results related to a fabricated aortic TEHV-scaffold and the developed cell culture systems are presented and discussed. Attempts to establish viable cell lines from ovine cardiac tissue are also reported.
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LIST OF ABBREVIATIONS
BD  =  Biodegradable
BPM  =  Beats Per Minute
BR  =  Bioreactor
CAD  =  Computer Aided Design
CFD  =  Computational Fluid Dynamics
CT  =  Computed Tomography
DLPLA  =  Poly dl-lactide Acid
DMEM  =  Dulbecco’s Modified Eagle Medium
DSCP  =  Dual Stereo Camera Photogrammetry
EC  =  Endothelial Cell
ECM  =  Extra Cellular Matrix
FDM  =  Fused Deposit Modelling
GAG  =  Glycosaminoglycan
HWA  =  Hot Wire Anemometry
LDA  =  Laser Doppler Anemometry
LPLA  =  Poly l-lactide Acid
MRI  =  Magnetic Resonance Imaging
MVP  =  Mitral Valve Prolapse
NMR  =  Nuclear Magnetic Resonance
NSF  =  National Science Foundation
P3HB  =  Poly (3-hydroxy butyrate)
P4HB  =  Poly (4-hydroxy butyrate)
PBR  =  Pulsatile Bioreactor
PBS  =  Phosphate Buffered Saline
PCL  =  Poly ε-caprolactone
PDCV  =  Proportional Directional Control Valve
PDO  =  Polydioxanone
PGA  =  Poly-glycolic Acid
PHA  =  Poly-hydroxy Alkanoate
PIV  =  Particle Image Velocimetry
PLA  =  Poly-lactide Acid
PS  =  Prototype System
RBC  =  Red Blood Cell
SLE  =  Systemic Lupus Erythematosus
SMC  =  Smooth Muscle Cell
TE  =  Tissue Engineering
TEE  =  Trans Esophageal Echocardiography
TEHV  =  Tissue Engineered Heart Valve
TMC  =  Tri-methylene
TTE  =  Trans Thoracic Echocardiography
VAD  =  Ventricle Assist Devices
VHD  =  Valvular Heart Disease

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INTRODUCTION

Cardiovascular disease is among the leading causes of death in the world today. According to the National Heart Foundation, one patient dies prematurely from cardiovascular disease every ten minutes in Australia. In 1999, cardiovascular disease caused over 50,000 deaths, or 40% of all deaths in Australia\(^1\). Heart valve defects are one of the major causes of cardiovascular disease and their surgical replacement is considered routine and effective. These procedures account for more than 80% of related surgery cases in Australia. Unfortunately, these valve replacements are at present associated with many deficiencies.

Deficiencies are primarily due to poor haemodynamic performance of the replacement valve. In the early fifties Charles Hufnagel implanted the first artificial heart valve in a human. Since that time research and development of a biocompatible, haemodynamically efficient and structurally sound prosthetic heart valve has been under continual development. There have been many modifications and engineering improvements since, but an ideal mechanical heart valve has yet to emerge (Ellis et al., 1998). Major problems associated with tissue and mechanical heart valve replacements include thrombosis, haemolysis and valve deterioration. Despite these problems the prognosis for patients who receive a heart valve replacement is reasonable and they usually are able to live a relatively normal life after implantation. Nevertheless, it is important to note that to improve this prognosis, further research into heart valve replacements and cardiovascular disease in general is vital. Heart valve research is an area that still requires a great deal of advancement from a social, medical and financial point of view. Further progress in heart disease related research relies not only with medical practitioners, but also with material scientists, engineers and chemists.

\(^{1}\) www.heartfoundation.com.au
Currently, clinically used replacement valves are constructed from synthetic materials or xenogenic/allogenic tissues. The “Holy Grail” of a valve substitute would be a non-obstructive, non-thrombogenic, durable, living structure that may easily be implanted into the recipient. The current inability of valve replacements to grow within a recipient is one important missing characteristic of current prosthetic valves. This limitation is most detrimental in young patients because successive surgery is needed to replace implanted valves that do not grow with the patient (O’Brien et al., 1999). Other important characteristics of an ideal replacement valve include a prompt and complete closure, resistance to infection and a quiet operating mechanism. These desired characteristics are only partly addressed by current prosthetic heart valves and there is a need for a new generation of valve replacements that are based on a functional copy of the healthy native heart valve. In theory, this replacement valve could be grown from the patient’s own cells on a valve shaped scaffold and an autologous source of cells would avoid rejection by the patient after implantation (Shinoka et al., 1995).

Tissue Engineering (TE) techniques may be used to grow heart valves from cells in vitro on biodegradable scaffolds in a Bioreactor (BR). BRs are constantly being developed in order to create an ideal environment for tissue growth. Environmental factors such as fluid-flow and pressure cycles have an important influence on cell differentiation and behaviour during cell culture and tissue production. These factors are crucial to the growth of cardiac tissue in particular and BRs need to provide these environmental factors to mimic the in vivo environment. One of the difficulties associated with growing cardiac tissue in a BR is to determine the optimal system conditions. Another difficulty is to monitor the hydrodynamic performance during all stages of tissue-development. At present, no studies with BRs for TE applications in relation to flow characteristics have been reported. This represents a large gap in the knowledge required for the efficient use of BR-systems for culturing Tissue Engineered Heart Valves (TEHVs) and/or other tissue constructs. In spite of the importance of these parameters, the dynamic stresses applied to developing tissues are poorly understood. Although a number of studies have concluded that cyclic flow is beneficial to tissue development, they do not verify measurements of the stresses produced in a BR (Sodian et al., 1999 and 2000abc; Hoerstrup et al., 2000a). As a result, the assessment of tissue
cultured in vitro in BRs may be based on incorrect assumptions. Most previous work was directed toward the assessment of the tissue without determining if these features were obtained under controlled conditions. Furthermore, in the field of BR design, only a small number of innovative alternatives have been proposed. Optimisation of tissue development and an understanding of the biomechanical requirements of engineered tissues grown in vitro is still in an early exploratory stage (Hoerstrup et al., 2000b; Dumont et al., 2001). In summary, it appears that new and innovative research is required.

In this study two BR systems have been proposed. Both BRs are bio-mimetic systems that may be used to engineer different types of tissue. Moreover, both BR-systems can be used to monitor hydrodynamic performance - before, during and after a specific cell cultivation period. A detailed model representing the hydrodynamic characteristics of TEHVs may be obtained that can provide a better understanding of flow related valve complications (Wright and Temple, 1971; Chadran and Cabell, 1984). Besides TEHVs, other tissue replacements may be dynamically tested with the proposed systems. Both systems provide a chamber that can be used to analyse decellularized biomatrices, native heart valves and heart valve replacements. In this study, the verification of these systems has been performed and may improve the understanding of engineering tissues under various flow conditions. The overall aim of this investigation was to investigate the characteristics of generated fluid-flows. Specifically, this study investigated the dynamic performance of BR-systems to provide data for future investigations related to TE applications.

The major outcomes of the experimental procedures of the current study are summarized as follows:

- The hydrodynamic performance of the Prototype System (PS) was investigated to verify its operating principle. In the designed circulatory system the pulsatile flow conditions created by the PS were determined and verified, with the aid of Laser Doppler Anemometry (LDA). Throughout this experiment the pump rate was maintained at 80 beats per minute (BPM). The measurements were taken in
the growth chamber, at twelve downstream locations inside a silicone tubular construct with an internal diameter of 8 mm. At these locations the velocity profiles inside the construct were similar in shape and magnitude to the input provided by the driver. Shear stresses $\leq 172$ Pa and axial velocities of 7 m/s were measured during the acceleration and peak phase in the test-tube. The prototype made from Plexiglass demonstrated adequate performance in the laboratory, however, shortcomings such as autoclave procedures and access to the TE-construct inside the BR resulted in a second BR-system: i.e. the Pulsatile Bioreactor (PBR).

The PBR had the same dimensions and used the same working principle as the PS. To verify the performance of both drivers, the produced flow rates in the PBR using the Proportional Directional Control Valve (PDCV) and the pulse duplicator were measured and compared. During the dynamic evaluation of the system, pulse rates ranging from 60 – 160 BPM and inlet pressures from 150 - 760 mmHg were investigated. Using the PDCV, flow rates ranging from $0.5 \times 10^3$ – $1.5 \times 10^3$ ml/min were investigated, on the other hand a maximum flow rate of $6.33 \times 10^3$ ml/min, was generated by the pulse duplicator.
1.1 Literature Overview

This section provides an overview of previous investigations relevant to the present study. The major elements, conclusion and justification of the literature overview are divided into five categories (I-V) as shown below:

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I  Formulation of the problem

During the past four decades, approximately three million prosthetic heart valves have been implanted worldwide. These prosthetic devices have since been subjected to many modifications and engineering improvements, but the ideal heart valve has yet to emerge (Ellis et al., 1998). The most comprehensive reviews associated with the problems related to tissue and mechanical prosthetic heart valve devices were carried out by Wieting et al. (1966), Wright and Temple, (1971) and Magilligan et al. (1980). These studies demonstrated that the major problems associated with the clinical use of these devices include thrombosis, haemolysis, tissue overgrowth, infection (endocarditis), calcification, valve wear, and excessive pressure gradients. The problems with most existing mechanical heart valve replacements have been well documented and a number of alternative designs have been suggested and dynamically examined by Chadran and Cabell, (1984) Teijeira and Mikhail, (1992) Morsi and Sakhaeimanesh, (2000).
Mechanical valves:
One of the main problems with all mechanical valves is the increased risk of blood clotting (thrombosis). When blood clots occur in the heart, there is a high risk of a heart attack. According to Caro et al., (1978), appearance of these blood clots is strongly influenced by the altered local fluid mechanics caused by the implanted mechanical heart valve. Recent evidence (DeWood, 1980) has established that a major proportion of shear stresses associated with thrombosis occur as a result of atherosclerotic plaque in a stenosis. Another problem with most mechanical valves is that there is a gap between the disc edge and the inside wall of the housing, that prevents jamming (sticking) between the disc and the housing. The size of this gap determines the leakage flow during the closed phase of the valve cycle. These leakage gaps may lead to increased haemolysis due to the high shear stresses within the gap flow and within the turbulent mixing region of the backflow jet. Knott et al., (1988) reported that these leakage jet velocities are three to five times higher than the peak forward flow velocities. Critical shear stresses on human red blood cells (RBC) has been extensively investigated by Blackshear et al., (1965) and Sallam et al., (1984). The findings from these studies indicate that shear stresses in the range of 1500 - 4000 dynes/cm$^2$ caused lethal damage to RBCs. However, much lower levels of red cell destruction are possible if the total exposure time is low. Shear stresses as low as 500 dynes/cm$^2$ may be clinically important, and shear stresses in the order of 10 dynes/cm$^2$ may actually destroy red cells in the vicinity of a foreign surface such as an implanted mechanical heart valve.

Tissue valves:
Tissue valves can be divided into three different types. The first type discussed is a valve transplanted from a human cadaver called an allograft. These valves tend to have exceptionally good haemodynamic profiles and a low incidence of thromboembolic complications and do not require chronic anticoagulation (O’Brien et al., 1987). However, such allografts are unable to grow, remodel or exhibit active metabolic functions and their usual degeneration cannot be attributed to immunologic responses alone (Michell et al., 1998). A second type of biological valve replacement is obtained through the Ross procedure (Ross, 1967) has become widely accepted. In this procedure, the abnormal aortic valve is removed and the patient's own pulmonary valve
is transplanted into the aortic position. A homograft pulmonary valve is then used to replace the patient's pulmonary valve. The third type of valve is made from animal valve tissue (typically porcine) or from of non-valve tissue such as bovine pericardium. All different types of tissue valve have low rates of thromboembolism and do not require anticoagulation therapy (Schoen et al., 1992). Stroke and bleeding problems rarely occur with tissue valves but valve failure with structural dysfunction due to progressive tissue deterioration as a result of calcification is a serious disadvantage that undermines the attractiveness of tissue valve substitutes. Borttolotti et al., (1987) reported that the degradation mechanisms of bio-prosthetic tissue valves are progressive and the rate of failure is time dependent. Generally, tissue valves need replacement within ten to fifteen years. Bovine pericardial valves suffer from poor durability and usually perform worse than porcine valves.

II Tissue Engineering (TE)

A new promising alternative that may overcome the shortcomings associated with current heart valves substitutes is a TE approach to grow heart valve replacements. The technique focuses on growing a functionally identical copy of a healthy native heart valve in vitro.

The reconstruction of living tissues was pioneered by Bell and his colleagues in the early eighties (Bell et al., 1981). Years later the developments in this field and the definition of TE as “an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain or improve tissue or organ functions”, were summarised by Langer and Vacanti (1993). Using TE it may be possible to grow TEHVs that are superior to existing prostheses. TEHVs may be grown in different environments i.e. in vivo and in vitro, according to two strategies. The first strategy utilizes acellular, natural biomatrices, while the second TEHV strategy uses degradable polymeric scaffolds shaped into heart valve geometries.
- **Using biomatrices:**
The most extensive studies using natural bio-matrices as a scaffold to culture TEHVs, were carried out by Bader et al., (1998); O’Brien et al., (1999) and Steinhoff et al., (2000). Bader and colleagues used decellularized porcine aortic valves and repopulated the scaffolds with cultured cells. In order to remove the native cells from a biomatrix, a similar approach was presented by Steinhoff et al., (2000). The major difference in this study was that ovine pulmonary valves were used. Additionally, Steinhoff et al. (2000) implanted its TEHV constructs into lambs. O’Brien et al., (1999) successfully used a non-detergent-based decellularization process that conditioned porcine aortic valve tissue but did not repopulate the biomatrices with cells prior to implantation into female sheep.

- **Using polymer scaffolds:**
The second strategy used biodegradable polymers to create TEHVs. Most of the initial research in this area was performed by Shinoka and colleagues in 1995, who successfully constructed a TEHV-leaflet from woven and non-woven polyglycolic acid (PGA) mesh sheets. They also fabricated leaflets from polyglactin sheets sandwiched between PGA mesh sheets. These materials were stiff, thick, and non-pliable and did not form non-stenotic, tri-leaflet heart valves. Also, fibrous PGA meshes had insufficient strength to withstand in vivo flow conditions. According to a study conducted by Jockenhoevel et al., (2001) the PGA polymer demonstrated problems such as toxic degradation and an inflammatory response. This group is now investigating the use of fibrin gel as an alternative scaffold material. Sodian et al., (2000b) investigated several other naturally occurring thermoplastic polymers such as; PHA, P4HB, and PHO. These polymers have been used to fabricate different TEHV-scaffolds and were used in several experiments. The outcomes of these experiments demonstrated significant differences in cell-attachment, mechanical strength and tissue-composition. It was earlier suggested by Sodian et al., (1999) that the combination of biodegradable polymers, such as PGA and PHA into one scaffold may lead to the creation of an identical copy of a functional native valve. Results demonstrated a nearly confluent cell layer around the complete...
scaffold and leaflet-cells were oriented in the flow direction after four days of culturing. Similar approaches have been presented by Hørstrup et al., (2000a) and Stock et al., (2000). These groups used a P4HB-coating on a PGA-scaffold, while a second type of scaffold was fabricated from PHO, PGA and PDO.

Industrial processing of 3D heart valve scaffolds was introduced as desirable and described in detail by Hutmacher et al., (2001). This team used a rapid prototyping technology known as Fused Deposit Modelling (FDM) to process a biodegradable polymer (poly ε-caprolactone) into a 3D scaffold. An additional cell culture study carried out by the same group demonstrated that after the scaffold was fully 3D-filled, seeded fibroblasts and osteoblast-like cells proliferated, differentiated and produced cellular tissue over a period of three to four weeks of static in vitro culture.

III Specific Studies related to the research theme

Twenty years ago it was recognized that cultured cells would grow more like their in vivo counterparts if their in vitro environment was not a static culture dish but a dynamic physiologic environment. Hence, a BR may provide this environment. One of the most basic BRs used is the flask system described by Bursac et al., (1999) and was used to culture cardiac tissues for one week. In the same year, Vunjak-Novakovic (1999) described a BR that mimicked the weightlessness of space and 3D liver tissues appeared to grow more naturally, i.e. tissue developed more naturally in microgravity, forming spherical clusters of functioning liver cells. Schwartz (1992) and Prewett (1993) described BRs that were rotated and operated in a horizontal plane and were used to engineer cartilage and cardiac tissues in Earth-based studies. An interesting outcome from these studies was that the dynamic environment simulated in these rotating vessels resulted in developed tissues that were metabolically and mechanically functional. Moreover, the engineered tissues grown in rotating vessels were structurally and functionally superior to constructs developed in either static or mixed flasks. It appears
that environmental factors, such as flow and pressure, have an important impact on cell differentiation and behaviour in *in vitro* cell culture systems. This is particularly important for cardiac tissue, as this type of tissue is constantly subjected to changing environmental factors *in vivo*. Various investigators have investigated experimental systems, able to provide changing environmental factors. Benbrahin et al., (1994) presented a ‘vascular simulating device’- a compliant tubular system that enabled the study of vascular cells under physiologic flow and pressure conditions. Niklason et al., (1997) established a system that created pulsatile flow under sterile conditions for long-term experiments to engineer an artificial blood vessel. This resulted in formation of solid vascular tissue able to withstand *in vivo* conditions.

The first described BR suitable for growth of TEHVs in a vertical position was developed by Hoerstup et al., (2000b) who used a respirator pump that converted air pressure into a fluid-flow through a fibrillating membrane. With this pump, flow rates from 50 - 2000 ml/min could be transmitted to the tissue constructs. Sodian et al., (2001) developed a pulsatile BR driven by a dual-phase control ventilator, able to condition tissue engineered patches *in vitro* for the use in cardiovascular surgery. A piston-system that generated the pulsatile flow for growing heart valves was described by Dumont et al., (2001). With this system, different types of settings provided a wide range of physiological flows that could be applied to developing tissue. No tissue was grown during the study carried out by Dumont (2001) but the BR was tested for the biocompatibility of its system components with two different sterilisation techniques. Sarraf et al., (2002) described a micro-stepping motor used to drive a peristaltic pump that was able to expose the construct to a cyclic load. Furthermore, a description of a system that could measure reaction forces of connective tissues using a force transducer was provided. It is unclear whether each of these two systems can be integrated with each other.
Measurement techniques:
To justify the dynamic performance of BRs and heart valves in vitro, different measurement techniques have been used. This was determined either experimentally or theoretically. Computer models have been used to build patient-parameterised models able to give detailed predictions on flow shears and recirculation zones of cardiac systems under various conditions. Moreover, to obtain real-time data directly different experimental techniques have been used by various researchers.

The first technique reviewed in this section is thermal anemometry i.e. using hotwires and hot-films. Both techniques rely on the principal that the electrical resistance of a metal conductor is a function of its temperature when used as a resistance thermometer, provided with enough current to heat the thermometers above the temperature of the surrounding fluid, the convective heat loss to the fluid can be related to the real-time velocity of the fluid (George and Taulbee, 1990).

Particle Image Velocimetry (PIV) is a planar measurement technique wherein a pulsed laser light sheet is used to illuminate a flow field seeded with tracer particles small enough to accurately follow the flow in 2D and 3D. The positions of the particles are recorded on either photographic film or by digital cameras at each instant the light sheet is pulsed. This system was used to investigate the pulsatile flow characteristics of a cerebrovascular aneurysm at Nanyang Technological University and Tan Tock Seng Hospital in Singapore (Yu et al., 1997). Later Lim and colleagues have also used this technique in 1998 (Lim et al., 1998). In their study four different kinds of prosthetic aortic heart valves were compared under steady flow conditions.

Flow visualisation has been widely used. This technique uses a dye to visualize the flow through valvular constructs to determine dynamic performance. Vieira et al., (1999) used this technique to study flow disturbances in two types of bio-prostheses.

The Laser Doppler Anemometer, or LDA, is a widely accepted tool for fluid dynamic investigations in gases and liquids and has been used as such for more than three decades. It is a well-established technique that provides information about flow velocity.
and the application of this technique for the evaluation of heart valve function was documented by Chew et al., (1983); Hanle et al., (1986); Yoganatan et al., (1989) and Morsi et al., (2000).

Dual camera stereo photogrammetry (DCSP) can be applied to record the \textit{in vitro} leaflet motion of bioprosthetic heart valves using digital cameras. By recording the mechanical deformation of the leaflets, it is possible to analyse the dynamic stresses and strains in the leaflets during opening and closing phases. Lo and Vesely used this technique in 1995 to quantify irregular surface geometry of aortic heart valves and also performed stress analyses under static and dynamic conditions. Gao et al., (2000) developed the study further by evaluating the performance of bioprosthetic heart valves.

\section*{IV Conclusions based on the reviewed literature}

By reviewing the literature associated with the engineering of cardiac structures it is apparent that, although some attempts have promising results, the optimal conditions to grow cardiac structures have not yet been determined. With great precaution it can be suggested that the more closely in \textit{vivo} circumstances inside a culture system is mimicked, the more likely it is that cultured cells will proliferate, differentiate and produce matrix comparable to native cardiac valves. By understanding the ideal settings of a BR-system it may be possible to grow a tissue substitute with similar performance and structural and mechanical properties as a native cardiac valve.

However, the ideal settings for growing cardiac structures are highly dependent on the design of the BR and especially on the system that drives it. Different drivers have been proposed by investigators to mimic \textit{in vivo} conditions. None of the systems currently appear able to generate flow-rates comparable to the flow generated by a native heart. Furthermore, the literature did not refer to any BR-designs that enabled culture of blood vessels and heart valves simultaneously (Niklason et al., 1997; Huerstrup et al., 2000b).
Both heart valves and blood vessels are closely integrated and in vivo studies and would make such a BR design ‘multifunctional’.

In addition, a measurement system that determines and evaluates the dynamic performance of the BR system during the different stages of a cultivation-period was desirable (Chew et al., 1983; Lim et al., 1998; Gao et al., 2000). Using such a measurement system, it would be possible to determine the output generated by the input settings of the driver.

V Justification of this study

Based on the conclusions formed from the literature review, two ‘multifunctional’ BR-systems were developed. Both systems were able to supply adjustable fluid flows to decellularized biomatrices, native heart valves and existing heart valve replacement prostheses. In addition, other structures such as tubes and patches can also be fixed and tested in the proposed BRs. In order to provide the correct dynamic forces, two drivers were proposed to be used: a pulse duplicator able to mimic cardiac cycles closely and an electronic Proportional Directional Control Valve (PDCV).

To determine the output generated by the input settings of the driver during the different stages of a cell-cultivation period, a suitable measurement system was identified and used, namely Laser Doppler Anemometry (LDA). With the aid of LDA, the produced shear stresses and velocities were measured and it was observed that the developed prototype BR produced axial velocities of 7 m/s and shear stresses up to 172 Pa. In addition, the generated flow rates under various settings of both drivers were recorded and compared to in vivo conditions. Flow rates $\leq 6.33 \times 10^3$ ml/min were investigated during these simulations.
1.2 Thesis structure

The initial aim of this research was to determine and compare the dynamic performance of two designed and developed BR-systems, able to support cardiac tissue culture. The BR-systems were compatible with a TE approach that may overcome the existing problems with cardiac valve substitutes. A Prototype System (PS) was introduced and the generated output (fluid-velocity, shear stresses) was measured with a LDA technique. In addition to this, a second system model was designed and developed; i.e. the Pulsatile Bioreactor (PBR). The driver of the PBR was compared to the driver used in the PS-design and both produced flow rates were determined under various settings (pressure and pulse rate). Finally, primary results related to an aortic heart valve scaffold and cell culture were investigated.

There are seven chapters in this thesis and each chapter deals with a specific area of the investigation process. However, chapter two is not a primary part of the presented work and was intended only to provide background information to underline the importance of the experimental procedures carried out in this investigation. Details of this chapter are attached as appendices. In summary, the objectives of the chapters are as follows:

Chapter 2.
Provides an introduction and overview on the heart and its valves, anatomy and function followed by an overview of valvular heart disease (VHD). To detect VHD in its early stages, the most common used non-invasive detection techniques are presented and discussed. Finally, a review of commercially available heart valve replacements is presented. The review also includes a discussion of some problems associated with these devices in clinical use.
Chapter 3

This chapter reviews fundamental aspects of a polymer TE approach that has the potential to overcome the problems associated with current heart valve replacement technology. It highlights the three main integrated components required to TE a heart valve: i.e. scaffold materials, cell-types/sources and \textit{in vitro} culture environments. These sections are reviewed and their relevance to TEHVs discussed. The specific aim for the experimental procedures presented in this chapter was to seed cells on a manually fabricated, porous, biodegradable, tri-leaflet heart valve scaffold.

Chapter 4

Details on the design and development of the PS are discussed in this experimental design chapter. Furthermore a modified version of the PS is presented with particular focus on culturing TEHVs. Both system models were designed to subject constructs to various types of flows and pressures. The final sections of this chapter were used to describe the performance of the drivers of the two developed system models under various settings.

Chapter 5

A wide range of measurement techniques can be used to determine the hydrodynamic performance of BRs and heart valves. In this chapter the most commonly used techniques were reviewed and one technique was selected to determine the characteristics of the PS design (LDA). A second technique (Flow Visualisation) was chosen to determine the dynamic performance of a heart-valve scaffold under various flows inside the growth chamber of the PBR.
Chapter 6

This chapter describes the outline of the experimental set-ups, used to obtain data from the techniques described in previous chapters. In the current study several types of experimental procedures were carried out: LDA-measurements, flow-rate-measurements, cell-culture-protocols, 3-day-observation-periods and a flow-visualisation-procedure. Finally, this chapter discusses and reviews the results from each of these procedures.

Chapter 7

This concluding chapter reports the experimental results of this study. The major outcomes are summarized, limitations discussed and suggestions for further research were proposed that may achieve the ultimate objective: culturing a TEHV in vitro.
CHAPTER 2

HEART DISEASE AND ARTIFICIAL HEART VALVES

2.1 Introduction

Each implant of a heart valve prosthesis is associated with some complications. Either
the recipient requires anticoagulation therapy for their lifetime or the prosthesis requires
surgically replacement after a certain amount of time. This issue is a balance of
relieving the haemodynamic burden of a faulty natural valve and the inherent
imperfections of a prosthesis. Unfortunately, there is no prosthesis to date used to
replace an abnormal cardiac valve that performs as a normal functioning heart
valve. This chapter describes the major problems associated with the implantation of
cardiac valve prostheses. The performance of an implant and the use of reliable
detection methods of valve insufficiency are vital for the patient. The most common
non-invasive techniques used to evaluate valve function are also discussed.

2.2 Anatomy

The heart is situated in the middle of the chest with its long axis oriented from the left
upper abdominal quadrant to the right shoulder. The weight and size of the heart
depends on age, sex, weight, and general nutrition. The adult male human heart weighs
approximately 325 grams and the female heart weighs approximately 275 grams.
The heart consists of four chambers: two atria and two ventricles (Figure 2.1). The atria receive blood from the body via the major veins. The superior and inferior vena cava delivers oxygen-depleted blood from the body to the right atria, while the pulmonary vein delivers freshly re-oxygenated blood from the lungs to the left atria. Blood passes from the atria to the ventricles through the atrioventricular valves. Blood from the right atria flows to the right ventricle, which then pumps the oxygen depleted blood to the lungs to be re-oxygenated. Blood from the left atria passes through the mitral valve, into the left ventricle, which then pumps the blood through the aortic valve with an average mean flow rate of 5 l/min to the rest of the body.

Two types of valves exist in the human heart: bicuspid and tricuspid. The main function of both types of valves is to regulate blood flow through the heart and the valves generally serve three sub-functions: (a) prevent regurgitation of blood from one chamber to another, (b) permit rapid flow without imposing resistance on that flow, and (c) withstand high-pressure loads.

Figure 2.1
Cross-section of a human heart with directions of blood-flow

(Figure obtained from www.heartlab.rri.on.ca)
Basic valve anatomy:
The sequence of events producing a heartbeat is known as the cardiac cycle. During the cycle, each of the four chambers goes through a contraction, called the systole, and a relaxation, called the diastole. In the first phase of the cycle both atria contract, the right first, followed almost instantly by the left atria. This contraction fills the relaxed ventricles with blood. When the ventricles contract, blood is expelled to the lungs and the rest of the body. As they do so, the atria relax and are filled once again by the veins. This cycle lasts, on an average, six-sevenths of a second.

The pressure created by the heart's contraction varies from point to point in the heart and great vessels. Blood returning from the right atrium through veins is under a relatively low pressure of 1 – 2 mmHg. The right ventricle, which delivers blood to the lungs, boosts the pressure to about 20 mmHg during systole. Blood returning from the lungs to the left atrium is once again at a low pressure, rising with contraction to 3-4 mmHg. The left ventricle delivers blood to the body with considerable force. It raises the pressure to about 120 mmHg with contraction, the same as the pressure in the arteries of the body. Between beats, the flow of blood into the capillaries lowers the pressure in the arteries to about 80 mmHg.

The four valves function in the following manners:

- The mitral valve is located between the left atrium and the left ventricle. It is the only valve with two flaps (cusps).
- The tricuspid valve is located on the right side of the heart, between the right atrium and right ventricle. It is made up of three cusps.
- The aortic valve is located on the left side of the heart and opens to allow blood to leave the heart from the left ventricle into the aorta, which is the main artery of the body. It closes to prevent blood from flowing back into the left ventricle.
- The pulmonary valve is situated on the right side of the heart, between the right ventricle and pulmonary artery. It allows blood to exit the heart and enter the lungs via the pulmonary artery. It closes to prevent blood from flowing back into the right ventricle.
Although all four valves have similar tissue structure and function, the aortic valve best demonstrates the principles. Aortic valve cusps open against the aortic wall during systole and close rapidly and completely under minimal reverse pressure, rendering the closed valve fully competent throughout diastole. As these cusps cycle, there are substantial and repetitive changes in size and shape. In particular, the aortic valve cusps have nearly 50% greater area in diastole than systole. This requires complex and cyclical structural rearrangements (Sauren et al., 1980).

The heart valves have a highly layered complex structure and highly specialized, functionally adapted cells and extra cellular matrix (ECM) (Schoen, 1997). A cross-sectional view of a heart valve cusp is shown in Figure 2.2.

The layers are:

- The *ventricularis*, facing the inflow surface is predominantly collagenous with radially aligned elastic fibers.
- The centrally located *spongiosa* is composed of loosely arranged collagen and glycosaminoglycans (GAG’s)
- The *fibrosa*, facing the outflow surface is composed predominantly of circumferentially aligned, densely packed collagen fibers. They are largely arranged parallel to the cuspal free edge (Hoffman-Kim, 2002).

*Figure 2.2*

*Composition of an aortic cusp*

(Figure adapted from: Hoffman-Kim D, 2002)
Interstitial cells populate the matrix of heart valves and express a variety of phenotypes. A proportion of these express smooth muscle alpha actin (Taylor et al., 2000). Of the three different layers, the fibrosa provides the primary strength. The spongiosa appears to lubricate relative movement between the two fibrous layers and dissipate energy by acting as a shock absorber during closure. The elastin of the ventricularis enables the cusps to decrease surface area when the valve is open but stretch to form a large coaptation area when backpressure is applied. Interstitial cells maintain the extracellular matrix. Sufficiently thin to be perfused from the heart's blood, normal human aortic (and other) valve cusps are predominantly avascular. Although the pressure differential across the closed valve induces a large load on the cusps, the fibrous network within the cusps effectively transfers the resultant stresses to the aortic wall and annulus, a ring of tissue that surrounds and supports the aortic orifice (Schoen, 1997).
2.3 Valvular Heart Disease (VHD)

As described in the previous section, four valves control blood flow to and from the body through the heart i.e. the aortic valve, the pulmonary valve, the tricuspid valve, and the mitral valve. Patients with VHD have a malfunction of one or more of these valves. Each of these valves may malfunction because of a birth defect, infection, disease, or trauma. When the malfunction reaches a level of severity so that it interferes with blood flow, an individual will have heart palpitations, fainting spells, and/or difficulty breathing. These symptoms may progressively worsen and can result in death, unless the damaged valve is replaced (Cheitlin, 1991). There are several types of VHDs with distinct symptoms and treatments. These are:

- Mitral valve prolapse (displacement)
- Mitral valve insufficiency (regurgitation)
- Mitral valve stenosis (narrowing)
- Aortic valve insufficiency
- Aortic valve stenosis
- Tricuspid valve insufficiency
- Tricuspid valve stenosis
- Pulmonary valve stenosis
- Pulmonary valve insufficiency

VHD is a non-specific, all-encompassing term for various diseases affecting the heart valves and can be classified into two general categories: congenital and acquired. Congenital VHD is present from birth, and occurs in about 0.6% of non-premature live births. It can be caused by chromosomal abnormalities, such as trisomy 18 or trisomy 21 (Down’s syndrome). In most cases, the causes of congenital valvular disease are unknown. Acquired VHD is more common than congenital VHD. Acquired VHD is generally caused by a disease or injury to the heart, which affects the individual at some point in their lifetime. An autoimmune disorder related to a streptococcus bacterium, acute rheumatic fever, may cause valvular stenosis due to calcification of the
valves. Other causes of VHD include tumors that develop in the heart muscle, injury to the chest and systemic lupus erythematosus (SLE), an autoimmune disease.

From a social, medical and financial point of view, cardiovascular disease and VHD in particular, has a global impact. According to the American Heart Foundation cardiovascular diseases cause 12 million deaths per year worldwide. This accounts for almost 50% of all deaths in the world. 300,000 procedures for heart valve repair or replacement are performed per year and finally, heart valves are currently a $260 billion industry in the US alone. ²

Two different ways of treatment of VHD are currently possible: medical, with drug therapy or surgical, with valve repair or replacement. There are two main types of faulty valve that may or may not require valvular replacement surgery. These involve valves that do not close properly and leak blood into another quadrant of the heart (regurgitation) or valves that are calcified and don’t open properly (stenosis). Valvular regurgitation cause the heart to work less efficiently because it has to pump some blood twice, and usually results in an enlargement of the heart chambers because there is more blood to pump. However, in severe cases the heart is not strong enough to compensate for the efficiency loss and it results in congestive heart failure. Valvular stenosis is a cause of high blood pressure in the heart because blood builds up behind the closed valve and forces the cardiac muscle to work harder to pump blood through the heart. The heart usually compensates by growing a thicker layer of muscle. By disrupting the flow and pressure dynamics of the entire cardiac cycle, valvular disease can ultimately cause secondary heart failure. In extreme VHD cases, valvular replacement surgery has become a viable option.

² www.americanheart.org
2.4 Detection of VHD

In general, detection methods for VHD can be divided into invasive and non-invasive techniques. Noninvasive imaging techniques have been used increasingly during the past decade for the evaluation of VHD and currently these techniques have almost completely replaced invasive detection methods for the diagnosis and assessment of the severity of VHD (Chetlin, 1991). This section discusses different non-invasive techniques and their role in the assessment of valvular disease. A brief overview is presented on the X-ray principle followed by Computed Tomography (CT) and Magnetic Resonance Imaging (MRI). Echocardiography is the most common technique used for detection of VHD and its relevance to different valvular complications, both native and prosthetic is also discussed. Each of the techniques discussed possess specific features concerning working-principles, visualisation-quality, and accuracy.

Methods:
Cardiac auscultation, using a stethoscope, to distinguish sounds recognized as a sign of health or of disease, remains the most widely used primary method of screening for VHD. From the evaluation of the auscultation procedure the physician can recommend a secondary detection method in order to assess the valvular disorder in detail. Several non-invasive techniques are available to diagnose VHD without the help of an invasive technique. However, combinations of both types of techniques can be a useful help to obtain a detailed diagnosis of the suspected VHD (Chetlin et al., 1997). Examples of these combinations are: catheterisation (invasive), echocardiography (non-invasive) and the use of angiocardiography i.e. use of x-rays following the injection of a radiopaque substance. Preferably the detection of VHD is not preformed as a semi-invasive investigation and can be avoided in selected cases. Each of the techniques discussed in this section are based on different principles and each has different advantages and disadvantages. Moreover, some of these techniques are also useful for determining the performance of implanted prosthetic heart valves or TEHV-replacements.
2.4.1 X-Ray Principle

X-ray technology was invented by accident when in 1895 a German physicist, Wilhelm Roentgen, discovered X-rays while experimenting with electron beams in a gas discharge tube. Roentgen's remarkable discovery precipitated one of the most important advancements in the history of human imaging. With X-rays broken bones, cavities and swallowed objects may be detected with extraordinary ease. Modified X-ray procedures may also be used to examine softer tissue, such as the lungs, blood vessels or the intestines.

The chest X-ray provides information about the size and configuration of the heart and great vessels, as well as pulmonary vasculature, and pleural effusions. Cardiac chamber dilation, rather than wall thickening is generally perceived as an alteration in cardiac silhouette. Although current X-ray methods are not directly used for the detection of VHD, it's able to detect abnormalities in the heart and great vessels and assist in the assessment of valvular disease. The working principle is the base for the computed tomography-scanning technique that is particularly useful in the detection and assessment of valvular diseases.

2.4.2 Computed Tomography (CT)

Computed Tomography (CT) is based on the X-ray principle i.e. as x-rays pass through the body they are absorbed or attenuated (weakened) at differing levels creating a matrix or profile of X-ray beams of different strength.

CT imaging, also known as "CAT scanning" (Computed Axial Tomography), was developed in 1973 when the X-ray-based CT was introduced by Hounsfield. This technique is currently available at over 30,000 locations throughout the world. CAT scans take the idea of conventional X-ray imaging to a new level. Instead of finding the outline of bones and organs, a CAT scan provides a full three-dimensional computer model of a patient's internal organs. CT has been the basis for interventional work such as CT guided biopsy and minimally invasive therapy. The obtained images are also used...
as a basis for radiotherapy, cancer treatment planning, and to determine how a tumor is responding to treatment. The image provided with this technique provides both good soft tissue resolution (contrast) as well as high spatial resolution using radiation. For this reason, CT-scanning is contraindicated to assess valvular abnormalities on patients during pregnancy.³

2.4.3 Magnetic Resonance Imaging (MRI)

Magnetic Resonance Imaging (MRI) has rapidly gained acceptance as an accurate, reproducible, non-invasive method for optimal assessment of structural and functional parameters in patients with VHD. Due to the development of newer and faster techniques its clinical role is gradually expanding, making detection of valvular disease simpler and clearer without moving the patient. MRI is based on the principles of Nuclear Magnetic Resonance (NMR), a spectroscopic technique used to obtain microscopic chemical and physical information about molecules. MRI began as a tomographic imaging technique that produced an image of the NMR signal in a thin slice through the human body. MRI has advanced beyond a tomographic imaging technique into a volume imaging technique.

Paul Lauterbur first demonstrated MRI in small test tube samples in 1973. He used a back projection technique similar to that used in CT. Since then several improvements have been made, bringing the images of the scans closer to real-time. Finally, in 1987 a technique called echo-planar imaging was used to perform real-time movie imaging of a single cardiac cycle (Chapman et al., 1987). In that same year Charles Dumoulin introduced Magnetic Resonance Angiography (MRA) that allowed the imaging of flowing blood without the use of contrast agents.

MRI for the detection of Valvular Heart Disease.

Exact visualization of valve morphology is possible with the cross-sectional imaging modalities, using MRI and CT. These techniques may be used, if other non-invasive

³ www.imaginis.com/radiotherapy
imaging modalities, such as echocardiography (section 2.4.4) fail or provide only limited information. The main advantages of MRI compared to CT in the diagnosis of VHD, are the absence of radiation exposure and the possibility of quantitative evaluation of valve function using flow measurements. Furthermore, MRI has the capability to detect the presence of stenotic and regurgitant lesions. However, MRI instrumentation is substantially more expensive and not as widely available. A major restriction associated with this technique is that it cannot be used in patients with any metallic prosthetic devices such as pacemakers or stents.

2.4.4 Echocardiography

Echocardiography uses ultrasound to image the heart and great vessels. It is widely regarded as the technique of choice for evaluation of suspected VHD. An ultrasonic transducer transmits and receives the ultrasound waves. It is placed on the patients’ chest wall and moved around to view different heart structures. Ultrasound waves are reflected only when they reach the edge of two structures with different densities. The reflected waves produce a moving image of the edges of heart structures.

Echocardiography is used for the determination of a wide range of heart related problems but, in particular, diseases that affect heart valves i.e. presence of aneurysms, clots, tumors and vegetations (bacterial growths) on valves. In Appendix A1 an overview is presented on how echocardiography may be used to determine and assess common valvular diseases, both within native or implanted prosthetic heart valves (Cheitlin et al., 1997). In general, echocardiography can be divided into four sub-techniques:

- M-mode
- TEE (Trans Esophageal Echocardiography)
- 2-D
- Doppler

Each of these techniques is derived from the same principle: the "Doppler effect," defined as a measured change in the frequency of sound or light waves caused by the
motion of the source or the observer. The sub-technique M-mode is a one-dimensional view of a small section of the heart as it moves while a 2-D echocardiogram produces a moving two-dimensional slice of the heart. Doppler ultrasound is used to evaluate the velocity and turbulence of blood flow in the heart. The trans esophageal echocardiography (TEE) approach uses a special ultrasound transducer that is inserted in a patient's esophagus. With this technique it is possible to image the heart from a different orientation not seen through the conventional chest-wall approach. Unlike trans thoracic echocardiography (TTE), where the transducer is placed on the patient’s chest, TEE positions the transducer behind the heart. In general, echocardiography often provides a definitive diagnosis and may use the need for catheterisation in some cases.

Echocardiography and prostheses
The clinical use of different types of prostheses are associated with different risks. Therefore, evaluations should be tailored to the patient's clinical situation and type of prosthesis. However, the evaluation of an implanted prosthetic heart valve is difficult even in the best of circumstances. In some patients with known prosthetic valve dysfunction, re-evaluation is indicated even in the absence of a changing clinical situation. “In some cases re-operation may be dictated by echocardiographic findings alone” (Cheitlin et al., 1997). Figure 2.3 shows how the 2-D echocardiography-technique visualizes a bioprosthetic aortic valve in vivo.

Figure 2.3
Bioprosthetic aortic valve in vivo: white arrows represent struts of valve
(Figure obtained from: www2.umdnj.edu/~shindler/prosthetic_valves.html)
**Murmurs**

Heart murmurs are produced by turbulent blood flow and are an indication of stenotic/regurgitant valve disease or acquired/congenital cardiovascular defects (Chetlin et al., 1997). In valvular and other congenital forms of heart disease, a murmur is usually the major evidence of the abnormality, although some haemodynamically significant regurgitant lesions may be silent. In patients with ambiguous clinical findings, the echocardiogram is the preferred test because it may provide a definitive diagnosis. In some patients the Doppler echocardiogram is the only non-invasive method capable of identifying the cause of a heart murmur. In the evaluation of heart murmurs, the purpose of performing a Doppler echocardiogram is to:

- Define the primary lesion and its etiology and judge its severity.
- Define haemodynamics.
- Detect coexisting abnormalities.
- Detect lesions secondary to the primary lesion.
- Evaluate cardiac size and function.
- Establish a reference point for future observations.
- Re-evaluate the patient after an intervention.

As valuable as echocardiography may be, the basic cardiovascular evaluation is still the most appropriate method to screen for cardiac disease and will usually establish the clinical diagnosis. “Echocardiography should not be used to replace the cardiovascular examination but can be helpful in determining the etiology and severity of lesions, particularly in paediatric or elderly patients” (Chetlin et al., 1997).
2.4.5 Discussion

The first line of diagnostic intervention in the determination of a VHD is still and will continue to be cardiac auscultation. By using a stethoscope, systolic clicks may easily be defined and from here a visualisation technique for further examination can be recommended.

By reviewing the available visualisation techniques it was concluded that the best and most common method currently used is echocardiography. This technique makes it possible to detect a wide range of heart valve and heart valve-related diseases (Chetlin et al., 1997). Newer techniques such as CT and MRI may eventually replace this technique because of superior image quality, unrestricted viewing angles and the possibility of making 3D reconstructions from 2D images. In comparison to CT-scanning, MRI has the advantage that it is not limited to the axial plane. Current limitations of MRI are that it can not visualize implanted metallic prosthetic heart valves because of their magnetic field. Another restriction is the cost of an MRI apparatus, which is not comparable to the cost of an echocardiography apparatus.

Although CT and MRI evaluation of patients with VHD is almost never performed as a first line of diagnostic intervention, their performance does provide important morphologic and physiologic information concerning the etiology and status of the valvular dysfunction. Evaluation of the heart chambers and aortic artery size as well as ventricular wall thickness provide the basis for diagnosing and analysing the severity of VHD. For assessment of stenosis severity, measurement of trans-valvular pressure gradient is an appropriate measure and MRI may not confer any benefits over echocardiography.

Ultra fast CT and MRI generate high-resolution cardiac images. Ultra fast CT requires intravenous injection of X-ray contrast media while MRI does not. However, it is widely accepted that both technologies can be used to evaluate a wide range of features. These include: cardiac chamber and aortic vessel dimensions, intracardiac and extracardiac masses, ventricular hypertrophy, left ventricular mass, congenital heart disease, regional and global left ventricular function and right ventricular function.
Specifically, MRI is highly useful for detection and semi-quantitation of valvular regurgitation while ultra fast CT is not. Another major disadvantage with CT is that radiation can harm foetal tissue. Although both techniques can detect aortic and mitral valve stenosis and assess coronary artery bypass graft status, ultra fast CT is the preferred method.

A summary of the advantages and disadvantages of currently available non-invasive techniques for the assessment of valvular disease is presented in Table 2.1.

**Table 2.1**

*Non-invasive techniques for the assessment of VHD*

<table>
<thead>
<tr>
<th>Technique</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stethoscope</td>
<td>• Quick</td>
<td>• Not accurate</td>
</tr>
<tr>
<td></td>
<td>• Cheap</td>
<td>• No visualisation</td>
</tr>
<tr>
<td>CT-scan</td>
<td>• 3D visualisation</td>
<td>• Limited to one plane</td>
</tr>
<tr>
<td></td>
<td>• High contrast</td>
<td>• Use of radiation</td>
</tr>
<tr>
<td>MRI-scan</td>
<td>• No radiation</td>
<td>• No prosthetic valves</td>
</tr>
<tr>
<td></td>
<td>• No contrast agent</td>
<td>• Expensive</td>
</tr>
<tr>
<td></td>
<td>• Quantitative measurements</td>
<td></td>
</tr>
<tr>
<td>Echocardiography</td>
<td>• Wide range of VHD</td>
<td>• No 3D visualisation</td>
</tr>
<tr>
<td></td>
<td>• Cheap</td>
<td>• Not very accurate</td>
</tr>
</tbody>
</table>
2.5 Problems with artificial heart valves

Current available heart valve substitutes can be divided into two groups - mechanical and biological. The mechanical replacements may further be subdivided depending on the type of occluder, while the type of tissue is used to classify the biological substitutes. This section provides a brief overview of the currently used replacements (Table 2.2). Each type of replacement used in cardiovascular surgery is something of a compromise. The problems associated with the clinical use of mechanical and biological prostheses are compared and discussed in this chapter. Ideal replacement valve requirements are reviewed and discussed in the last part of this section (section 2.5.4). In general, assessment of the haemodynamic performance of both types of heart valve substitutes are based on three main criteria;

- The replacement should function efficiently and present a minimum load to the heart.
- The substitute should be durable and maintain its efficiency for the patient's lifespan.
- The replacement should not cause damage to molecular or cellular blood components or stimulate blood clotting.

Table 2.2
Overview of heart valve substitutes

<table>
<thead>
<tr>
<th>Mechanical Valves</th>
<th>Tissue Valves</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ball Valves</td>
<td>Animal Tissue Valves (Xenografts)</td>
</tr>
<tr>
<td>Disk Valves</td>
<td></td>
</tr>
<tr>
<td>Single Leaflet Disk Valves</td>
<td>Human Tissue Valves (Homografts, Autografts, Ross Procedure)</td>
</tr>
<tr>
<td>Bileaflet Disk Valves</td>
<td></td>
</tr>
</tbody>
</table>
2.5.1 Mechanical Valves

Many prosthetic heart valves have been implanted worldwide during the last decades. Although these valves have undergone many improvements, the ideal mechanical heart valve has not yet been developed (Ellis et al., 1998). As stated in the introduction chapter, problems associated with mechanical prosthetic heart valves include thrombosis, haemolysis, tissue overgrowth, infection (endocarditis) and excessive pressure gradients (Wright and Temple 1971; Magilligan et al., 1980). The problems with most of the existing heart valves are well documented in the literature and alternative designs have been suggested and hydro-dynamically examined by many researchers (Chadran and Cabell, 1984). An overview of the evolution of the mechanical heart valve is presented in Appendix A2. In this section, an overview of the literature is presented on how two of the most common complications, thrombosis and haemolysis, are related to the clinical use of mechanical valve prostheses.

Thrombosis:

All clinically used mechanical valves have one main problem in common i.e. the increased risk of blood clotting. It has been suggested that the locally altered fluid mechanics increase shear-stresses and strongly influence the creation of blood clots. When blood clots occur in the heart, there is a high risk of a heart attack (Caro et al., 1978). More recent evidence indicates that a major proportion of shear stresses are associated with thrombosis occurring in altered flow fields, such as an atherosclerotic plaque in a stenosis (DeWood et al., 1980). Furthermore, several studies have suggested that rupture of an arterial plaque initiates thrombus formation (Alpert, 1989). The initial cause of plaque disruption is still unknown. It is known, however, that the endothelium has an abnormal response when exposed to turbulent flow. It is also believed that turbulent flow contributes to the activation and deposition of platelets that contribute to blood clotting. Furthermore, some investigators have suggested that haemodynamic forces have the potential to activate endothelial cells, which in turn are able to accommodate changing physiological conditions (Gimbrone et al., 1989). Therefore it has been hypothesised that a major cause of thrombosis may be directly associated with mechanical heart valves. As a result, to prevent blood clots, mechanical valve recipients must take anti-coagulant drugs (eg. sodium warfarin) for their lifetime. This effectively
turns patients into borderline haemophiliacs. The anti-coagulant used may also cause birth defects in the first trimester of foetal development, and rendering mechanical valves unsuitable for women of childbearing age. Another problem with most mechanical valves is that they have a gap between the disc edge and the housing’s inside wall to prevent jamming between the disc and the housing. The size of this gap is a determinant of the regurgitation during the closed phase of the valve cycle. These leakage gaps may lead to increased haemolysis due to the high shear stresses with the gap flow and within the turbulent mixing region of the backflow jet (Knott et al., 1988). It has been reported that the leakage jet velocities are three to five times higher than the peak forward flow velocities.

Haemolysis

Destruction of red blood cells (RBCs) is a condition associated with the clinical use of mechanical heart valves. An erythrocyte (RBC) consists of flexible membrane and haemoglobin, which endows blood with its large capacity for carrying oxygen. The RBC is capable of extreme distortion and is able to deform into an infinite variety of shapes without stretching its membrane. However, with very severe deformation as occurs when RBCs are exposed to a high shear stress, the membrane will become tense and stretched, lose its flexibility and may consequently rupture. The RBC loses its haemoglobin, through the ruptured membrane, a process known as haemolysis.

Haemolysis occurs in intensely turbulent flow such as the downstream area of a mechanical heart valve. Several experiments have been conducted to investigate the magnitude and duration of the produced shear stresses required to haemolyse RBCs. Shear stresses in the range of 1500 to 4000 dynes/cm² have been shown to cause lethal damage to RBCs (Blackshear et al., 1965; Sallam and Wang, 1984). Lower levels of RBC destruction, are possible if the total exposure time is low. Sublethal damage can reduce both the elasticity of the RBC membrane and the lifetime of the RBC itself. Chronic conditions can be a precursor to anaemia (deficiency of RBCs) and therefore shear stresses as low as 500 dynes/cm² may be clinically important. Bulk forward-flow velocity and turbulent shear stress studies have been used extensively to investigate valves (Figure 2.4). While improvements in valve design have been introduced to
reduce turbulence or alter flow-velocity contours, most of these changes have produced insignificant differences in currently used mechanical heart valves. Leakage patterns of mechanical heart valves have been studied as these patterns relate to hinge mechanisms and to haemolysis. Very high backflow with turbulent stresses in the order of 9000 dynes/cm² have been documented in a variety of tilting-disc designs, well above values believed to cause RBC damage. From a haemodynamic point of view, leakage through mechanical valves during the closure phase is substantially more important than that observed in forward flow (Knott et al., 1988; Ellis et al., 1998). For future valve designs, an understanding of the influence of the leakage gap and hinge dimensions is crucial to the improvement of haemodynamic performance and minimization of haemolysis and/or thromboembolic events.

Figure 2.4
Hypothetical shear fields and red cell path lines through a bi-leaflet heart valve

(Figure from: http://www.ctdigest.com/May99/2_rev2/2_rev2.html.)
2.5.2 Biological Valves

Bio-prosthetic valve leaflets are fabricated from a combination of chemically treated xenogenic tissue and/or synthetic materials. The valve-frames are usually flexible in the axial direction but effectively rigid in the plane of the sewing ring in order to maintain position. The type of tissue is used to classify tissue valves and this originates from either animal or human tissue. The type of tissue used can be either valve tissue or non-valve tissue. Human tissue valves, transplanted from another person are called homografts, while autografts are valves transferred from one position to another within the same patient. The most common autograft procedure involves transferring the pulmonary valve to the aortic position, called the Ross Procedure (Ross, 1967). In general, tissue valves have better haemodynamic performance than mechanical replacements, although their limited durability is a major drawback (Borttolotti et al., 1987). In this section the problems related to the clinical use of three types of tissue valves are discussed A. Homografts, B. Autografts and C. Xenografts.

- **A. Homografts/Allografts**: Homografts or Allografts are human tissue valves. After death, the valve is removed treated with antibiotics and transplanted into the recipient. There are usually no problems with rejection of the valve and patients do not require any type of immunosuppressive therapy. Homograft valves are donated by the donor family and then preserved in liquid nitrogen (cryopreserved) until needed. These valves tend to have exceptionally good haemodynamic profiles, a low incidence of thromboembolic complications and do not require chronic anticoagulation (Borttolotti et al., 1987). Such valves are especially efficacious for replacing those excised because of endocarditis (O’Brien et al., 1987; Tuna et al., 1990). Cryopreserved allografts are unable to grow, remodel, or exhibit active metabolic functions and their usual degeneration cannot be attributed to immunologic responses. As with heart transplants, homograft availability is limited by a lack of suitable donors.
B. Autografts (Ross Procedure): Autografts are valves taken from the same patient in which the valve is implanted. The most common autograft procedure is the Ross procedure developed by Donald Ross in the sixties and has become widely accepted (Ross, 1967). The Ross procedure is used in patients with diseased aortic valves. The abnormal aortic valve is removed and the patient's own pulmonary valve is transplanted to the aortic position. A homologous pulmonary valve is then used to replace the patient's pulmonary valve (Figure 2.5). The main advantage of the Ross procedure is that the patient receives a living valve in the aortic position. The hope is that in children, the valve will continue to grow as the child grows older. Other potential benefits are better haemodynamics (there is essentially no pressure drop across the valve) and better durability.

Figure 2.5
Schematic of the Ross procedure
(Figure adapted from: Kouchoukos et al., 1994)
However, it remains unclear whether the durability of valves implanted by the Ross procedure is better when compared to porcine or pericardial valves (David et al., 1996). The Ross-procedure is a technically difficult procedure for a surgeon and involves considerable skill and time. The pulmonary valve must be sculpted to fit the aortic root and the pulmonary homograft must similarly be shaped to fit the pulmonary root. Special measurements must be made to fit the transplanted pulmonary valve into the aortic root. There are many potential complications in less skilled hands; the most common one is leakage of the valve after the procedure. However, many patients have small amounts of aortic regurgitation and some have moderate or even severe amounts and require a second operation for valve replacement. Other potential complications include stenosis of the coronary artery, right-sided endocarditis (since a prosthetic valve has now been implanted in the pulmonary position) as well as the usual complications of valve replacement (David et al., 1996).

- **C. Animal Tissue Valves (Heterografts, Xenografts)**

Animal tissue valves are called xenografts from the Latin prefix "Xeno-" for foreign or heterografts. Xenografts may be of valve tissue, typically porcine valve tissue, or they can be of non-valve tissue, eg. bovine pericardium. The term heterograft has the same meaning but the prefix comes from a different root, "hetero-" meaning "different".

All three types of tissue valves discussed are sterilised with glutaraldehyde before human use and maintain a low rate of thromboembolism without anticoagulation. Stroke and bleeding problems rarely occur with these types of valves. However, valve failure with structural dysfunction due to progressive tissue deterioration (including calcification and non-calcific damage) is a serious disadvantage that undermines the attractiveness of tissue valve substitutes (Schoen et al., 1992). Moreover, the calcification-process in bio-prosthetic valves is accelerated in children and young adults. The degradation mechanisms of bio-prosthetic valves are progressive and the rate of failure is time dependent. They usually need replacement within ten to fifteen years or sooner in younger patients. Bovine pericardial valves suffer from poor
durability, and usually perform significantly worse than porcine xenografts (Hammermeister et al., 1993). There is also a concern for the transmission of prion diseases i.e. BSE (cattle) and scrapie (sheep). There are currently no tests available for the diseases and the diseases are uniformly fatal. The long-term mortality of patients with tissue valves replacements do not differ significantly compared to those with implanted mechanical valves. Comparison between mechanical valves and bio-prostheses from in vivo trials such as the Edinburgh trial and the Veteran trial demonstrated that the mechanical-valve and bio-prostheses groups did not differ for long-term mortality or total valve-related complications. Other important complications, including valve infection (endocarditis) and non-structural dysfunction, affect both tissue and mechanical valves (Schoen and Levy, 1999).

2.5.3 Mechanical versus Biological

Most of the clinically used valves are not yet ideal, but patients with implanted valves can lead a relatively normal life. During the past three decades more than 80 different prosthetic valves have been trialled and currently about 20 of these are still in clinical use. A twelve-year comparative study of mechanical vs. bio-prosthetic valves found that approximately one third of all heart valve replacement recipients had prosthesis-related problems within 10 years of surgery (Bloomfield et al., 1991). Despite all the research and development efforts, there are still no ideal manufactured valves, particularly when comparing the haemodynamic performance. Appendix A3 summarizes the respective advantages and disadvantages of mechanical valves, homografts, xenografts, and bioprosthetic valves.
2.5.4 Discussion: The ideal Replacement

Heart valve prostheses have been used successfully for the treatment of VHD, and it cannot be disputed that hundreds of thousands of lives have been saved and extended by their use. However, many currently used heart valve replacements are associated with problems directly related to the design of the valve. Therefore, it may be useful to describe some of the design goals for an ideal repair material or replacement valve. The design goals for an ideal valve replacement may be divided into basic design goals and other desirable characteristics as shown in Table 2.3.

<table>
<thead>
<tr>
<th><strong>Basic design goals</strong></th>
<th><strong>Other desirable characteristics</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Prompt and complete closure</td>
<td>Repair of cumulative injury</td>
</tr>
<tr>
<td>Non-obstructive</td>
<td>Provide ongoing remodeling</td>
</tr>
<tr>
<td>Non-thrombogenic</td>
<td>Grow in maturing recipients</td>
</tr>
<tr>
<td>Non-haemolytic</td>
<td>Tissue engineered</td>
</tr>
<tr>
<td>Last the lifetime of a patient</td>
<td>Not annoying to the patient (noise free)</td>
</tr>
<tr>
<td>Chemically inert</td>
<td>Infection resistant</td>
</tr>
</tbody>
</table>

The ultimate valvular replacement would be a device that incorporates the basic design goals with the other desirable characteristics. Reviewing the points presented in Table 2.3, it is apparent that biomedical engineers will need to focus on a biological substitute, to meet demands such as self-repair and growth. Besides these demands, limited
durability of currently used biological replacements in general presents a major
drawback for clinical use (Borttolotti et al., 1987). Furthermore, all current biological
heart valve substitutes are unable to grow, repair, or remodel within the recipient
(Mitchell et al., 1998). This universal limitation is most detrimental to young patients in
need of heart valve replacements because successive surgery is required to replace the
implanted valves that cannot grow with the child (O’Brien et al., 1999). One solution
that may meet all of these requirements is to grow an identical copy of a healthy valve
with cells from the recipient. Using this strategy, many different fields that include
design, engineering, biology and medicine need to be combined as a ‘multidisciplinary’
approach.
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 \textit{in vitro}, mimicking an \textit{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) \textit{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 immunogenicity 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 decellularization 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 19th century, research into synthesized materials such as glycolic acid and other \( \alpha \)-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 elicit 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 occurs 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 to 0 °C. The polymer is processed at the lowest possible temperature to prevent depolymerisation 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 metabolism 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.

![Image not available-See printed version](image-url)

**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, were investigated for TEHV scaffold fabrication. As described in the previous section, all naturally thermoplastic polymers possess 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.

(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.
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 bioabsorbable, 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. Furthermore, 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 cardiomyocytes 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 CO₂ 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.
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 O₂, CO₂, N₂) 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 \textit{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 \textit{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.

\textbf{Figures 3.10}

\textit{Various Bioreactors:}
\textit{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-con structs subjected to recirculated culture media though a closed loop (Hoerstrup 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 (Hoerstrup 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).

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 (Hoeerstrup 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 respectively. 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 TEHV was functioning 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 TEHV 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 TEHV is presented in *Appendix A5.*
CHAPTER 4

DEVELOPMENT OF THE PBR-APPARATUS

4.1 Introduction

As reviewed in the previous chapter, the investigation undertaken at Swinburne University focused on a polymer-strategy. Three main elements were required to grow TEHVś in vitro: cells, scaffold and a simulated in vivo environment. A review of the literature demonstrated that in Pulsatile Bioreactor (PBR)-design only a small number of innovative alternatives have been proposed. Optimisation of tissue development and an understanding of the biomechanical requirements of engineered tissues cultured in vitro is still at an early stage (Lanza et al., 1999; Hoerstrup et al., 2000b). In this chapter the concept, design and development of a Prototype System (PS) and a second model, the PBR, are described and discussed. Both models were capable of long term in vitro culture of cardiac constructs. Furthermore, these systems were capable of providing physiological pressures and flow of nutrient medium for engineering both arteries and heart valves. In addition, the development of the system may allow further research in TE at Swinburne University. The final two sections of this chapter describe two experimental procedures:

- The generated mean flow rates in the PBR have been estimated and are compared to the performance of the PS.
- 72-hour endurance tests were conducted, to verify the functionality of a manually fabricated BD scaffold. The scaffold was subjected to different sub-physiological-flows generated in the PBR.
Although several groups have reported encouraging results from TEHV-constructs, a completely bio-mimetic heart valve construct remains elusive. Current attempts have failed to produce replacements comparable to native tissue valves (Sodian et al., 1999; Stock et al., 2000). One key issue yet to be resolved is the optimal conditions under which TE constructs should be developed. The conditions under which the TE constructs are cultured and developed are vital determinants of the quality of the final product. Research has shown that pulsatile flow applied during culture leads to an improved development of TE constructs (Sodian et al., 2000abc). Therefore the use of ‘PBRs’ that provide pulsatile flow of nutrient media for the development and culture of TE construct, is essential for proper development of the tissue. The ultimate aim of a PBR is to provide an environment that as closely as possible mimics the natural in vivo conditions. These types of PBRs have been largely designed with little effort being directed into mimicking in vivo conditions. As stated in the introduction chapter, the review of the literature did not find reference to any similar research where fluid forces and haemodynamic conditions created by PBRs were analysed.
4.2 Design of the Prototype System (PS)

The Prototype System (PS) was versatile in its use and compact in design. The dimensions of the BR are 150 mm in diameter and 230 mm in height. The material of construction is acrylic-plastic, which is inexpensive, strong and easy to sterilize with ethylene oxide. The PS was comprised of three main circular compartments (Figure 4.1).

![Cross-sectional view of the Prototype System](image)

The air-chamber (1) is filled with air by an air pump, with a certain pressure cycle. The pressure differential fibrillates the silicon rubber diaphragm (2). The diaphragm is located between chamber 1 and 3 and attached like a drum-skin. The pressure-chamber (3) can be filled with blood or culture media, which flows to the growth-chamber or tissue perfusion-chamber (4) due to the fibrillating membrane. The tangential position of the two inlets as well as the sphere-shape of the pressure-chamber should improve the mixture of the growth medium through the inlets. With this design there are no dead angles in the pressure-chamber and this chamber has a volume of approximately 220 ml. The TE construct may be secured and cultured in the tissue perfusion-chamber. The design of this chamber can be altered to suit multiple types of TE-constructs, making this PS ‘multifunctional’. The software programs ACAD 2000 and Solid Works were used to fabricate the technical drawings (Appendices 6 and 7).
To enhance the circulation of growth-medium through the TE-construct, outlets were placed halfway along the perfusion-chamber. After the systolic phase the silicon diaphragm returned to equilibrium, transferring the growth-medium from the medium-reservoir through the two inlets on the outside of the pressure-chamber. A one-way valve, located between the pressure chamber and perfusion chamber, prevented backflow from the perfusion-chamber to the pressure-chamber and ensured a constant fluid circulation through the system. To maintain a pulsatile flow a bi-leaflet aortic valve was used, which had a low interference and good haemodynamic characteristics.

**Multifunctional Design Parameters:**
The major advancement in the design of this BR over existing BRs was the incorporation of a multifunctional design feature. Various types of TE-constructs may be cultured in this single BR by simple exchange of the connections in the perfusion chamber (Figure 4.2). These exchangeable parts are divided into two major areas (1) the artery-parts are subdivided for different diameters of arteries and (2) heart-valves parts. These connections (Figure 4.3) can be easily screwed into the top of the pressure chamber. Moreover, it may be possible to grow arteries in vertical position, from 5 - 12 mm internal diameter. Valvular constructs, ranging from a 20 – 28 mm internal diameter may be fixed above the connection part or a placed inside a tubular construct.

![Figure 4.2](image)

**Figure 4.2**

Various types of TE constructs:
A: Aortic Valve, B: Artery, C: Coronary artery
The top-part of the TE-constructs may be attached to an acrylic-plastic disk. Different acrylic-plastic hollow tubes on this disk, corresponding to the size of the connection parts, will secure a uniform fluid flow through the whole construct. More tubes on the disk surface allow the attachment of branches of the construct. Figure 4.4 shows the described PS in a test set-up.
4.3 Design of a Pulsatile Bioreactor (PBR)

The design of the Prototype System (PS) and data obtained from the initial measurements (presented and discussed in Chapter 6) demonstrates the potential to culture TE-constructs. However, some improvements need to be made in order to achieve the overall aim of this investigation. This section describes the development of a second system model, the Pulsatile Bioreactor (PBR), that is able to provide constructs with long-term housing for growth and development.

4.3.1 Methodology

As a consequence of the many experimental procedures carried out with the PS, problems related to the design were discovered and a PBR was designed and developed. This methodology-section is divided into two phases;

- The choice of material and how to secure the tissue-construct.
- The design of the sealing mechanism of the reactor.

The measurements related to the performance of the PS demonstrated that the principle of converting air pressure into a fluid-flow by a silicon membrane was possible. Acrylic plastic, the material used to fabricate the prototype did not fulfil the desired criteria. Ethylene oxide, a gas used for the sterilizing-process was not longer available at Swinburne University and therefore autoclaving was used. This resulted in the formation of tiny cracks in the various glued layers of the pressure chamber after several periods of autoclaving. The disassembly of the device for measurements and exchanging different constructs also did not improve the performance. Eventually, leaking made this prototype unreliable and sterility could no longer be guaranteed. These problems required a new approach in the choice of materials and improved accessibility to the construct. A more robust material to fabricate the reactor and a different system to access the TE-construct was necessary.
4.3.1.1 First Phase

It was concluded that acrylic plastic was not a reliable option for the current investigation and alternatives were investigated. Stainless steel was selected to fabricate the fluid and air chamber of the PBR. From the different types of stainless steel, Fe316 was chosen. This material is strong, easy to process, can be autoclaved and is chemically resistant. A cross-sectional view of the stainless steel component is presented in Figure 4.5.

The growth chamber is made of bio-glass, which is autoclavable and used in standard chemical applications. The basic design and function, as described in the previous section, did not change in comparison to the PS. Two perfusion chambers were used: The first perfusion chamber has a single screw top that allows moving an 8 mm stainless steel bar with an exchangeable connection part to move up and down to culture different sizes of tubular constructs. To fixate this part a removable stainless steel construct on the outside of the PBR was developed to hold the bar. A second growth chamber was constructed that did not need to use this crane-construct for fixation. The top has a concentric ground glass inlet and is shaped to hold a removable glass rod used for sealing and adjusting different lengths of TE-constructs (Figure 4.9). The walls of this growth chamber are thin and were designed for better visualisation and to improve to ease of conducting measurements. The base of both glass parts has a standard flange that improved exchangeability. The acrylic-plastic disk for fixation, as described in section 4.2, was not required with this design. Therefore, this device is less complicated and more user friendly.
To guarantee a continuous flow through the TE construct a check valve was used. In this study it was decided to use an exchangeable valve, which was easily screwed inside the top of the fluid chamber. Inside this part the ATS bi-leaflet aortic valve (type: 29mm Aortic500FA29, as used in the PS) was embedded. The main advantage compared to the PS was that with this design, simulation with or without this check valve could be made. This is particularly important when culturing valvular constructs to determine their functionality. The check-valve is located just before the exchangeable connection part. This valve is biocompatible, but more importantly, it has a low interference on the fluid flow when compared to other valves. In Figures 4.7b and 4.7d, the hydrodynamic performance from a single leaflet heart valve is compared to the bi-leaflet valve. From these figures a significant difference can be observed.

The perfusion chamber was fixated on the fluid chamber using two fixation rings with six stainless steel screws each. These rings are screwed into the outside of the fluid chamber, as shown in Figure 4.5. This provided a good result in terms of sealing but was rather time consuming and unsafe in terms of locating the rings. The twelve stainless steel screws could damage the growth chamber, if not fixated correctly. Therefore it was decided to redesign this part of the PBR, which resulted in a design where the sealing principle was totally different to the one described in this paragraph.
Figure 4.6

Housing for the bi-leaflet check valve used in the PBR

(a) (b)

(c) (d)

Figures 4.7

Comparison of speed distribution between single and bi-leaflet heart valve

a: single leaflet heart valve
b: Speed distribution of a single-leaflet heart valve
c: Bi-leaflet heart valve
d: speed distribution of a Bi-leaflet heart valve
4.3.1.2 Second Phase

To overcome the accessibility problem, the method of connecting the perfusion chamber to the fluid chamber was changed. The perfusion chamber could now be easily clicked onto the BR using a standard dry seal flange (Schott) (Barteld Sc. Industries, Heidelberg). In the authors’ opinion, this is one of the biggest advantages for researchers who may use this device for future tissue culture. The stainless steel parts were adapted to make this redesign possible, as presented in Figure 4.8.

![Figure 4.8](image)

**Figure 4.8**

*Cross-sectional view of the PBR after Phase Two*

Although the second phase of the PBR was redesigned and finalized on paper continuing problems prevented the actual fabrication of this device. The time factor, money to carry out the adjustments and most importantly; the lack of skill on the work-floor made the search for alternatives necessary. Due to these reasons the necessary adjustments were made outside the University. J.F.M Engineering, a machining factory made the necessary adaptations and the stainless steel welding. With these changes further improvements were not needed. Tests, as described in section 4.5, were carried out to verify the functionality of the device. Technical drawings of the PBR and a description of the different parts are presented in Appendices A6 and A7.
Figure 4.9

3-D view of the PBR

Glass concentric insert (left) allows fixation of different sizes and heights of tubular constructs. The exchange-part on the bottom with the location of outlet-holes for the fluid during operation (white arrow).
4.4 The New Pulsatile Pump System

To generate adjustable fluid flows inside BRs many different systems have been proposed (Niklason and Langer 1997; Sarraf et al., 2002). The pulsatile flow generated in the PS was activated by a pneumatic pulsatile pump with adjustable vacuum and positive pressure. This pump was designed at Waseda University, Japan (1994) and used in the first part of this study. The pulsatile blood pump is pneumatically driven by the compact driver consisting of a vacuum pump, buffer tanks, regulators and a solenoid valve. The block diagram of the pneumatic circuits for the compact driver is presented in Figure 4.10.

\[ \text{Figure 4.10} \]

*Block diagram of the pneumatic circuits for the compact driver*

(Figure from: Owida A, 2000)

Compressed air was supplied to the compact driver through a 2-μm air filter. Both the vacuum air pressure and the compressed air pressure are adjustable. A pump driving frequency as well as a systolic duration was set by the controller box that propagated an electronic operating signal to the solenoid valve. In order to indicate the period of each cycle, an encoder communication cable was connected between the controller of the pneumatic pump driver and the external input box of the measurement system. With this system air pressures may be generated up to 760-mmHg positive and 760 mmHg.
vacuum. A further description of this system used in the experimental set-up is presented in the following section.

The system has a small amount of vacuum as part of the backpressure exerted from fluid flow, which mimics in vivo conditions. However, this system has some limitations for use in tissue culture and therefore an alternative system had to be developed. The pneumatic pulsatile pump will eventually be stationed in a laboratory where long term in vitro cell culture can be investigated. This system has dimensions of 1 x 0.7 m and is noisy during operation. Since the system needs to operate for long periods during cell culture, there is the danger of overheating the vacuum pump.

A suitable alternative needs to fulfil some basic demands in order to mimic in vivo conditions as closely as possible. Firstly, it must be able to create enough fluid flow through the construct in order to sustain the metabolism of the growing tissue, which is comparable to the natural flow and pressure in vivo. One average heartbeat generates 70 ml and with a rate of 70 BPM the heart ejects a volume of approximately 5 l/min. This is a requirement that a new driver system needs to fulfil. Another need was the creation of cyclic stress with an adjustable peak pressure (similar to that in a heart beat) and an adjustable frequency (BPM). Without these features it is not possible to produce the optimal conditions for engineering tissue in vitro. Furthermore, the system should be compact, reliable and durable. In the following section a description of a system that is able to apply several types of fluid flow for a prolonged period is described. The working principle of the different components is also provided.

**Proportional 5/3 way valve:**
The system used in this investigation was an electronic proportional directional control valve, a PDCV, as shown in Figure 4.11 (FESTO type MPYE-5-3B). With this system it was possible to provide a physiological flow for long-term cultivation of cells. Furthermore, the system is novel for this application and has not previously been described in the literature. With a PDCV, the valve-slide stroke is controlled proportionally to a specified set point. The analogue electrical input signal produces a
step-less variation of the flow rate. The PDCV thus controls the flow rate in regard to its magnitude (continuous throttle-valve function) and direction (5/3-way function).

Figure 4.11
The Proportional Directional Control Valve (PDCV)

(Obtained from: FESTO users manual 2001 for product-type; MPYE-5-3B)

In industrial applications the PDCV is used to control the speed and position of a pneumatic cylinder. In the current study a different application is presented, which describes its control of different types of pressures and flows in the PBR. Its mechanism is simple, durable and easy to maintain. The working principle may be divided into two phases i.e. inflation and deflation-phases (Figure 4.12 a). To inflate, pressurized air is directed from (1) to (2) through the valve and this pressurizes the air chamber of the PBR. To deflate, air is coming back from the air chamber and flows from (2) to (3) through the valve. In this phase incoming air is diverted to the exhaust (4). The system can exert pressures to the PBR up to 7600 mmHg and can generate an airflow rate up to 700 l/min at $P_{\text{nominal}}$ (4560 mmHg).
In Figure 4.12b the pneumatic schedule is shown of the switching function of the valve in its neutral position. The valve is controlled electrically by a 40V/3A LAB power supply combined with a function generator (BWD Australia). The set-up is shown in Figure 4.13. Output \( U_w \) moves the valve from left to right to create inflation and deflation. The controller unit has an analogue set point value input of \( V_{set} \): 0 - 10 V DC or \( I_{set} \): 4 - 20 mA. With this function generator a frequency (BPM) can be generated up to \( 10^2 \) Hertz (6000 BPM), which is the limit frequency of the PDCV. Three different output signals can be subjected to the PBR; (A) block, (B) triangle and (C) sinusoidal waves (Figure 4.14). Inlet pressure to the reactor can be adjusted by changing the amplitude (D). A vacuum, as seen in native heart cycles, cannot be created with these inputs. A vacuum generator synchronized with the PDCV connected to the exhaust 4, may rectify this.
**Figure 4.13**  
Controller box for the PDCV

**Figure 4.14**  
Types of output on the controller box:  
A - block, B - triangle,  
C - sinusoid and D - setting of the amplitude (min-max).
4.5 Testing the PBR in combination with a manually fabricated TE scaffold

The first test carried out with the developed PBR was an endurance test. The aim was to determine if the system functioned over a fixed period without leaking. In addition, the valvular PCL scaffold (Figure 3.8) was subjected to an endurance test in the PBR. During the endurance-test, sub-physiological flow rates at super physiological pressures were generated for a period of 72 hours. This test should give a clear indication if the scaffold is able to withstand super physiological pressures for long exposure times, without loss of its functionality. Therefore, in this experiment the bi-leaflet check valve, was not used (section 4.3).

Experimental Set-up:
In figure 4.15 the regulator (c) connected to the Proportional Directional Control Valve (PDCV) (a) is shown. For extra control and safety of the system, a control valve was placed between the outlet and the air chamber of the PBR (b) the PDCV was connected to the PBR and controlled with the electrical control unit. Compressed air was provided to the regulator, which filters the air and adjusts differences in air pressure. Beside this it also functioned as a valve. In the presented set-up a maximum pressure of 760 mmHg could be generated, using the PDCV. This was the direct boundary condition of the system and maximum pressure occurred at the peak of the sinusoid pressure wave. In this set-up the amplitude was set to 80%. This was equivalent to 300 mmHg, assuming that the silicone membrane translated the air-pressure into fluid-pressure with at least 50% ratio. The heartbeat rate was set to 70 BPM, which resulted in a flow rate of 1150 ml/min. Two other mean flow rates were also investigated: 600 ml and 850 ml per minute with similar pressures. The tests were conducted over a period of 72h and the circulation media used during this experiment was water.
Figure 4.15
Experimental set-up of the PDCV: a; valve, b; check-valve, c; regulator.

Figure 4.16
4.6 Testing the Proportional Flow Valve Parameters

A flowmeter was used to measure the input parameters generated by the proportional flow valve (PDCV). The pulse-wave electromagnetic flow meter (model MFV-1200, Nihon Koden, Japan) was able to measure flow rates ranging from 0.2 ml/min to 19.99 ml/min. This type of flow meter was initially designed for medical applications to provide easy, accurate blood flow measurement as well as stroke volume measurement.

In this investigation the mean flow rates though the system generated by the PDCV were measured and compared. Pressures ranging from 0 - 760 mmHg and heart beat rates from 60 – 160 BPM were applied to the PBR in order to determine the generated mean flow rates. Also, three different simulations (runs) were conducted with and without the bi-leaflet check valve, to determine the effect on the flow rate. During these runs the functionality of the PCL scaffold (Figure 3.8) was observed under different pressures.

A second experiment was carried out under similar conditions. The pulse duplicator used in the PS in the first part of this investigation (section 4.4) was connected to the PBR to compare its performance to the PDCV. Positive and vacuum pressures ranging from 0 - 760 mmHg and rates from 60 – 160 BPM were applied to the PBR to determine the mean flow rates.

**Experimental Set-up:**
The PBR was attached to a fluid reservoir with silicon tubing and connected to the PDCV (Figure 4.16). Different characteristics were investigated by varying the settings with the control box (1). The probe (5) was placed between the PBR (3) and the fluid reservoir (4). Results were displayed on the flow meter (2). After calibration of the measurement system, the experiments were conducted under the following conditions: Each measurement was taken at 1 min intervals until the fluid-flow was fully developed. Water was used as the circulation media and the fluid reservoir contained a volume of 650ml prior to the experiments. In each run a sinusoid pressure wave was used.
- For the first simulation the maximum air pressure was set at 1 bar (760 mmHg) and during the experiment the amplitude was increased incrementally by 10% (76 mmHg) from 0 - 100% using beat rates of 60,70,80,100,120,140 and 160 BPM. From these runs it was observed that up till 40% no reliable fluid flow could be measured (Figure 6.13).

- Therefore a second simulation was conducted with a maximum inlet pressure of 380 mmHg from 60% - 100% of the inlet pressure to obtain flow data under lower pressures.

- The third simulation was conducted without the bi-leaflet check valve using a similar inlet-pressure to that from the first run (760 mmHg). The results of these runs are shown in Figures 6.14 and 6.15.

The same set-up was used to investigate five different combinations using a positive vacuum pressure ranging from 150 – 760 mmHg with the pulse duplicator. These inputs were applied to the PBR to determine the generated flow rates in the system. These results are shown in Figure 6.16.
CHAPTER 5

MEASUREMENT TECHNIQUES

The developed Prototype System (PS) was evaluated to determine the produced flow characteristics. This was achieved by choosing from several available techniques that included Dual Camera Stereo Photogrammetry, Particle Image Velocimetry and Laser Doppler Anemometry. These techniques are able to provide detailed information on shear-stress distribution, velocity and flow patterns. Many studies on existing heart valves replacements have been performed to gain better understanding of their haemodynamic performance (Chew et al., 1983; Lim et al., 1998). However, a review of the literature showed that no hydrodynamic research has been carried out on Bioreactors (BRs) with the described techniques. This chapter presents the different measurement techniques that can provide the hydrodynamic data for this study. The following section summarizes these techniques and discusses the selection of the most suitable technique.

5.1 Measurement systems

Morphology and function are directly related in the cardiovascular system. Altered flow conditions, such as separation and recirculation zones, low and oscillatory shear stress, play an important role in the development of cardiac tissue. In turn each of these flow conditions are modified by arterial wall changes such as intimal thickening or changes in performance of heart valves (Alpert, 1989). A detailed understanding of the local haemodynamic environment, the influence of flow patterns on the long-term adaptation of cardiac tissue can be determined either experimentally or theoretically. Experimental methods such as Computational Fluid Dynamics (CFD) may be used to build patient-parameterised models able to give detailed predictions on factors such as flow shears and recirculation zones in the cardiac system. The CFD-programme is used at IRIS, Swinburne University in related projects to the current investigation. Specifically, a
virtual haemodynamic research carried out to investigate cardiac tissue offers different options to investigators: **first**, an understanding of the correlation between any modification of cardiac tissue and the associated alteration of flow patterns, with its effect on long-term results; **second**, the basis for detailed planning, before performing procedures in a patient; **finally**, the possibility to alter parameters such as geometries in order to study or predict end-results of vascular procedures.

Hot-wire anemometry (HWA) and Laser Doppler Anemometry (LDA) are experimental techniques used for the measurement of instantaneous flow fields around prosthetic heart valves and Ventricular Assist Devices (VAD). Also, recent advances in particle image velocimetry (PIV) have made this technique a useful tool for flow measurements. However, there is currently no single measurement technique that can provide all the desired information (George and Taulbee, 1990) and each of these techniques has advantages and limitations in different environments (Morsi, 1996). In the next subsections 5 different experimental techniques are reviewed that may be useful in this study.

### 5.1.1 Thermal Anemometry Techniques

Thermal anemometry techniques use two primary categories of transducers: hotwires and hot-films. Both rely on the principle that the electrical resistance of a metal conductor is a function of its temperature and may be used as a resistance thermometer. When provided with enough current to heat the conductor above the temperature of the surrounding fluid, the convective heat loss to the fluid can be related to the velocity of the fluid (George and Taulbee, 1990). For liquids, hot-film probes are normally used. In general, hot-film probes are less effective than hot wires for several reasons. Hot-film probes have more complicated directional problems than hot-wires and their frequency response tends to be limited due to heat loss to the backing material (George and Taulbee, 1990).
The sensor element used in HWA may be either a thin wire or metal film laid over glass support and coated to protect the film. The sensor element is heated by an electronic circuit that supplies the power to the element. The main component of the hot wire anemometry device is a miniature metal element heated by an electrical current and inserted into the flow under investigation. Preliminary calibration is performed to establish the electrical resistance as a function of flow velocity.

There are some limitations and a number of sources of error in this technique that cannot be ignored in applications. Primarily, problems in HWA techniques arise because the transducer converts the measured vector quantities into scalar output maps. Since heat transfer from most common configurations can achieve a specific value for a variety of flow directions, the ability to distinguish direction is lost (Morsi, 1996). For instance, a wire cannot distinguish any of the components of velocity in a plane perpendicular to it. This basic problem with HWA in high-density turbulent flow results in large difference between the LDA and the HWA results in the jet measurements. These effects become progressively more important as the turbulence intensity rises above 50%. Such levels of turbulence intensity are quite common in shear flows close to walls and it is very likely that these will occur with artificial heart valve applications.

HWA requires calibration prior to a measurement and the type of calibration may introduce further errors. Calibration based on the frequency response is desirable but it is extremely difficult to generate well-characterised flow fields at frequencies > 1 KHz. Unless the calibrations are done immediately before and after testing the absolute level of the measurements is ≤ 0.2%. The sequel representation of signals is another concern in HWA. The cause of deceptive signals may be related to eddy shedding from the probe, vibration of the probe support or the hot wire, electronic noise, temperature fluctuations and the drift in the electronics (Morsi, 1996).
5.1.2 Particle Imaging Velocimetry (PIV)

In the majority of fluid research experiments lasers have been used to provide the quantitative data (Chew et al., 1983). However, the PIV-technique is capable of providing full-field measurements of velocities, flow and stresses (Figure 5.1) and PIV may be used to study flow behaviour around valve constructs inside BRs. PIV has been used to investigate the pulsatile flow characteristics of cerebrovascular aneurysm at Nanyang Technological University and Tan Tock Seng Hospital in Singapore (Yu et al., 1997). It has the advantage over laser-techniques in that it is not necessary to obtain the velocity and turbulence fields point by point. For example, using this system the flow profile during cultivation of a TEHV can be directly determined and compared to native heart valves or other prostheses in terms of stresses and flow fields. Similar studies have been reported by Lim et al., (1998) using four different prosthetic aortic heart valves under steady flow conditions.

![Image not available-See printed version](source: www.dantec.com)

**Figure 5.1**

*Typical plot obtained with PIV: Results of flow in artery with aneurism, a time record through the cycle of the pulse is also possible*

(Source: www.dantec.com)
Working principle:
PIV is a planar measurement technique where a pulsed laser light sheet illuminates a flow field seeded with tracer particles small enough to accurately follow the flow in 2D and 3D. The positions of the particles are recorded on either photographic film or with digital cameras at each instant the light sheet is pulsed. Data processing consists of either determining the average displacement of the particles over a small integration region in the image or determining the individual particle displacements between pulses of the light sheet. Determination of the time interval between light sheet pulses then permits computation of the flow velocity.

Figure 5.2
Basic Set-Up of the PIV-system
(Figure obtained from: www.lavision.de/products/piv)

Figure 5.2 shows the configuration of a typical PIV experiment where a double pulsed Nd: YAG laser was used to provide the pulsed light sheet illumination. A CCD camera was used to record the positions of particles entrained in the flow that was oriented 90 degrees to the plane of the light sheet. Depending on the type of camera used and the particle concentration either particle tracking or correlation processing can be used to produce the velocity vector map. When a low particle concentration is used, the individual particle displacement can be determined. If high particle concentrations are used, correlation processing is usually the technique of choice. In some instances, particle tracking can be used after correlation processing to provide "super resolution" particle velocity maps. The accuracy of velocity measurement depends on the
uncertainly in the measurement of displacement and the time interval. In many cases the principal source of uncertainly in locating the centre of an image is due to irregularities in the shape of the images. This is caused by factors such as irregular shaped particles, lens aberrations, film grain noise or video pixel noise of refraction of the light rays in the fluid. Accuracy and good special resolution require very small markers and when examining a PIV image, pairing may be difficult if the image density is high (many images per spot). When the image density is low, most of the integration spots are empty. Successful measurements occur when the images of one particle reside in the measurement window. If more than two images are present, it is difficult to determine the correct pair. If there only two images from different particles an erroneous measurement will result.

5.1.3 Laser Doppler Anemometry (LDA)

The Laser Doppler Anemometer (LDA) is a widely applied tool for fluid dynamic investigations of gases and liquids that has been used for more than three decades. This technique provides information on flow velocity. Its non-intrusive principle and directional sensitivity make it very suitable for applications with reversing flow, chemically reacting or high-temperature media and rotating machinery, where physical sensors are difficult or impossible to use. It requires tracer particles in the flow. The application of Laser Doppler techniques in the evaluation of heart valve function has been well documented (Chew et al., 1983; Hanle et al., 1986 and Yoganathan et al., 1989). The LDA advantages include non-intrusive measurement, high spatial and temporal resolution, calibration is not required and the ability to measure in reversing flows.

4 Most of the materials presented relating to the LDA-application are taken from other sources within the Swinburne Modelling and Simulation Research-Group (Owida, 2000).
The basic configuration of an LDA consists of:

- A continuous wave laser
- Transmitting optics, including a beam splitter and a focusing lens
- Receiving optics, comprising a focusing lens and a photo detector
- A signal conditioner and a signal processor.

Advanced systems may include transverse systems and angular encoders. The laser beam is divided into two and the focusing lens induces the two beams to intersect. The photodetector receives light scattered from tracer particles moving through the intersection volume and converts light intensity into an electrical current. The signal conditioner and signal processing removes noise from the signal, extracts the Doppler frequency and hence the velocity information. With a known wavelength of the laser light and a known angle between the intersecting beams, a conversion factor between the Doppler frequency and the velocity can be calculated. The tracer particles scatter light in all directions, with the highest intensity observed in forward scatter i.e. away from the laser. Much less light is scattered in other directions, but direct back scatter is often used, because it allows integration of the transmitting and receiving optics in a single head. This is much simpler to handle than several heads, which must be carefully aligned with each other. The addition of one or two more beam pairs of different wavelengths to the transmitting optics and one or two photo detectors and interference filters permits two or all three velocity components to be measured. Each velocity component also requires an extra signal processor channel. The basic configuration gives the same output for opposite velocities of the same magnitude. In order to distinguish between positive and negative flow direction, frequency shift is employed. An acoustic-optical modulator - the "Bragg cell" - in the transmitting optics introduces a fixed frequency difference between the two beams. The resulting output frequency is the Doppler frequency plus the frequency shift. Modern LDA optics employs optical fibres to guide the laser light from the often-bulky laser to compact probes and to guide the scattered light to the photo detectors. Modern signal processors use correlation or FFT algorithms efficiently to determine the Doppler frequency from the often-noisy signals received from the photo detectors. The linear relationship between velocity and
output, the insensitivity to temperature and pressure variations, the ability to measure in reversing flows, in supersonic flows, in chemically reacting flows and in rotating machinery (where, for example, turbine blades or engine pistons are present in the measuring volume during parts of the cycle), makes LDA the first choice in many applications where hot-wire anemometry would be difficult or impossible to use. The advantages of this optical flow measurement technique over techniques such as PIV are the high spatial and temporal resolution.

Basic Principles of LDA
Although many different LDA instruments are available, the dual-scatter LDA has become most general because of its simplicity. A laser beam is split and the resulting two equal intensity beams are brought to a focused crossing at a given angle by the focusing lens, the two beams being coherent to each other in the volume of their intersection. A small particle traversing this volume reflects light from the laser beams and scatters it; the light consists of two components, one corresponding to each beam. Both components will have a Doppler shift due to the velocity of the particle, however, the shift also depends on the direction of the light beam. Since the two beams are at an angle, the two components of scattered light have different Doppler shifts, which is directly proportional to the velocity of the particle.

A fringe model may also explain the dual scattered method of measuring velocity. In the region where the beams cross, the wavefronts are quasi-planer. When two coherent laser beams cross they will interfere in the volume of intersection, creating a fringe pattern. These fringes are parallel to the bisector of the beams. A particle going through these dark and light patterns will scatter light and intensity will vary, as shown in Figure 5.3.
Intensity across the laser beam is made to follow a Gaussian distribution (the intensity varies within a Gaussian envelope). If $d_f$ is the distances between fringes and $t_1$ is the time for the particle to go from one fringe to the next, then the velocity component, $V$, normal to the fringes, of the particle is given by:

$$V = \frac{d_f}{t_1} = d_f \times F$$

(5.1)

$$d_f = \frac{\lambda}{2\sin\frac{\theta}{2}}$$

(5.2)

Where $F$ is the frequency of the fluctuation, $\lambda$ is the laser wavelength and $\theta$ is the laser beam intersection angle. $F$ is obtained as the frequency of the photodetector output signal. The frequency $F$ is the same as the Doppler frequency. This simple model provides the correct expression for the velocity of the flow and requires no calibration.
The optics collecting scattered light could be placed in any direction. If the optics are placed as shown in Figure 5.4, the system is then referred to as the forward scatter mode and provides the best signal quality. In the forward scatter mode, optical access to the flow field is required for both transmitted and scattered light. On the other hand, in the case of the backscatter mode, all the optics are kept on one side of the flow field and hence need only one optical access. However, the signal intensity is much less in this mode than in the forward scatter mode.

**Figure 5.4**

*A LDA set-up with forward scatter receiving configuration*

(Figure obtained from: Owida A, 2000)
5.1.4 Dual Camera Stereo Photogrammetry (DCSP)

Dual Camera Stereo Photogrammetry (DCSP) may be used to record the leaflet motion in bioprosthetic heart valves with digital cameras. Although it cannot be directly used to determine forces produced in the PS, the technique may be used to verify these forces when they are applied to a valvular construct. By recording the mechanical deformation of the leaflets, it is possible to analyse the dynamic stresses and strains in the leaflets during the opening and closing phases. To obtain the data two digital cameras are used, each positioned as shown in Figure 5.5. True 3D displacements ($\Delta X$, $\Delta Y$, $\Delta Z$) may be estimated from a pair of 2D displacements ($(\Delta X, \Delta Y)$, as seen from the left and right camera respectively.

![Image not available—See printed version](image.png)

**Figure 5.5**

*Fundamentals of stereovision*

(Figure from: www.me.umn.edu/courses/)

**Working principle:**

The exact loading conditions under physiological operations should be determined. The valve leaflet motion with mechanical deformation under the given loading condition should also be acquired and the material properties of the valve leaflet should be determined so that the stress strain distribution within the valve leaflet can be calculated. Finally, the stress-strain behaviour should be related both to the macrostructure of the valve and the microstructure of the leaflet to determine possible
failure mechanisms (Gao et al., 2000). 3D evaluation is only possible in the area covered by both cameras. Due to the perspective distortion, each camera covers a trapezoid region of the light sheet (Figure 5.6). 3D evaluation requires a numerical model, describing how objects in space are mapped onto the CCD-chip of each camera. Parameters for this numerical model are determined through camera calibration.

Optical markers have been used as a convenient testing tool for tissue strain analysis. Combined with the stereo imaging technique, the stereo photogrammetry method was first used in the seventies (Clark and Missirlis). More recently Lo and Vesely (1995) used this technique to quantify irregular surface geometry of aortic heart valves and to perform stress analysis under static and quasi-static conditions. This study was further developed by Gao et al., (2000) and Sony XC-77 CCD cameras were used that acquire 30 frames per second. These frames were used to evaluate the performance of bioprosthetic heart valves. The leaflet of the test valve was marked with 80 India ink dots to form a fan shaped matrix. From the acquired image sequences, 3D coordinates of the marker matrix were derived and the surface contour and the local mean and Gaussian curvatures at each opening and closing phase during one cardiac cycle were reconstructed (Gao et al., 2000).
5.1.5 Flow Visualisation

Another technique used to provide information related to the performance of heart valves in vitro, is flow visualisation. Flow visualisation involves obtaining a clear image of physical flow fields and capturing the image on sketch, photograph, or other video storage device for display or further processing (Freymuth, 1993). For these purposes tracers, correct illumination and an appropriate image capture system are necessary. This technique is particularly useful for evaluation of valves because of its simplicity and low cost.

Flow visualisation is not as widely used as HWA, PIV, LDA and DSCP. However, flow visualisation has been used to determine the hydrodynamic performance of two types of bioprostheses (porcine and bovine pericardium) under steady flow conditions using dye-injection and a single lens reflex photo camera (Veira et al., 2002). Owida (2000) used the same technique to investigate the flow fields generated by a jellyfish valve prosthesis under different flow rates.
5.2 Discussion: Selection of Techniques

In this section two techniques were selected, one to determine the hydrodynamic performance of the prototype system (PS) and the second to determine the performance of the PCL-scaffold under various flows.

With thermal anemometers, the measuring devices are introduced inside the flow medium to make the measurements. Many errors may occur due to the interference and variations of the flow physical parameters such as temperature, density and pressure. Thermal anemometers are not suitable for measurements in high-density turbulent flows. PIV is an increasingly popular method for making measurements over an entire plane instantaneously and may be used to determine the performance of both the prototype and the PCL-scaffold. However, PIV is still in the primary stages of development for the measurement of the performance of artificial heart valves. LDA is a non-contact optical instrument for the investigation of fluid flow structure in gases and liquids. An advantage of this technique is that LDA measures the desired component of the velocity directly in any complex flow field without interfering with the flow structure. It was concluded that LDA was the most suitable technique for the current investigation.

DSCP can only be applied for measurements on moving structures such as heart valve leaflets. In comparison to flow visualisation, this technique is time-consuming and more costly, although more detailed data can be obtained. Reviewing all the advantages and disadvantages of these techniques it was decided that the flow visualisation technique was best suited for this investigation.
5.3 Experimental Procedure with LDA

LDA was selected to determine the flow characteristics of the PS. This section describes the Aerometric two-component LDA system set-up for the experimental measurements. Data was obtained on the produced fluid flow through the silicon test-tube inside the perfusion chamber of the PS.

5.3.1 The Aerometric Two-Component LDA System Installation

The system was installed and aligned according to the Aerometric LDA manual (1997) (Figure 5.6). The 5W Spectra-Physics Stabilite 2000 Argon laser was mounted on the 1.8 metre long Dantec 55X98 straight mounting bench. The laser was operated in the fundamental TEM$_{00}$ mode, so that the laser power was centred on the optical axis and the maximum power intensity was in the centre. The overall output power of the laser source was adjusted to 3W to ensure sufficient light intensity. A Spectra-Physics 404 Power meter was used to measure the power output from the laser.

The fibre drive was set onto the bench with the input aperture facing the output aperture on the laser. After installing the beam shield into the input aperture of the fibre drive, the fibre drive was moved towards the laser until the beam shield contacted the laser. The Bragg cell cable was attached between the ‘Bragg-cell out’ connector on the rear of the signal processor to the ‘processor 40MHz IN’ terminal on the back of the Fibre Drive. The Fibre Drive internal optics was pre-aligned at the factory. Only the external steering mirrors and couples required adjustment to bring the output beams into alignment. The output power of the four beams from the probe was determined to have minimum 50mW by using the power meter. The receiving fibre of the probe was terminated with a plug fitted to the Receiver Module. The Receiver Module was connected to the ”High Voltage Out” and “Signal In BSCs” receptacles on the back panel of the Real Time Signal Analyser (RSA).
At the onset of each cycle, generated signals were sent from the synchronized output of the controller of the pneumatic pump driver (section 4.4) to the encoder input of the RSA External input (Aerometric Inc.) through a purpose designed encoder cable. The external output was connected directly to the Real-Time Signal Analyser (RSA) I/O card inside the computer. This allowed synchronisation between the trigger signals of the pneumatic pump driver and the arrival time clock register of the encoder. It defined how the arrival time clock would be incremented and reset.

A data interface board was inserted in a sixteen-bit slot on a Compaq S720 computer that received the data from the signal processor and transferred it into memory. Finally, the specialized software package Data-View acquired and stored the data then converted it into velocity values and subsequently displayed the information.

### 5.3.2 Traversing Measurement

After allowing 1 hour for the laser system to warm up, the fibre optic probe was aligned in a particular position where two green beams were parallel to the x-axis to measure the axial velocity component, \( U \), and the blue beams were perpendicular to the x-axis to measure tangential velocity component \( V \). It was essential to position the measuring volume (the point of four beams crossing) accurately inside the silicon tube that was fixed inside the PS. This was not applicable to the measurements on the outflow of the perfusion chamber of the PS, since there was no refraction from the solid surface that has to be penetrated. Once the measuring volume reaches any solid surface, the high voltage from the photomultiplier could be overloaded, and the red light for monitoring the high voltage on the front panel of the signal processor will turn on automatically. Using this reaction, the fibre optic probe was slowly traversed towards the surface-wall of the silicon tube, until the high voltage overload red lights were turned indicating that the measuring volume reached the 8 mm external diameter of the test-tube. Assuming this to be a reference point on the silicon tube surface, the probe was then traversed 4 mm towards the inside of the tube (x-axis), which was on the centreline of the silicon tube. This system was now ready to make the first measurement (Figure 5.7).
Figure 5.7

Crossing of the laser beams inside the silicone tube during the first measurement

A total of twelve axial positions across the 80 mm long silicon tube were measured as well as 126 points on the right side of the perfusion chamber. The basic set-up is presented in Figure 5.8.

Figure 5.8

Set-up of the LDA-system used in the current investigation

(Figure obtained from: Owida A, 2000)
5.3.3 Obtaining the Data from the Measurements

To obtain hydrodynamic data from the developed prototype system (PS), a set-up was needed. The following section describes this set-up and the subsequent data processing.

The PS was placed in a square glass tank filled with water. Small seeding particles with a high ratio of refractive indices were used in the circulating fluid. During the systolic phase, circulating water with the particles left the pressure-chamber of the PS and entered the tissue growth-chamber through the 8 mm silicon tube. In this set-up the PS was functioning in “shortcut-mode”, a closed loop system with the outlets directly connected to the inlets. This ensured that no seeding particles flowed into the water tank to create false measurements. A schematic of the set-up for the flow testing is shown in Figure 5.9. During the diastolic phase, circulation fluid from the tissue growth chamber re-entered the pressure-chamber and closed the fluid circle. The pulsatile flow generated in the PS was activated by air-driven respirator (section 4.4). The driver was placed outside the tank and connected to the PS by an air hose. By adjusting the stoke volume, the stroke rate (BPM) and the inspiration/expiration time of the pump, various pulsatile flows and hydrodynamic stresses were transmitted through the silicon tube. Throughout this experiment the pump rate was maintained at 80 beats/min with a mean flow rate of 6 l/min.

![Figure 5.9](image_url)

*Figure 5.9*

*PS set-up for the LDA measurements*
Particle Seeding:
Seeding is an important consideration when using the LDA technique since the seeding particles are considered to be actual velocity probes. Physical and geometric parameters of the particles influence the quality of signals obtained from the photodetector of the LDA system. The particles should be small enough to track the flow accurately, but large enough to scatter sufficient light for the correct operation of the photodetector and the signal processor.

Another important physical parameter is the refractive index of a particle. In this study, 5µm silver coated pearl particles were used because of the high reflectance portion of their index of refraction. These particles were found to track the flow accurately and to generate sufficient light scattering for detection by the photodetector and signal processor.

Signal processing
LDA measurements depend on the ability of how a signal processor extracts the frequency information and is related to flowing velocity from the photodetector output. Laser Doppler signal processors can be divided into two categories: continuous and burst mode. The burst mode processor is usually either a frequency counter or a Fourier-signal analyser.

The fundamental problem with continuous LDA processing occurs when more than one particle is in the beam at a single moment. The phase of the overall signal from the entire particle is determined by the aggregated phase of all the particles in the scattering volume at a single moment. When a particle leaves that volume, the phase of the aggregate signal changes. These phase changes replicate velocity fluctuations, and generally cannot be distinguished from the fluid flow and consequently phase fluctuations from the particle add noise to the data. Another problem with continuously mode processing of LDA signals is the dropout due to phase fluctuations, which cause the amplitude of the Doppler signal to reach zero intermittently (Owida, 2000).
Considering the difficulties of continuous mode processing, burst mode processing appears to have some unique advantages, as there are no phase fluctuations. The signal processor of the Aerometrics LDA system used for this study is a recently developed digital burst spectrum analyser (BSA). Based on a discrete Fourier transform method, the processor offers speed and automation with outstanding performance. Signals can be detected, processed and validated simultaneously and continuously. The BSA processor has the potential capability to improve both data rates, which correspond to velocity measurements per second, and accuracy, with the ability to distinguish the dominant frequency from background noise, compared to other techniques. Processing is completed in 100 nanoseconds, so the potential data rate is ten million frequency measurements per second, which is fast enough to deal with the most complex flows. Additionally, there is no dead time. The processor reviews portions of the incoming signal that have already been processed, thus overlapping segments of the signal card used. The maximum transfer rate to the interface card is one million frequencies (or particle velocity) reading per second, irrespective of the number of samples used in each signal (Owida, 2000).

Data processing
Processing of LDA data is achieved on digital computers with the development of high-speed analogue-to-digital converters with large storage capabilities and digitising rates up to 100MHz and faster. A suitable software package acquires and stores the signal processor outputs and converts them into velocity values. The desired statistical flow measurement results such as mean velocity, RMS velocity and turbulence intensity may be displayed and stored.

In the set-up, a software package Data-View was used for acquiring and analysing data from signal analysers and acquisition devices. The software simplifies system set-up including hardware diagnostics, processor set-up, optics configuration documentation and flow field mapping. Simultaneous real-time histogram display of the velocity is a key feature of Data-View that helps to optimise system performance and gives a quick display of statistics to ensure that valid data is collected.
The internal data structure of Data-View is divided into several levels. Raw data from the burst spectrum analysers (BSAs) is stored in the hardware level. This is the base level for data in the data-view program. The next level, verified, contains the validated data from the hardware level. The data in the verified level is converted from the hardware level to discrete information (velocity, size). The following level is sub-ranged and verified data is passed though software filters for the sub-ranged level. The highest level in Data-View is the coincident level. If more than one channel of velocity or size is present, they can be compared with each other. Measurements made at the same time (representing the same particle) are passed to the coincident level. Data-View stores only the hardware level, info in the higher levels is calculated from this level and then displayed (Owida, 2000).

5.3.4 Uncertainly Associated with LDA

LDA is becoming one of the most common diagnostic proceedings for the quantitative investigation of flows. The technique is based on the phenomena that light scattered by the particles in the flow is Doppler shifted. The frequency of this Doppler shift is directly proportional to the velocity of the particles. There are however, a number of sources of errors and in the following section an overview is presented.

One of the most important sources of error is that that the velocity of the seeding particles in not equal to the velocity of the fluid flow and therefore unrealistic data is obtained. This problem can be resolved by carefully selecting a seeding particle that will track the flow accurately. It is therefore important to know what the relationship is between the particle and the fluid velocities. Many authors assume that particles used for a particular application will lead to negligible errors. Measurements of turbulent kinetic energy are unlikely to be affected when using micrometer sized seeding particles as the bulk of turbulent energy occurs at relatively low frequencies even in laboratory flows (Morsi, 1996).
Errors that occur when two seeding particles are present in the measuring volume is another concern. This is because the two particles have similar Doppler frequencies shifts so that the wavelength of the beat frequency could be much longer than the time of sampling. However, this error can be considered negligible (Dinterfass, 1989). The use of counter type processors can introduce further uncertainty. This due to the fact that amplitude discrimination is used by the counter processor to validate burst detection. These processors are unable to correctly determine velocity with acceptable accuracy when two or more particles of comparable size are illuminated by the two sources. The two sinusoidal signals could be mixed and will produce invalid velocity measurements. Another source of error is the velocity bias in LDA, which is well described in the literature. It arises from the fact that LDA measures particle statistics rather than fluid velocity directly. The number of particles registered by the measuring system associated with a particular level of velocity will depend on the velocity level. Several methods of dealing with this problem have been proposed. One such method uses “inverse velocity weighting of individual velocity realisations”. Another method is the use of residence weighting applied to individual realisations. A third option is the “sample and weighting method”, which weights each realisation with the time to the next realisation.

Measurement frequency of LDA bursts, which depends primarily on laser wavelength and the cross-angle of the beams, can introduce errors. Although the error associated with wavelength for the Ion Argon laser is negligible, the beam-crossing angle (which is determined by the beam spacing and the focal length of the lens) can introduce an uncertainty of about 1% in the real velocity. However, the error due to uncertainty of the optical configuration is constant and affects all measurements equally. Fringe bias is another source of error in LDA. A common test of Doppler burst quality is that preset minimum number of fringes must be present in the burst with a signal level above some arbitrary signal threshold. In flows with considerable changes in the direction of the velocity vector, occurrences approaching a direction parallel to the fringes will lead to insufficient fringes being present in the burst. The validation circuit will reject these occurrences and a reduction of its effect has been achieved by frequency shifting.
5.4 Flow Visualisation

The main focus of this study was the design and development of a BR-system. Also, LDA was used to measure of the main flow patterns created in the PS. In this section another technique is described to visualize the functionality of a manually fabricated heart valve scaffold (Figure 3.8), used in this investigation.

For complex flow fields, single-point measurements, such as those obtained from HWA or LDA, do not allow for qualitative information on the flow structure, and hence a detailed flow visualisation would be useful. Flow visualisation is an essential tool needed to help understand the complex phenomena associated with most fluid flows and has been successfully applied in biology and medicine (Freymuth, 1993). To study the flow pattern generated by the PCL-scaffold under different flow rates it was necessary to find a method for visualizing the general behaviour of the flow through this scaffold. The results obtained using flow visualisation are presented for different mean flow rates in the following sections.

Experimental Set-up:
The same set-up was used as described in section 4.6 and shown in Figure 4.16. In this experimental procedure the PBR was driven by the pulse duplicator. To visualize the flow, powder or dye was used to determine the flow-behaviour through the scaffold under the different generated pulsatile conditions. After a number of attempts, with various dyes, a dye was chosen that provided most satisfactory results. The dye chosen was Indigo carmine (Australian food colour # 132) and was used at a concentration of 1.6g/100ml. For each run the dye was added to the fluid reservoir after the system was functioning for 1 minute at a preset flow rate. This to produce ideal flow through the system before beginning the experiments.

Images of the different flows through the scaffold were captured with a digital camera (Sony DSC P7, 30 frames/second) and are presented in Figures 6.18 – 6.22. During this
experiment the bi-leaflet check valve was removed from the PBR. Five different settings were investigated using the pulse duplicator as the driver for the system (Table 5.1).

**Table 5.1**

*Flow rates investigated with the flow visualisation technique*

<table>
<thead>
<tr>
<th>Run</th>
<th>Flow rate in litre/min</th>
<th>Beat rate</th>
<th>Vacuum pressure</th>
<th>Driver pressure</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.72</td>
<td>80</td>
<td>150 mmHg</td>
<td>750 mmHg</td>
</tr>
<tr>
<td>2</td>
<td>2.88</td>
<td>80</td>
<td>300 mmHg</td>
<td>600 mmHg</td>
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<td>600 mmHg</td>
<td>300 mmHg</td>
</tr>
<tr>
<td>5</td>
<td>3.25</td>
<td>80</td>
<td>750 mmHg</td>
<td>150 mmHg</td>
</tr>
</tbody>
</table>
CHAPTER 6

EXPERIMENTAL DATA

6.1 Introduction

During this investigation several techniques were used to verify the hydrodynamic performance of the proposed BR-systems. Shear stresses, velocities and fluctuations were determined, as well as flow rates under different settings. These experiments were undertaken to specify the characteristics of the designed and developed systems and to evaluate the performance of a non-seeded heart valve scaffold made from Poly ε-caprolactone (PCL). Flow visualisation was used to visualize the performance of PCL-scaffold under various flows.

The following sections describe how both a flow visualisation technique and the LDA technique was used. Results of the mean flow rates generated by the Proportional Directional Control Valve (PDCV) and the pulse duplicator are presented. 72-hour endurance tests are reviewed and outcomes are discussed.

6.2 Review of the Experimental Data

Several techniques were used in this study to acquire information on the performance of the PS and the PBR. All the conducted experiments were carried out in vitro.
6.2.1 LDA Measurements

The flow field measurements were carried out in vitro under pulsatile conditions with different pulse rates (Chapter 5). The 2D LDA-system was used to determine the velocities and shear stress distributions in the PS driven by the pulse duplicator. Measurements inside the silicon test-tube and inside the growth/perfusion-chamber were taken at a beat rate of 80 BPM. The following section provides an overview how these measurements were conducted.

Velocities and turbulent stresses inside the fixated silicone tube and growth chamber of the PS were measured under pulsatile flow. A computer controlled pulse duplicator was used as the driver in this system. One velocity measurement upstream in outflow of the growth chamber was conducted first to ensure that the system was working and the flow reached a fully developed status. Corresponding measurements were then taken. In the PS 12 centre line positions were measured all in the vertical position, (y-axis) each 5 mm from each other, along the 80 mm long silicon test-tube. Furthermore, 126 points were measured inside the perfusion chamber to determine the velocities and stresses. Areas of interest were the outside of the fixated tube and at the outlet to the reservoir. The positions of these measurements in the reactor are presented in Figure 6.1. The fluid-cycle was divided into five time phases, the Acceleration phase (0-0.06 sec), Peak Phase (0.06-0.27 sec.), De-acceleration phase (0.27-0.34 sec.), Back-flow phase (0.34-0.36 sec.) and the Close phase (0.36-0.73 sec.). Each phase represented an average of measurements taken during a specific time-period controlled by the synchronizer. To obtain measurements, a functional bi-leaflet aortic heart valve was used in the PS to guarantee a continuous fluid flow with new seeding particles.
Figure 6.1
Location of measured points (mm) in the PS-growth chamber
6.2.1.1 Silicone Test-tube

The results of the velocity measurements are presented as the axial mean velocities plotted against time obtained from 12 points on the centreline of the test-tube (Figure 6.1). The velocity profile for each phase was obtained by measuring the velocity of the fluid flow along the length of the tubular construct during operation. Four different beat rates are presented; 80 BPM in point 1 of the tube and respectively 120, 160 and 200 BPM for point 12 in the tube. These plots are shown in Figures 6.2 – 6.4.

![Figure 6.2](image)

*Figure 6.2*

*Velocity profile measured at point 1 in the tube, 10mm above point of origin*

**Velocity**

For 80 BPM, the height of the test-tube (mm) is plotted on the x-axis and the velocity (m/sec) on the y-axis (Figure 6.3). From this figure it can be observed that the velocities during the different phases do not significantly change along the height of the test-tube. Axial Velocities from –2.6 m/s to 7 m/s were found during this simulation.
In Figures 6.4 a-c pulsating waveforms are presented for point 12 in the test-tube. The fluid flows were significantly different compared to the one generated at 80 BPM in point 1. The maximum velocity decreased as well as the cycle time, when the beat rate was increased from 120 to 200 BPM. Furthermore, it was observed that the fluctuation in the peak phase increased when the beat rate was increased.
Shear stresses

The shear stresses along the 12 measured points in the test-tube are presented in Figures 6.5a to 6.5e. Comparing the measured points it was observed that point 1 of the test-tube was subjected to the highest shear stresses in the peak and de-acceleration phase (147 and 172 Pa). The stress distribution showed a decreasing trend-line for the peak phase along the height of the test-tube. For the back-flow phase (Figure 6.5d), an opposite trend was observed. Starting from 53 Pa in point 1 the shear stress increased to 138 Pa in point 12.
Axial fluctuation

Figures 6.6 a-e demonstrated the variation of the non-dimensional axial velocity along the test-tube during five different phases in the cycle. Results show that the ratio \( \left( \frac{U_{\text{max}}}{\bar{U}(t)} \right) \) reached maximum values in the de-acceleration phase from point 1 to 6 and then gradually decreased from points 6 to 12 (Figure 6.6c). Note that the ratio \( \left( \frac{U_{\text{max}}}{\bar{U}(t)} \right) \) increased from point 9 to 12 in the close phase when compared to the first points in that phase (Figure 6.6e).
Figures 6.6 a - e

Velocity fluctuation $\left(U_{\text{max}} / \overline{U}(t)\right)$ in the different phases along the height of the tube.
6.2.1.2 Perfusion Chamber

To determine the velocity, the fluctuations and stresses in the right side of the perfusion chamber, two time phases of the cycle are presented; (1) time phase 0.12 to 0.14 seconds and (2) 0.38 to 0.4 seconds of the full cycle (0 – 0.73 seconds).

Velocity vectors

Figures 6.7 a and b show the mean velocity vectors inside the perfusion chamber during time phases 0.12-0.14 and 0.38-0.4 seconds. These phases represent the peak-phase and the close phase in the time cycle. It can be observed that in figure 6.7a all the velocity vectors are pointed to the outflow of the perfusion chamber. The mean velocity at the outflow is 2 m/s. However, the time phase from 0.38–0.4 seconds shows velocity vectors from the outflow backwards into the perfusion chamber, which does not correspond with the closed phase. Figure 6.7b implies that the flow was still in the backflow phase somewhere between time phase 0.34-0.36 seconds.

![Velocity Vectors inside Perfusion chamber](image1)

(a) 

![Velocity Vectors inside Perfusion chamber](image2)

(b) 

*Figures 6.7 a - b*

*Velocity vectors at two different time phases*
Tangential velocity

Figures 6.8 a and b show the different tangential velocities found during the two different time phases. As shown in figure 6.8a, the maximum velocity towards the outflow of the chamber is 1.19 m/s ($x,y: 45,60$), while velocities from 1.98 m/s in the other direction are observed, just above the outflow ($x,y: 45,78$). Figure 6.8b shows a reversed situation: In this figure negative velocities of -0.273 m/s close to the outside of the test-tube ($x,y: 12,58$) were found while positive velocities from 0.165 m/s (directed towards the outflow) were observed.

Figures 6.8 a – b

Tangential velocities at 2 different time phases
Shear stress
Reviewing Figure 6.9a, it was observed that the shear stresses during the peak phase fluctuated between $-5.9 \times 10^{-4}$ to $14 \times 10^{-4}$ Pa. Specifically, for this time-phase, a concentration of peak shear stresses was found at the position: $(x,y: 47,28)$. For the time phase 0.38-0.4 seconds (Figure 6.9b) shear stresses from $13 \times 10^{-2}$ to $-40 \times 10^{-2}$ Pa were observed in the area between the outflow and test-tube.

*Figures 6.9 a - b*
Shear stresses at two different time phases
Axial velocity flux

In the peak phase, a fluctuation \( \left( U_{\text{max}} / \bar{U}(t) \right) \) throughout the whole chamber was observed. This flux was particularly high in the region close to the test-tube, as seen in Figure 6.10a, \((x,y): 12,65\). At the same location, maximal axial fluctuations were observed for the time phase 0.38-0.4 seconds. Note that very small fluctuations were found in the outer regions of the perfusion chamber during the specified time-period (Figure 6.10b).

Figures 6.10 a - b
Axial velocity fluctuation at two different time phases
Tangential velocity flux

Reviewing Figures 6.11 a - b it can be noticed that the tangential velocity flux \( (V_{\text{max}}/\bar{V}(t)) \) showed a similar pattern to that found with the axial velocity flux for the two presented time phases. Note that in Figure 6.11b a tangential velocity flux of 0.13 m/s was found which is considerably lower compared to the axial velocity flux (maximum value of 0.72 m/s).

Figures 6.11 a - b
Tangential velocity fluctuation at two different time phases
Phase difference

The velocity vector plots shown in Figure 6.7 did not show the correct phase for that specific time frame. Therefore the measurements taken from point 1 (pink dots) and point 70 as shown in Figure 6.1 (located at the outflow of the perfusion chamber shown as the blue dots) were plotted in one graph (Figure 6.12). From this graph it is observed that at time phase 0.38 - 0.4 seconds is not the actual close phase. The graph shows that the flow is still in the backflow phase as suggested before. The phase difference is 0.06 seconds, which means that the time a particle needs to travel from point 1 to point 70. Specifically, the distance that a particle covered can be determined by using the first law of movement (\( s = v \cdot t \)). During the simulation the average outflow-velocity was 2 m/s at peak-phase (Figure 6.7a). Using the formula it was calculated that the particle covered a distance of 120 mm from point 1 to point 70. For the peak phase (0.12-0.14 seconds), however, the phase difference does not have any affect on the results, since the actual flow was still in that phase (Figure 6.12).

![Phase Difference Between Points 1 and 70]

*Figure 6.12*
Phase difference between points 1 and 70
6.2.2 Generated Mean Flow Rates

During this experiment 170 different combinations of the system-settings (amplitude on controller box, beat rate and inlet-pressure) were used to generate the different mean flow rates. These flow rates were recorded with an electromagnetic flow meter. After calibration of the measurement system the experiments were conducted under the following conditions: Each measurement was taken after 1 minute when the flow was fully developed. Water was used as the circulation media and the fluid reservoir contained a volume of 650 ml prior to all the experiments. In all runs a sinusoid pressure wave was used. In the related graphs the mean flow rate is plotted on the $y$-axis while the amplitude is plotted on the $x$-axis.

- For the first simulation (run 1) maximum air pressure was set to 760 mmHg and during the experiment the amplitude was increased incremental by 10% (76 mmHg) from 0 to 100% using seven different beat rates of 60,70,80,100,120,140 and 160 BPM. The results for these beat rates are presented in graph 6.13. Reviewing the graph, it was directly observed that up till 40% no fluid flow is measured. From this setting all the different beat rates follow a similar pattern; up till 80 – 90% of the amplitude, an increase in mean flow rate is observed. Maximum mean flow-rate of 1.5 l/min was measured, at a beat rate of 60 and 80% pf the maximum inlet-pressure.
Figure 6.13
Measured mean flow rates with the PDCV, inlet-pressure of 760 mmHg.
Using the same approach as in the first run a second run was conducted with a maximum inlet pressure of 380 mmHg, ranging from 60% - 100% of the maximum inlet pressure (Figure 6.14). As seen from this graph, similar trend lines were obtained as seen in the first run, except for the fact that for the beat rates 60 to 80. Note that a decrease of mean flow was observed at 80% while from 90% an increase is found. A maximum flow rate of 1.17 litres/min was observed at 60 BPM by 80%.

**Figure 6.14**

*Mean flow characteristics with an inlet pressure of 380 mmHg*
- The third simulation (run 3) was carried out without the use of the bi-leaflet check valve and with the same inlet-pressure as the first run (760 mmHg). The results of this run are presented in Figure 6.15. It was observed that the absence of the check valve decreased the flow rates with an average decrease of 22%, compared to the simulation with the use of this valve.

**Figure 6.15**

*Mean flow characteristics: inlet pressure of 760 mmHg, no check valve*
Pulse duplicator:
For the second part of this experiment the PBR was driven by the pulse duplicator. This device was used to drive the prototype for the LDA measurements and a description is presented in section 4.4 of this thesis. In order to carry out the experiments a similar set-up was used as in the first part of this experiment. 120 combinations were investigated with beat rates ranging from 60 – 160 BPM and inlet pressures ranging from –760 to 760 mmHg. The results are presented in Figure 6.16.

Figure 6.16
Measured mean flow rates: PBR driven by pulse duplicator, simulation with system check valve
Reviewing the results (Figure 6.16), flow rates increased significantly compared to simulations carried out with the PDCV.

- Using a pressure ratio of 1 (positive pressure is equal to the vacuum pressure, 450/450 mmHg) generated the highest flow rate for all different beat rates. Using that setting, flow rates were observed to the maximum flowrate of 6.33 l/min.

- For the setting 750/150 and 600/300 an increase of the fluid in the reservoir of respectively 150 ml and 200 ml was observed.

6.2.3 72-hour tests under 3 sub-physiological flows

The valvular scaffold, fabricated from PCL, was subjected to three mean flow rates (1150, 800 and 600 ml/min) for periods of 72-hours in the PBR. During these tests the PBR was driven by the PDCV. It was observed that during these tests the scaffold kept functioning (opening and closing were synchronous to the generated pulse). Furthermore, no visual deficiencies in shape, strength or composition were observed after conditioning of the valvular scaffold.

It was concluded that the valvular scaffold successfully withstood the pressures and flows over these three-day-periods using water as a circulation fluid. The performance of the scaffold under various flows was visualised by a flow visualisation technique, as described in section 5.1.4. The PBR showed no leakage after the 72-hour tests and the PDCV did not overheat.
6.2.4 Poly ε-caprolactone Scaffold

During this study the functionality of a porous PCL aortic heart valve scaffold was visualised. The valvular scaffold possesses a porosity of 84% that may facilitate cell-ingrowth. In order to fabricate the scaffold with the desired porosity, a salt leaching technique was used.

- The scaffold was subjected to three endurance tests as described in section 4.5. The results demonstrated that the scaffold was still functional and no visual changes were observed during the tests. After removal of the scaffold from the PBR, no macroscopic changes in shape, strength or composition were evident. Therefore, it was concluded that the PCL scaffold successfully withstood the applied pressures and flows over a period of 72h using water as a circulation fluid.

- The PCL scaffold was also subjected to 170 different flow rates (section 4.6). The controller-box of the PDCV was used to generate these different flow rates. The general observation from this test was that up till 40% of the maximum inlet-pressure applied to the PBR no movement was seen in the leaflets of the valvular scaffold. At 50% little vibrations were detectable but these were difficult to see. From 60% to 100% clear movement (fully opening and closing) of the PCL-leaflets was observed.

Finally, the non-seeded PCL-scaffold was used in a flow visualisation experiment (section 5.1.4). In this experiment the scaffold was placed in the PBR. The system was driven by the pulse duplicator to visualise the hydrodynamic performance under various flow rates. The results of this experimental procedure are presented in Figures 6.19 – 6.22. No bi-leaflet check valve was used during these measurements. Therefore, the used construct functioned as an independent valve and the outcomes of the flow visualisation procedure were fully related to the hydrodynamic performance of the valvular scaffold itself.
- As shown in Figure 6.18, the dye used for the visualisation-procedure fully penetrated the porous scaffold as a result of the high ratio of the positive pressure/vacuum pressure. A similar pattern was observed for the 600/300 ratio (Figure 6.19). This was not observed for the mean flow rate of 5.92 l/min, as shown in Figure 6.20. The leaflets of the valvular scaffold did not open properly compared to the movements of a native heart valve leaflets (Figure 6.17).

Figure 6.17
Distal view of closure to opening sequence of a native aortic heart valve in vivo

Figure 6.18
Flow rate of 1.7 l/min, 750/150 mmHg positive/vacuum pressure

Figure 6.19
Flow rate of 2.65 l/min, 600/300-mmHg-positive/vacuum pressure
The used flow visualisation technique in this study provided an inexpensive and practical method for capturing images of the flow fields associated with the PCL scaffold. However, the results demonstrated that the valve-leaflets do not have enough flexibility compared to native valve-leaflets. Specifically, the leaflets appeared to be too
stiff for correct opening and closing. However, after reviewing the literature, populating this scaffold with cells should improve the valve functionality. In theory the flexibility of the leaflets will increase as the PCL degrades and the seeded cells will replace of the structure of the valvular construct (Shinoka et al., 1995).

6.3 Discussion

The LDA-experiments showed that the PS is able to condition TE-constructs. From a design point of view, the principle of converting air into a fluid flow is feasible. The developed PBR, driven by the PDCV was capable of providing different types of haemodynamic flows and can be used for long-term culture of cardiovascular constructs under in vivo physiological conditions.

The PDCV was able to generate different mean flows, but its range is limited. Reviewing the data it appears that the PDCV could not create flows that exceed the values measured in vivo, at least not with the dimensions and/or the set-up of the present driver. Another observation was that when the applied pressures to the PBR exceeded 70% of the maximum pressure, the membrane of the PBR did not return to its equilibrium and pressurised air was trapped in the air chamber. As a result, circulation media rose in the fluid reservoir. A similar pattern was made when the pulse duplicator was used as the driver. Specifically, this phenomenon was observed for the pressure ratio’s 750/150 and 600/300 (Figure 6.23). Figure 6.23 demonstrates that there was not significant vacuum pressure available to remove the trapped air from the air chamber before the next cycle starts. The flow-rates generated by the pulsatile duplicator compared to the PDCV showed that a significant vacuum pressure is essential to generate higher flow rates.
The experimental sections of the investigation provided some useful information. Specifically, results related to the tested valvular scaffold showed that this construct was able to maintain its function under long-term exposure to sub-physiological flows. The flow visualisation procedure, demonstrated that the leaflets of the tested valvular scaffold were too stiff. When pressure ratios with a high positive pressure were used, the construct did not function as a native valve, due to fluid that penetrated the side of the valve (Figures 6.18 and 6.19). This was expected as the construct was porous and non-seeded. By altering these parameters, performance and functionality of the scaffold under generated flows should improve significantly. It should also be considered that by combining different biodegradable materials may improve the overall-performance of the construct (Sodian et al., 1999).

During this study, two developed BR-systems were tested by the two selected drivers and a valve-scaffold was tested under various flows. It was concluded that improvements are feasible after reviewing the results presented in this thesis. Using a vacuum pressure, synchronized with the PDCV, allows the generation of higher flow rates in the BR. Hospitals and medical centres have standard vacuum pressure lines that may be used for this application.
CHAPTER 7

CONCLUSIONS AND RECOMMENDATIONS FOR FURTHER RESEARCH

7.1 Introduction

In this thesis an overview was provided on how a polymer based TE approach may overcome the current limitations associated with heart valve replacements. Two BR systems were designed and developed to measure the hydrodynamic performance of valve constructs using various measurement techniques. Moreover, different types of constructs may be cultured and/or visualized under various types of flows. Besides polymer based TEHVs, the system may also provide housing to decellularized biomatrices, native heart valves and existing heart valve replacements. In addition, a detailed picture of the hydrodynamic characteristics of heart valves can be obtained to provide a better understanding of flow related valve complications (Wright and Temple, 1971; Chadran and Cabell, 1984). These systems may prove useful as a guideline for future investigations related to TE applications.

Furthermore, both systems could be easily and conveniently converted to suite various types of TE-constructs (heart valves, arteries etc.), by a simple adaption of the connection parts. These parts are located in the tissue growth chamber (PS) or by replacing the insert from the top (PBR). What remains unclear is whether the same culture conditions will be optimal for various types of cardiac components, and whether the flow, pressure and pulse rate needs to be tailored to the type of tissue being engineered.
The study has demonstrated that future scaffold designs for TE constructs such as TEHVs and/or blood vessels can be tested for a wide range of features including mechanical strength, degradation and hydrodynamic performance. Subsequently, these constructs may be cultured (long-term) inside the same BR, using TE strategies. The results presented in this study indicated that the second proposed driver, the Proportional Directional Control Valve (PDCV), has great potential for engineering cardiac tissues from a fluid dynamics point of view. With this device many different types of flows can be generated in the BR. The conclusions of this investigation may be summarized as follows:

1) The results obtained with LDA showed that the developed PS is able to generate pulsatile fluid flows. In addition, a phase difference of 0.06 seconds was found in this simulation. The driver used in this system was able to create flow rates up to 6.33 l/min.

2) By measuring the mean flow rates generated in the PBR driven by the PDCV, it was found that the absence of a vacuum pump restricts the range of fluid flow from 50 – 1500 ml/min, and therefore did not fulfill the basic requirements of 5 l/min.

3) After reviewing the operation of the PBR, it was observed that by using pressure ratios with a low vacuum pressure or no vacuum pressure at all, resulted in an increase in the fluid reservoir (due to the absence of a vacuum-pump). The membrane did not return to its equilibrium and may, after long simulation periods, become permanently stretched. This affects the hydrodynamic performance of the BR system and may cause leaks or contamination of the engineered tissue.
4) The attempts to culture cells during this study resulted in negative outcomes. All the cell isolations were contaminated after 3 – 8 days in culture.

5) According to the outcomes of the 3-day endurance tests using three sub-physiological flows, the PBR functioned as expected and the porous PCL scaffold demonstrated appropriate strength in the mimicked \textit{in vivo} conditions. However, mechanical strength tests should be considered for a more accurate assessment of the construct.

6) The flow visualisation of the PCL scaffold demonstrated that the scaffold, in its current format, was not able to mimic the performance of a native aortic valve. Seeding the scaffold with cells from primary cell lines may improve the hydrodynamic performance of the scaffold.
7.2 Limitations of this Investigation

Although valuable information was obtained during this investigation by the presented experimental procedures, some limitations were encountered. The limitations presented in this section should be considered for the continuation of the project.

- **Measurement techniques:**
  The main diagnostic technique used in this study was Laser Doppler Anemometry (LDA). However, this technique is based on point measurements and there was always the question of whether the data obtained presented the complete instantaneous picture of the flow in the PS. In addition, the measurements in the test-tube were conducted on the centre-line and it remains unclear if the walls of this construct were subjected to the measured data. Hence it may be argued that Particle Image Velocimetry (PIV) can provide a better presentation of the results. The design of the PBR was based on the outcomes of the LDA-results from the PS. These results showed that the principle of converting air pressure into a fluid pressure with a silicone membrane was possible. However, the fact that the PBR had the same dimensions and working-principle may not prove that the hydrodynamic performance was similar to that generated in the PS.

- **Scaffold:**
  To fabricate the PCL scaffolds, a salt leaching technique was used. The initial objective was to use Fused Deposition Modelling; a processing-technique is able to create reproducible scaffolds and generate more comparable results as the produced scaffolds are identical. Furthermore, the shape of the currently used scaffolds is cylindrical, which is not the case with native valves. In addition a more sophisticated leaflet design with crimped, expandable surfaces may be required but cannot be made with the salt leaching technique. Although no TE-experiments were carried out in the current study it is known that the PCL scaffold material, had a degradation time of more than 24 months (Appendix
A4). For TE applications a shorter degradation time is necessary. Time constraints did not allow further investigation of other materials in this study.

- **Cell seeding:**
  It was a great restriction that the cell seeding experiments in this study could not be continued, due to time constraints. Once successful however, the different elements for a polymeric TE approach presented in this thesis could be combined. By combining these elements, the first TE attempt at Swinburne University would be a reality.
7.3 Future Work and Recommendations

Review of the literature associated on TEHVs demonstrated that progress has been made in recent years, both with polymeric scaffolds and decellularized biomatrices strategies (O’Brien et al., 1999; Sodian et al., 1999 and Hoerstrup et al., 2000a). However, it is still uncertain if one of these two methods will yield the optimal solution. TEHVs clearly have the potential to improve our ability to treat VHD and to abolish many of the undesirable characteristics found with current heart valve replacement options. Additional research and improvements are needed, before TEHVs constructed from both polymeric scaffolds and decellularized biomatrices are available for clinical use.

The results obtained from this study are preliminary and may contribute to the overall research program. From these results future researchers may use the developed system(s) to gain further understanding of the TE process. The following nine recommendations are provided for future studies related to this investigation:

1. **Measurement techniques:** The use of other measurement techniques such as Particle Image Velocimetry should be considered to allow a complete map of flow through the developed PBR. This technique should also be considered when seeded structures are investigated to record their performance during different stages of development.

2. **Driver:** the PDCV should be synchronized with a vacuum pump. This will expand the range of flow rates, shear stresses and pressures that can be applied to the tissue constructs in the PBR.

3. **Cell source and culture:** Most experiments to date have used endothelial cells from the carotid artery and myofibroblasts, but these cell populations are difficult and impractical to obtain for human clinical applications. Other cell sources such as the peripheral vein, mesenchymal stem cells, and dermal
fibroblasts induced along appropriate differentiation pathways may be considered for future TEHV experiments.

4. **Cell seeding techniques:** High efficiencies are needed for cell seeding, and reliable, long-term labeling techniques are desirable to confirm whether cells populating the TEHV constructs are from the initial seeding or from ingrowth of surrounding tissue. All constructs that were used for TEHV-approaches in the reviewed literature were seeded *in vitro* under static conditions.

5. **Scaffold materials:** Much progress has been reported in this area, but further research is needed to evaluate new materials that take advantage of the wide range of properties of polymers. However, few attempts have been made to assess the materials in detailed toxicological studies *in vivo*, or investigations of degradation rate and mechanisms. Evaluation of the physio-mechanical properties has been published for only a small fraction of available polymers. The PCL scaffold used in this study had a degradation time of more than 24 months. To obtain results in future TE experiments, it is necessary to evaluate materials with a shorter degradation time.

6. **Scaffold Design:** The shape of the trileaflet heart valve structure may be improved to take physiological parameters into consideration. Currently, TEHV structures are cylindrical, but the distal end of native aortic valve includes bulges called the Sinuses of Valsalva. More sophisticated leaflet designs with crimped, expandable surfaces may be required for TEHVs to grow with the patient to prevent regurgitation and cause leaflets to touch at closure.

7. **Scaffold Processing Technique:** Although FDM techniques have the capability to fabricate reproducible scaffolds that fulfil the demands of a TEHV, the scaffold materials have to be compatible with the FDM process. This is part of the research activities currently being undertaken at Swinburne University.
8. **Longer-term studies**: Future studies need to be carried out to examine how the PCL-scaffold can withstand higher pressures and longer exposure times than the 72-hour experiments as described in section 4.5. In addition, larger experimental sample sizes and the use of circulation media other than water should be used.

9. **BR parameters**: Although the system presented in this study made it possible to determine the ideal requirements for tissue growth (magnitude, duration, and rates of change of flows and pressures), these parameters still need to be determined. Variables such as viscosity, concentration, and the addition of growth factors in a cell culture media that stimulate ECM formation should also be considered in future studies.
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APPENDICES
Natural valves

- **Valvular Stenosis**

  To assess stenosis of heart valves, prognostic information may be obtained from the haemodynamic response to exercise and/or delineation of morphological characteristics. Echocardiography can be used for both prosthetic and native heart valves and both the 2D and the Doppler technique in particular, can be effectively used to identify and quantify the severity of the stenosis. TEE and TTE- images can accurately quantify mitral stenosis, while Doppler measurement of transvalvular gradients can give an estimate of the valve area by the pressure half time. Pressure half time is the time needed for the pressure gradient across the mitral valve to decrease by 50 percent. The longer the pressure half time, the longer it takes for the gradient across the mitral valve to close. On the other hand aortic stenoses can be accurately quantified by Doppler measurements of instantaneous and mean transvalvular gradients, estimation of valve area by the continuity method, or determination of aortic valve resistance.

- **Valvular Regurgitation**

  Doppler echocardiography is the most sensitive technique available for detection of valve regurgitation. However, backflow disturbances are frequently detected in normal patients and if trivial, these disturbances should not be identified in the presence of a VHD. On the other hand, significant regurgitation may be silent on auscultation, most often, but not always, in unstable symptomatic patients.

  Precise assessment of the severity of regurgitant valvular lesions is difficult using any invasive or noninvasive technique, and no gold standard is available to judge relative accuracy. Doppler methods for detection of regurgitation are similar for all four native

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5 Most of the materials presented here are obtained from Cheitlin et al. (1997)
valves and prosthetic valves. Methods include assessment of regurgitant jet characteristics, effective regurgitant orifice area and measurement of regurgitant flow volume.

- **Mitral Valve Prolapse**
  Mitral Valve Prolapse (MVP) occurs when the heart valve leaflets are displaced, and regurgitation is a typical consequence. Physical examination seems the optimal method of diagnosing MVP, because echocardiography may detect systolic billowing of the leaflets, but is not representative of clinically relevant disease. By using a stethoscope pathological consequences of MVP such as, systolic clicks may be defined, valvular thickening assessed and the presence and severity of regurgitation can be determined. In patients with a non-ejection click and/or murmur, an echocardiogram is useful for diagnosis, particularly by identifying leaflet thickening. Routine repeated studies are of little value unless there is significant (nontrivial) mitral regurgitation or a change in symptoms or physical findings.

- **Infective Endocarditis**
  Echocardiography is a useful tool for the detection and characterization of the haemodynamic and pathological consequences of infection. These include valvular vegetations, regurgitant lesions, ventricular function, and associated abnormalities such as abscesses and shunts. However, the possibility of a false-negative examination such as the absence of a vegetation, may be assessed with echocardiography. False-positive studies like Lambl's excrescences, which is a condition where the damaged valve area is covered by fibrin and subsequently becomes lifted, or partially detached from the valve surface. Subsequently a layer of intimal cells covers the surface of the fibrin deposits. This should not be classified as a VHD. Other false-positive results can be obtained from non-infective vegetations or thrombi and in these cases the echocardiography findings should not replace clinical and microbiological diagnosis.
Prosthetic valves

- **Stenosis:**
  The assessment of prosthetic valve stenosis is best performed by combined echocardiography-Doppler techniques. The Doppler examination alone can be problematic because eccentric flowjets through the prosthesis may cause recording of falsely low velocities. This phenomenon is often seen in valves with central occluders. On the other hand, elevated trans-valvular velocities may be observed in some prosthetic valves due to pressure recovery that does not accurately represent the hemodynamic gradient. Trans-valvular gradients will vary with valve type and size even in the normally functioning prosthesis. Therefore, individual valve flow characteristics should be considered in the diagnosis of obstruction and re-evaluation may be particularly useful in individual patients.

- **Regurgitation:**
  Accurate determination of prosthetic valve regurgitation is often hampered by prosthetic shadowing. Therefore, the transesophageal approach may be particularly useful in the assessment of regurgitation. However, care should be taken to differentiate between the normal and central regurgitation of many mechanical prostheses and pathological para-valvular leaks. Therefore, the use of contrast injection may enhance the spectral recording of both regurgitant velocities as well as the extent of the regurgitant jet.

- **Endocarditis:**
  Diagnosis of prosthetic valve endocarditis by the transthoracic technique is more difficult than diagnosis of endocarditis of native valves because of the reverberations, attenuation, and other image artefacts related to both mechanical valves and bioprostheses. Particularly, in the case of a mechanical valves, TTE may be helpful only when there is a large or mobile vegetation or significant regurgitation. Therefore, the TTE-technique cannot be used to exclude the presence of small vegetations. These limitations may be diminished by the use of transesophageal recording techniques because of the superior imaging quality and posterior transducer position. Therefore,
transesophageal techniques have enhanced echocardiographic assessment of prosthetic valve infective endocarditis, particularly of the mitral valve. Doppler techniques provide important information about the functional consequences of endocarditis of prosthetic valves. Infection may cause leakage around the heart valve root, which is also known as a para-valvular leak.
APPENDIX A2

Evolution of Mechanical Heart Valves

The first clinically used replacement valve was an acrylic ball valve, where a caged ball was used for occlusion. In 1952, Charles Hufnagel used this artificial heart valve to correct aortic incompetence. This procedure was rarely performed until the heart-lung machine became available in 1953 and surgeons were first able to perform open-heart surgery under direct vision.

Several forms of the ball valve were created and used in valve replacement procedures, but were phased out from 1965 when the first disk valve was created by Kay and Donald Shiley. This valve was thought to have traits that the ball valve did not possess, but it also had problems with blood pressure drops, due poor haemodynamics inside the heart. A Japanese invention resulted in a second form of the disk valve i.e. a tilting disk that prevented falls in blood pressure. This new valve tended to wear quickly, and in 1969 a tilting disk valve with a floating disk made of Delrin was created that was more durable than its predecessor. In 1971, the Delrin disk was replaced by a disk of carbon pyrolite as carbon pyrolite does not react with the blood or become swollen and immobile as the Delrin disk did. In 1976, the carbon pyrolite disk was given a convexo-concave shape, that produced an aerodynamic profile allowing the valve to open and close quicker and with less space for blood to leak through. In 1977, a bi-leaflet valve, the "St. Jude Cardiac Valve Prosthesis," was introduced. This valve consisted of two disks, and was more efficient in controlling blood flow and resisting blood clotting and bacterial infection than the previous designs.

In addition to the 1977 St. Jude Cardiac Valve prosthesis, biological replacements manufactured from porcine or bovine tissue were introduced. These types of valves do not have conventional occluders as seen in mechanical replacements.
### APPENDIX A3

Advantages and Disadvantages of Existing Heart Valve Replacements

<table>
<thead>
<tr>
<th>Device Type</th>
<th>Example</th>
<th>Advantages</th>
<th>Disadvantages</th>
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<tbody>
<tr>
<td>Mechanical Prostheses</td>
<td>• Ball-in –cage</td>
<td>• Long-term durability</td>
<td>• Thromboembolism</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Reproducible manufacturing</td>
<td>• Lifelong anticoagulant therapy</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Risk of infection</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Noisy operation</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Turbulent flow</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Catastrophic failure</td>
</tr>
<tr>
<td>Homografts (Allografts)</td>
<td>• Cadaver valves</td>
<td>• Less thromboembolic</td>
<td>• Limited supply</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Lower risk of immune response, infection, or disease transmission</td>
<td>• Early failure in young people</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Degeneration &amp; calcification requires replacement in 5-15 years</td>
</tr>
<tr>
<td>Xenografts</td>
<td>• Porcine or bovine tissue valves</td>
<td>• Less thromboembolic</td>
<td>• Progressive degeneration &amp; limited durability</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Less immunogeticity after glutaraldehyde treatment</td>
<td>• Early failure in young people</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Lower risk of infection</td>
<td>• Possible disease transmission</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Greater supply</td>
<td></td>
</tr>
<tr>
<td>Bio-prosthetics</td>
<td>• Titanium stents covered with pericardium, etc.</td>
<td>• Less thromboembolic</td>
<td>• Catastrophic failure</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Improved durability</td>
<td>• Risk of infection, foreign body response</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Calcification</td>
</tr>
</tbody>
</table>
## APPENDIX A4

Properties of common BD Polymers

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Melting Point (°C)</th>
<th>Glass-Transition Temp (°C)</th>
<th>Modulus (Gpa)(^a)</th>
<th>Degradation Time (months)(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGA</td>
<td>225—230</td>
<td>35—40</td>
<td>7.0</td>
<td>6 to 12</td>
</tr>
<tr>
<td>LPLA</td>
<td>173—178</td>
<td>60—65</td>
<td>2.7</td>
<td>&gt;24</td>
</tr>
<tr>
<td>DLPLA</td>
<td>Amorphous</td>
<td>55—60</td>
<td>1.9</td>
<td>12 to 16</td>
</tr>
<tr>
<td>PCL</td>
<td>58—63</td>
<td>(—65)— (—60)</td>
<td>0.4</td>
<td>&gt;24</td>
</tr>
<tr>
<td>PDO</td>
<td>N/A</td>
<td>(—10)— 0</td>
<td>1.5</td>
<td>6 to 12</td>
</tr>
<tr>
<td>PGA-TMC</td>
<td>N/A</td>
<td>N/A</td>
<td>2.4</td>
<td>6 to 12</td>
</tr>
</tbody>
</table>

\(^a\) Tensile or flexural modulus.

\(^b\) Time to complete mass loss. Rate also depends on part geometry.

---

\(^6\) Source: www.devicelink.com
# APPENDIX A5

## Summary of Recent Experiments using PBRs for Culturing TEHVs

<table>
<thead>
<tr>
<th></th>
<th>Sodian et al. (1999)</th>
<th>Sodian et al. (2000a)</th>
<th>Sodian et al. (2000b)</th>
<th>Høerstrup et al. (2000a)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell source</strong></td>
<td>Mixed vascular cells from adult ovine artery</td>
<td>Mixed cells from intima, media, and adventitia of ovine carotid artery</td>
<td>Mixed population of fibroblasts, smooth muscle cells, and endothelial cells from ovine vascular artery</td>
<td>EC’s extracted from lamb carotid artery segments; myofibroblasts migrate out from minced segments and then expanded</td>
</tr>
<tr>
<td><strong>Scaffold Material</strong></td>
<td>Tri-leaflet scaffold with conduit wall from bi-layer of PGA (outer layer) and PHA (inner layer); leaflets from PGA-PHA-PGA sandwiches</td>
<td>Tri-leaflet scaffold constructed from salt leached PHA</td>
<td>Tri-leaflet scaffolds constructed from porous PGA, PHA, and P4HB</td>
<td>Tri-leaflet scaffolds from PGA coated with thin layer of P4HB.</td>
</tr>
<tr>
<td><strong>Bioreactor Settings</strong></td>
<td>From 140 ml/min and 10 mmHg systole to 350 ml/min and 13 mmHg systole</td>
<td>From 140 ml/min and 10 mmHg systole to 350 ml/min and 13 mmHg systole</td>
<td>100 ml/min for 1 hour</td>
<td>125 ml/min and 30-mmHg systole to 750 ml/min and 55-mHg systole.</td>
</tr>
<tr>
<td><strong>Observation Points</strong></td>
<td>1, 4, and 8 days</td>
<td>1, 4, and 8 days</td>
<td>1 day</td>
<td>4, 7, 14, 21 days</td>
</tr>
<tr>
<td>Evaluation Methods &amp; Results:</td>
<td>Leaflets for all TEHV open and close synchronously with fluid flow</td>
<td>TEHV function under sub- and super-physiological flow conditions; pliable constructs</td>
<td>Leaflets for all TEHV open and close synchronously with fluid flow</td>
<td>Valves open &amp; close synchronously under low &amp; high pressure</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>-------------------------------------------------</td>
<td>---------------------------------------------------------------------------------</td>
<td>-------------------------------------------------</td>
<td>-------------------------------------------------</td>
</tr>
<tr>
<td><strong>ESEM- cell attachment, surface morphology</strong></td>
<td>Nearly confluent cell layer; leaflet cells oriented in flow direction by day 4; cells on conduit wall form bridges in pores.</td>
<td>Cells grow into pores, confluent cell layer by day 4, cells oriented in direction of flow; control group = disoriented cells</td>
<td>Almost confluent cell layers by day 8 for PHA and P4HB constructs</td>
<td>Confluent surface by 7 days <em>in vitro</em>; smooth surface on both sides of TE leaflets <em>in vivo</em></td>
</tr>
<tr>
<td><strong>Mechanical strength</strong></td>
<td>Not known, however, ECG shows no pulmonary regurgitation.</td>
<td>Not known, however, ECM content shows collagen &amp; GAGs, but no elastin</td>
<td>Supra-physiological strength</td>
<td>Decrease over time until comparable to native vessel</td>
</tr>
<tr>
<td><strong>DNA &amp; 4-hydroxyproline assays: cell number &amp; collagen content</strong></td>
<td>More cells and collagen on constructs exposed to flow and with prolonged flow exposure than static conditioning</td>
<td>More cells and collagen on constructs exposed to flow and with prolonged flow exposure than static conditioning</td>
<td>More cells on PGA sheets than on PHA and P4HB constructs; significantly more collagen on PGA sheets than PHA &amp; P4HB</td>
<td>Collagen, elastin and GAG-content exceeded that of a native artery by day 30.</td>
</tr>
</tbody>
</table>
APPENDIX A6

Description of the different components of the PBR

Exploded 3D-view of the PBR-components
Description of the PBR-components

1: Air chamber
This chamber was conducted of stainless steel (Fe 316) and has a 1/8”G-connection to connect a FESTO 6mm air hose with an easy to click in FESTO connection.

2: The membrane
This part consists of a white silicone rubber sheet with a thickness of 0.7mm and is located between the fluid and air chamber.

3: Fluid chamber
This compartment was also constructed of stainless steel and did not differ much to the PS. Exceptions were in the sphere-shape and the position of the inlets. This part can be easily adapted if a second inlet is needed.

4: O-ring
This part of the PBR is located between the fluid and perfusion compartment. The ring prevents leakage between these compartments. The pressure applied for sealing is from a Dry Seal Flange: diameter 125-155mm (section 4.3.1.2). This part consists of a ring with an inner ring. The click mechanism located on the outside of the ring pushes the inner ring and the two flanges (compartment 2 and 3) together, to provide the sealed connection.

5: Perfusion chamber
This off the shelf part is available with a standard flange. The height and shape can be altered by a glass blower to culture different constructs. The compartment has one outlet that can be easily altered.
APPENDIX A7

Technical drawings of the PBR

Air-chamber
Fluid-chamber
Housing for Check-Valve